DETECTION OF *TOXOPLASMA GONDII* IN FRESH PRODUCE

by

DANAYA AMINA BETHEA

(Under the Direction of Ynes R. Ortega)

ABSTRACT

*Toxoplasma gondii* is an intracellular obligate protozoan parasite that causes toxoplasmosis in humans. This study aimed to detect and genotype *T. gondii* in fresh produce. Six PCR assays using various *Toxoplasma* gene targets were examined for their suitability in vegetable matrices. The SAG2 PCR was selected for this study. Collectively, DNA was extracted from 818 vegetable samples acquired from 16 markets in Atlanta, GA, U.S. and 5 in Lima, Peru. Nested polymerase chain reaction was conducted using characterized primers for *T. gondii* SAG2 locus. PCR products were purified and sequenced. Detection via PCR showed that 2.5% and 0.79% of U.S and Peru samples tested positive, respectively. The detection of *T. gondii* in fresh produce indicates that it may play a role in the prevalence of toxoplasmosis in the U.S and Peru. It also emphasizes the need to develop methodologies to prevent dissemination and inactivation of viable oocysts in the environment.

INDEX WORDS: *Toxoplasma gondii*, Fresh Produce, PCR, Genotyping, SAG2, Peru, Georgia
DETECTION OF *TOXOPLASMA GONDII* IN FRESH PRODUCE

by

DANAYA AMINA BETHEA

B.S., Clark Atlanta University, 2011

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2014
DETECTION OF *TOXOPLASMA GONDII* IN FRESH PRODUCE

by

DANAYA AMINA BETHEA

Major Professor: Ynes R. Ortega
Committee: Jennifer Cannon
Joseph F. Frank

Electronic Version Approved:

Julie Coffield
Interim Dean of the Graduate School
The University of Georgia
August 2014
DEDICATION

I would like to dedicate this manuscript to my family and friends, for their immeasurable love and support and my boyfriend Roderique John, for always being there for me. Most importantly, I would like to dedicate this work to my ancestors. Thank you for paving the way for me so that I am able to be where I am today. Ase. I love you all.

In loving memory of Julia Mae Williams and Michael Williams. We miss you.
ACKNOWLEDGEMENTS

I would like to thank Dr. Ynes Ortega for all of her guidance and support. This work would have been impossible to complete without your aid.

I would also like to thank my advisory committee, Dr. Jennifer Cannon and Dr. Joseph F. Frank for all of their suggestions and technical advice.

It is with my sincerest appreciation and gratitude that I thank Patricia Torres for her encouraging words and support. Thank you for helping me to become acclimated with the laboratory. It was a pleasure working with you!
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>2 DETECTION OF <em>TOXOPLASMA GONDII</em> IN FRESH PRODUCE</td>
<td>39</td>
</tr>
<tr>
<td>3 CONCLUSION</td>
<td>50</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>34</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1: PCR Primer Name & Sequence.................................................................43
Table 2: Toxoplasma Distribution by Market-Peru ...............................................45
Table 3: Toxoplasma Distribution by Vegetable-Peru...........................................46
Table 4: Toxoplasma Distribution by Season-Peru ..............................................46
Table 5: Toxoplasma Distribution by Market-U.S ................................................47
Table 6: Toxoplasma Distribution by Vegetable-U.S............................................47
Table 7: Toxoplasma Distribution by Season-U.S................................................48
LIST OF FIGURES

Page

Figure 1: Amplification of *Toxoplasma* spiked U.S. vegetable samples targeting the

BTUB gene .....................................................................................................................43

Figure 2: Amplification of *Toxoplasma* spiked U.S. vegetable samples targeting the

GRA6 gene ....................................................................................................................44

Figure 3: Amplification of *Toxoplasma* spiked vegetable samples targeting the

SAG3 gene ....................................................................................................................44

Figure 4: Amplification of *Toxoplasma* spiked U.S. vegetable samples targeting the

SAG2 gene ....................................................................................................................45

Figure 5: Amplification of *Toxoplasma* DNA using the SAG2 PCR ............................................46
CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

This study seeks to understand the role *Toxoplasma gondii* plays environmentally in the occurrence of fresh produce in the United States and Peru. The presence of *T. gondii* in undercooked and raw meats harvested for human consumption is widely known. Although the transmission of viable oocysts has been attributed to the consumption of contaminated fresh produce, along with other environmental elements, there is a lack of data supporting this. The study was conducted to contribute data on the novel topic and to investigate the possibility of *Toxoplasma* transmission via the consumption of contaminated vegetables. The findings in this study should prompt scientists and outreach facilities to continue research on the matter and develop more effective ways to prevent environmental contamination.

As stated previously there is a lack of information on the presence of *T. gondii* on fresh produce sold for human consumption. Hence, by nature, this study is novel. Both U.S and Peru samples were collected around the same time within a year. After DNA extraction, these samples were then pooled in sets of five based on vegetable type - basil, cilantro, green onion, lettuce, mesclun, parsley, Peruvian black mint, and spinach and from two different location- U.S. and Peru. Pooled samples were screened targeting the *Toxoplasma* SAG2 locus. The pooled samples that tested positive were then individualized and tested by triplicate. Individual positive samples were sequenced for ID confirmation and genotyping.

Although *T. gondii* oocysts can survive in inclement weather conditions and various climates, it thrives in warm weather and tropical climates. Hence, it is suspected that there will
be more positives present in samples collected during the warm seasonal months than those collected during the cold months. There may also be more positive samples found in Peru due to its tropical climate. Within the group of positive samples, those collected from local markets are expected to outnumber those that were collected from major markets.

*Toxoplasma gondii* is an intracellular obligate protozoan parasite that causes toxoplasmosis in humans. This study aimed to detect and genotype *T. gondii* in fresh produce. Six PCR assays using various gene targets were examined for their suitability in vegetable matrices. The SAG2 PCR was selected for this study. Collectively, DNA was extracted from 818 vegetable samples acquired from 16 markets in Atlanta, GA, U.S. and 5 in Lima, Peru. Nested polymerase chain reaction was conducted using characterized primers for *T. gondii* SAG2 locus. PCR products were purified and sequenced. Detection via PCR showed that 2.5% and 0.79% of U.S and Peru samples tested positive, respectively. The detection of *T. gondii* in fresh produce indicates that it may play a role in the prevalence of toxoplasmosis in the U.S and Peru. It also emphasizes the need to develop methodologies to prevent dissemination and inactivation of viable oocyst in the environment.

**Microorganism description**

The genus *Toxoplasma* belongs to the phylum Apicomplexa, class Coccidia, order Eucoccidiorida, and family Sarcocystidae[1]. *Toxoplasma* has been identified in various animal species and is likely able to infect all warm-blooded animals and humans [2-5], *T. gondii* is the only species described [6-8]. Before this discovery, species were classified based on the animals they were isolated from. The following *Toxoplasma* species, including the animals they were isolated from and the scientists who isolated them, have been described: *T. gondii* - > 200 mammals and birds (Nicolle & Manceux), *T. alencari* - *Leptodactylus ocellatus* (frog, Da Costa
& Pereira), *T. brumpti* - *Iguana tuberculate* (iguana, Coutelen), *T. colubri* (snakes *Coluber melanoleucus* and *Coluber vindiflavus*, Tibaldi), *T. hammondi* (house mouse with oocysts in the domestic cat, Frenkel & Dubey), *T. ranae* - *Rana pipens* (leopard frog, Levine & Nye) and *T. serpai* - *Bufo marinus* (toad, Scorza, Dagert & Itturiza Arocha) [9]. The aforementioned species were later either found to be the same as *T. gondii* or reclassified into other coccidian genera [9, 10].

There are however, three clonal lineages of *T. gondii* identified as type I, type II, and type III [4, 11-15]. Type II strains are the major source of human infections in the U.S. [4, 12, 16]. Type I strains have been associated with acute congenital toxoplasmosis in Europe. It has also been identified in immune-competent persons experiencing critical atypical ocular toxoplasmosis in the U.S. [16].

The genus *Toxoplasma* was derived from the Greek words toxo/toxon – bow, due to its crescent shape, and plasma-life/cell [17-19]. *T. gondii*, originally misidentified as *Leishmania*, was discovered in the rodent *Ctenodactylus gundi* in 1908 by researchers Nicolle and Manceaux in Tunis and by Splenore in a rabbit in Brazil [2, 9, 18]. Nicolle and Manceaux discovered the species in the North African rodent, *Ctenodactylus gondi* [17]. In 1923 the first congenital case of *T. gondii* was discovered in humans [20]. Almost twenty years later, the first fatal case of toxoplasmosis in a cat was discovered in 1942 [19]. Research concerning *T. gondii* infections in humans and animals continued because congenital infections did not vindicate the extensive infections in humans and animals as they rarely occurred. In 1954, Weinman and Chandler suggested that *T. gondii* infections may be contracted from the consumption of undercooked meat [18]. By 1965, Hutchinson determined that cat feces could be a source of *Toxoplasma*
infection [21]. The domestic cat was established as the definitive host for *Toxoplasma gondii* in the 1960’s[22] and in 1970 it was confirmed that oocysts were the infective agent in cat feces [21]. *Toxoplasma gondii* has a clonal lineage composed of three predominant genotypes identified as type I, type II, and type III [4, 11-15]. These genotypes are widely distributed geographically [11]. Analysis of isoenzyme profiles from isolates originating in France revealed a potentially deliberate association of different isoforms for a diverse amount of enzymes, indicating the presence of distinct strains [23]. Most of the strains identified in North America and Europe have been categorized by one of the genotypes [12, 14, 24]. The genotypes are also phenotypically and genetically similar with a 1% or less divergence [11, 12]. Only two alleles at each of the locus are present for most of the genes in majority of the *T. gondii* strains [11, 16, 23]. It has been revealed that type II strains have are more genetically diverse than other types and subtypes [25]. However, recombinant strains and exotic strains do exist. Recombinant strains contain mixtures of the two allele patterns found in the three clonal types, whereas, exotic strains are composed of unique polymorphisms. According to the calculations of Su et al. clonal lineages originated approximately 10,000 years ago and exotic strains 1 million years ago [11]. It is suggested that recombination amongst two distinct ancestral gene pools, A and E, created recombinant progeny, three of which dominate the world today [16, 23]. It has also been suggested that the predominance of these three lineages is due to a recent meiotic event - independent of sexual recombination- that resulted in a restricted amount of progeny obtaining a trait that allows them to parasitize the majority of warm – blooded vertebrates [11]. Interestingly, exotic strains did not originate from the same cross that produced the predominate clonal lineages [11]. Hence, suggesting that exotic strains, including those considered to have originated from the forest of French Guyana, depict more ancestral lineages [11, 23]. The parents
of the ancestral strains, are limited to the archetypal two-host life cycle and will probably be found in hosts from pre-Columbian times [11, 23].

*Toxoplasma gondii* infections are a major cause of morbidity and mortality in immunocompromised individuals, particularly AIDS patients and infants who are congenitally infected [26-31]. This parasite, along with *Listeria monocytogenes*, is the most important foodborne pathogen during pregnancy as infection can be fatal or cause long-term disease after birth [32]. *Toxoplasma gondii* causes the third highest number of deaths caused by foodborne pathogens [32] and is a leading cause of hospitalizations in the United States. It is also considered the third most common cause of fatal foodborne disease via pork ingestion [32]. Although there are many routes of infection for the parasite- (1) horizontally via oral ingestion of sporulated oocysts from the environment, (2) horizontally via oral digestion of tissue cysts present in raw/undercooked meat or viscera of the intermediate host, or (3) vertically, via the trans placental transmission of tachyzoites [33, 34].

**Life Cycle**

The life cycle of *Toxoplasma* is very unique. This tissue-cyst forming coccidium parasite with a heteroxenous life cycle can replicate indefinitely via sexual or a sexual subcycles [2, 23]. The three infectious stages of *T. gondii* are the tachyzoites, bradyzoites, and sporozoites [33]. Of the three stages present in the life cycle, the first two are result of asexual multiplication and can occur in both the intermediate and definitive hosts.

Initially, tachyzoites multiply rapidly via replicated endodyogeny in a variety of different host cells [2]. Tachyzoite, derived from the Greek word tachos, meaning speed, was a term made popular by scientist Frenkel to describe this rapidly dividing stage [33]. This was also the stage that Nicolle and Manceaux, found in the gundi rodent in 1909 [18]. These zoites are crescent
shaped and approximately 2 by 6µm with sharp anterior and spherical posterior ends [33]. The second stage of asexual development occurs upon the last multiplication of the tachyzoites in the host cells from which the formation of tissue cyst result [2]. Within these tissue cysts are bradyzoites or cystozoites, slowly dividing via endodyogeny [2, 6, 18, 33, 35, 36]. Bradyzoites, derived from the Greek word brady, meaning slow, was another term coined by Frenkel to portray the slow multiplication of these zoites within the tissue cyst [18, 33]. As the bradyzoites continue to divide, tissue cysts grow and remain intracellular [33]. Hence tissue cysts can range in size. Juvenile tissue cysts may be as small as 5µm in diameter while spheroidal tissue cyst in the brain can reach 70µm, and elongated intramuscular cyst can be as wide as 100µm in diameter [33]. Although tissue cysts grow in the visceral organs such as the lungs, liver, and kidneys, they are mostly found in the neural, central nervous system, and muscular tissues which include the eyes and skeletal/cardiac muscles [2, 33]. The thin and elastic tissue cyst wall is less than 0.5µm thick, enclosing hundreds of crescent shaped 7 x 1.5µm sized bradyzoites[37]. Host cell, parasite material, and granular material make up the tissue cyst wall [35, 36]. It is likely that unscathed tissue cyst do not cause harm to the host and can grow for the host’s entire life without triggering an inflammatory response [2, 33]. However, it is believed that tissue cysts can systematically break down, which results in the transformation of bradyzoites into tachyzoites that enter healthy host cells and eventually convert back to bradyzoites in these new host cells [8, 33, 38]. Research conducted by Remington and Melton revealed that bradyzoites are not digested via gastric juices although the cyst wall can be digested by pepsin or trypsin [18]. Hence, in terms of zoonosis, the tissue cyst development stage in which bradyzoites grow is important because it allows carnivorous hosts to become infected via consuming undercooked infected meat [18, 21].
Some differences among bradyzoites and tachyzoites include nucleus location and presence of amylopectin granules. There are many amylopectin granules in bradyzoites and little to none in tachyzoites. The nucleus is centrally located in tachyzoites and terminally in bradyzoites [18, 33]. Lastly, bradyzoites are not digested by gastric juices while tachyzoites are [18].

Interestingly, the bradyzoite cycle in cats is most successful as almost all cats fed tissue cysts shed oocysts, compared to the < 30% of cats that shed oocysts when fed tachyzoites or oocysts [33, 38]. Prepatent periods vary based on infection stage as well. Cats shed oocysts 3-10 days after bradyzoite ingestion, ≥18 days after ingestion of sporulated oocysts and ≥ 13 days after ingestion of tachyzoites [18, 33, 38].

The final infective stage of T. gondii is the sporozoite. The development of oocysts can only occur in the small intestine of the definitive host (felines) and results in the production of oocysts containing sporozoites [6, 18, 38]. Sexual reproduction is thought to be prompted by the latter stages of asexual reproduction within the definitive host [2, 38]. Gametogony occurs after the five morphologically unique asexual types of T. gondii, classified type A-E, develop in the intestinal epithelial cells or [2, 33] enterocytes [18, 33, 38]. After fertilization of the macrogametes by the microgametes, the zygote is formed that further differentiates when the oocyst wall develops and surrounds the parasite [33]. Eventually, the enterocytes rupture and release oocysts into the intestinal lumen, where they are excreted to the environment along with the cat faeces [2, 33]. Sporogeny occurs externally from the host, with sporulation occurring within 1-5 days of [33] excretion, depending on conditions such as aeration and temperature [6, 33]. Millions of oocysts are shed due to the copious amounts of multiplication that occurs within the intestines [22, 38, 39]. Unsporulated oocysts can range from subspherical to spherical in shape and are 10 x 12µm in diameter [33, 40]. Sporulated oocysts are ellipsoidal in shape and are 11 x
13µm in diameter with two sporocysts containing four sporozoites each that are 6-8 µm in size [33, 40]. Once sporulated, the oocysts can survive for up to 12 to 18 months depending on environmental and climatic conditions [22]. They can also survive in temperatures ranging from 4 to 37°C and can survive in soil for a maximum of two years [32]. Experimentally, oocysts were able to survive outdoors in Texas uncovered for 46 days with temperatures ranging from 6-36°C [26]. A study conducted in Kansas in which oocysts were buried in soil 3-6 cm deep revealed that oocysts survived for 18 months [26]. They are also very resistant to disinfectants and freezing, as it was shown that oocysts can survive in -21°C for 28 days. However, they can be killed at temperatures above 60°C and via ultraviolet rays [26].

**Epidemiology**

**Clinical Toxoplasmosis**

Susceptible Populations:

Toxoplasmosis is one of the most prevalent parasitic infectious diseases in animals and humans [41]. It is estimated that nearly one-third of the human population is infected with *T. gondii* [2, 32, 33, 42]. The prevalence rate of chronically infected adults is approximately 15 -85%, depending on geographic location [15, 33]. Interestingly, infections occur less often in cold climate areas compared to warm and humid areas [2]. Infected immune-competent individuals are usually asymptomatic with a few exceptions [3, 4, 42, 43]. However, toxoplasmosis can bare severe manifestations in immune-compromised individuals, particularly AIDS patients [2, 11-13, 15, 18, 22, 23, 33, 38, 42-46]. Generally, all populations are susceptible as *T. gondii* can be transmitted via consumption of raw or undercooked meat possessing viable tissue cysts or via the ingestion of food or water contaminated with oocysts shed from infected cats [2-4, 11, 15, 31, 38, 39, 46].
Clinical Description

As previously mentioned, immune-competent individuals normally do not show any symptoms of infection. However there are approximately 10-20% of *T. gondii* cases involving immune-competent individuals that are symptomatic [43]. In some cases they were shown to have lymphadenopathy and ocular toxoplasmosis [3, 4]. Toxoplasmosis is estimated to be responsible for 3-7% of clinically significant lymphadenopathy [43]. Symptoms associated with lymphadenopathy include fever malaise, night sweats, myalgia, sore throat, maculopapular rash, abdominal pain, and hepatosplenomegaly – all of which last a maximum of a year or dissipate within a few months [43]. Other common symptoms include myocarditis, polymyositisis, chorioretinitis along with other severe ocular infections [23, 43]. Acute chorioretinitis has the potential to cause blurred vision, Scotoma, photophobia, epiphora, or loss of central vision. Consequently, asymptomatic toxoplasmic chorioretinitis can cause partial or complete loss of vision [43]. In terms of other severe ocular infections, *T. gondii* is one of the main identifiable causes of uveitis. More than 85% of posterior uveitis cases in Southern Brazil were found to be due to *T. gondii* infection [43]. The most common development amongst immune-competent individuals is asymptomatic cervical lymphadenopathy [43]. Recent data also suggests that chronic *T. gondii* infection may make individuals vulnerable to the development of behavioral disorders including schizophrenia [47-49].

Prior to the Acquired Immunodeficiency Syndrome (AIDS) epidemic in the 1980s, the most susceptible population for *T. gondii* infection was perceived to be infants who could contract the disease congenitally [43, 44]. Recent data shows that the incidence of prenatal infections is seemingly low – 1 -120 infections per every 10,000 births [2]. Currently, *Toxoplasma* is the leading cause of neurological disease in AIDS patients [44]. This is also a drastic change from
circumstances in the past where T. gondii infections in adults were the consequence of organ transplants and blood transfusions [18]. AIDS patients affected by toxoplasmosis are at risk for developing fatal diseases like encephalitis, pneumonitis, and myocarditis [2, 33, 43, 46]. Unfortunately, many AIDS patients (10-30%) succumb to these diseases [2] as T. gondii infection is a primary cause of morbidity and mortality amongst this T-cell deficient group [13, 22, 45]. Toxoplastic encephalitis (TE), which was initially identified among patients with Hodgkin’s disease, is of worthy mention because it is the most common clinical diagnosis involving AIDS patients [38, 43]. Similar to other diseases caused by Toxoplasma, TE is the result of reactivation of a previously dormant T. gondii infection [12, 18, 33, 38, 43, 45]. For immune-compromised individuals with toxoplasmosis, tissue cysts provide a nesting place for the proliferation of various infections [43]. With the aid and wide distribution of prophylaxis like trimethoprim sulfamethoxazole, the incidence of toxoplasmosis has drastically declined [43, 45]. Prior to its use, pneumonia due to encephalitis was described in 5% of advanced AIDS cases with a mortality rate of 35% [43]. A decrease has also been observed in the incidence of central nervous system toxoplasmosis in a number of countries due to retro-antiviral therapies and immune recovery [2]. However, the use of prophylaxis is often discontinued due to unpleasant side effects experienced among patients [45]. Unfortunately, in countries where HIV treatment is unavailable, toxoplasmosis still a major problem [43].

**Outbreaks/Clinical Symptoms**

**Environmental Related Outbreaks**

In October of 1977 a toxoplasmosis outbreak involving 37 cases occurred in a riding stable in Dekalb County, GA [50]. Thirty-five of the thirty-seven cases suffered common symptoms such as myalgia, stiff neck, anorexia, rash, confusion, arthralgia, confusion, and hepatitis [50]. Less
common symptoms included fever, headache, and lymphadenopathy [50]. Three of the twenty-five cases that visited physicians were diagnosed with toxoplasmosis with incubation periods ranging from 4-21 days [50]. There were no common meals consumed by the infected, but five feral cats were found positive for *Toxoplasma*. Hence it was concluded that infection was transmitted via inhalation of dust made by movements of the horses [50]. During the same month of the previous year, six children and a mother of one of the children developed symptoms caused by *Toxoplasma* infection [51]. Common symptoms among infected were fever, leukocytosis, cough, pneumonitis, rash, nasal congestion and elevated levels of specific antibodies. Symptoms specific to some of the affected the children included nasal eosinophilia, lymphadenopathies, malaise, and chorioretinitis. Bilateral cervical lymphadenopathy was a distinct symptom of the infected mother [51]. Since majority of the affected were children, it was concluded that the mode of transmission was due to a specific activity conducted among the group i.e. playing in the yard or play area where seropositive cats were allowed to defecate [51]. Nine members of a family living on an Illinois farm tested serologically positive for *Toxoplasma* [52]. Six of the nine children had symptoms that included fever, pharyngitis, mild abdominal pain, conjunctivitis, maculopapular rash, and submandibular, anterior cervical, and inguinal adenopathy [52]. This family had a lot of contact with cats, and ingested drinking water from a stream on a farm inhabited by many cats [52]. A large outbreak of acute toxoplasmosis involving a battalion of U.S. Army soldiers occurred in the Panama Canal area from January 27-February 18, 1979 [53]. Of the 98 soldiers from one company, there were 31 cases of acute toxoplasmosis with incubation periods ranging from 5-18 days. The most prominent symptoms included fever, chills, headache, malaise, myalgia, stiff neck, abdominal pain, nausea/vomiting, and joint and eye pain. Less prominent symptoms included splenomegaly, hepatomegaly, atypical
lymphocytes, and absolute lymphocytosis. Lymphadenopathy was noted in axillary and anterior and posterior cervical lymph nodes. Since the battalion mostly ate from canned rations but drank from small streams, the probable source and cause of infection was ingestion of water from contaminated jungle streams containing oocysts shed from infected jungle cats [53]. From December - January 2004, there was a toxoplasmosis outbreak amongst thirty-three villagers in Patam, near the French Guinan border [54]. There were eleven cases of toxoplasmosis in all. Immunocompetent adults comprised of eight multivisceral infections, resulting in one death. Two cases, a neonate and fetus, were diagnosed with lethal congenital toxoplasmosis. One child was symptomatic. The symptoms, lasting 2-3 weeks, included fever, cough, headache, contractions, abdominal discomfort, splenomegaly, hepatic pain, myalgia, arthralgia, extreme clinical impairment, tachyarrhythmia, breathlessness, and hepatomegaly [54]. Although the outbreak was thought to be due to oocyst ingestion, there were no epidemiological sources that could be linked to the outbreak [54]. As shown from the reported cases, incidences of toxoplasmosis outbreaks including more than a single family or small group are seldom reported [54]. However, there are two large-scale toxoplasmosis outbreaks that have been documented. The first outbreak involved 100 individuals ranging from 6-83 years in age [55]. Ninety-four of these cases occurred amongst residents of the Greater Victoria area in British Columbia, Canada and six occurred amongst visitors. Symptoms of the infected were accounted for and diagnosed. Clinical diagnosis included retinitis (n=19) and lymphadenopathy (n=51). Four individuals had symptoms commonly experienced by those with toxoplasmosis (among immunosuppressed-prominent pneumonitis, and encephalomyelitis; among immunocompetent- lymphadenitis, asthenia, myalgia, and headache) eighteen individuals were asymptomatic, seven individuals experienced symptoms unrelated to toxoplasmosis and one would not supply information. It was
determined that this outbreak was likely caused by oocyst contamination in the municipal drinking water [51, 55]. The water could have become contaminated through numerous vectors. The water system that serves a minimum of 100,000 people was not filtered and used inadequate chemicals for initial disinfection. The surface water was also vulnerable to direct and indirect contamination. Felids, including domestic and feral cats along with cougars, are commonly seen in the watershed. It is quite possible that runoff containing oocysts contaminated the reservoir. It was estimated that between 2,894 and 7,718 persons were infected in this outbreak [55]. The second large outbreak occurred in North Rio de Janeiro State, Brazil [56]. Due to a previous study conducted in 1997 that revealed 61/74 people living in a very disadvantaged area of Campos dos Gpytacazes, North of Rio de Janeiro and 15/27 public school children had antibodies to \( T. gondii \) a more inclusive study was executed. Of the 1,436 people comprising of lower, middle, and upper socioeconomic classes, 84%, 62%, and 23% respectively, were seropositive [56]. The consumption of untreated water was ruled the most likely source of infection. Similar to the aforementioned outbreak, oocysts could have entered the stream via floods or runoff after intense rainfall. Based on the information from a created logistic model, drinking water from a faucet, lake, river, or stream is associated with \( T. gondii \) infection [56]. A conclusion that is probably true for all places that use a municipal water system, is the closer animals, particularly cats, are to human residents and the less space there is for the discarding of animal waste in urban and suburban locations, the higher the risk of water contamination with \( T. gondii \) oocysts [56].

Foodborne Related Outbreaks

As Demar et. al. so eloquently stated, significant routes of infection will differ among human populations as it depends on social culture, eating habits, and environmental factors [2, 54]. This
is supported by the estimates given for the *T. gondii* seroprevalence in the human population; there is great variation among different countries, geographic regions within a country and among different ethnic groups living in the same vicinity [2]. The reports above highlight the important role environmental factors play in transmitting the parasite to humans. There have also been a few documented outbreaks of toxoplasmosis caused by the consumption of raw/undercooked and contaminated meat. These outbreaks give insight on the eating habits and social culture of the affected group. There were two outbreaks in Korea due to ingestion of raw pork. During the first outbreak, which occurred in September of 1994, three of six people became sick after consuming raw boar viscera and pork [57]. After a month, the first patient went blind and was diagnosed with focal chorioretinitis. With the aid of chemotherapy, his vision was restored. The other two patients were diagnosed with toxoplasmic chorioretinitis. However, due to macular scarring, their blindness became permanent [57]. The second outbreak in Korea took place in Kangwha Gun, Kyunggi Do amongst five soldiers. All soldiers consumed raw liver and uncooked meat from a domestic pig. Many enlarged lymph nodes were noted among patients and toxoplasmic lymphadenitis was surmised [57]. Another food related outbreak involving seven family members, with five being symptomatic, occurred in June 1975. The most common symptoms included fatigue, malaise, fever, sore throat and headache [58]. Less common symptoms included splenomegaly, hepatomegaly, rash, and pharyngitis. Symptom manifestations became evident between seven and eighteen days [58]. One family member was diagnosed with retinochoroiditis, which took 129 days to develop. In this case, the prominent clinical development was lymphadenopathy in the posterior cervical area. Although not confirmed, the probable source of infection is ingestion of raw lamb meat [58]. It has been noted that lymphadenopathy occurs often among family members involved in common-source
outbreaks [59]. An outbreak, seemingly caused by the ingestion of raw goat’s milk, occurred in June 1978 and infected ten family members [60]. Interestingly, the family owned a large fenced vegetable garden in which cats were present. Statistical analysis revealed an association between infection and unwashed vegetable ingestion as well as an increased risk of infection due to gardening [60]. However, ingestion of raw goat’s milk is the most probable cause because all seropositive patients drank the raw milk, while none of the seronegative did. Of the ten patients, only one developed symptoms. These symptoms included occasional fever, tiredness, headache, decreased vision, right eye pain, and raised lesions on superior retina [60]. Unlike the previously mentioned food related outbreaks, infection was due to the ingestion of tachyzoites and not tissue cysts. This outbreak serves as a reminder as to why it is important to pasteurize milk before drinking it. Lastly, five medical students who did not know each other but ate rare hamburgers at the same place during the same night had acute lymphadenitic toxoplasmosis [61]. The most common symptoms included headache, fever, myalgia, and lymphadenopathy. Other symptoms included enlarged spleen, abdomen soreness, chills, fatigue, atypical lymphocytes, and fleeting erythematous macular rash [61]. As mentioned earlier, the likely cause of infection was ingestion of raw hamburgers. However, the risk of contracting toxoplasmosis via the ingestion of beef is not of major epidemiological concern because cattle are naturally resistant to infection [61, 62]. Hence, it is probable that cross-contamination could have occurred during the grinding process or while mixing other processed meats (i.e. pork or mutton) as filler [61, 62]. The butcher who supplied the meat to the school cafeteria, from which the students were infected, was interrogated. He denied using any other meat as filler and insisted that he only used pork on the same grinder as he uses beef at the end of the day after all beef is processed and grinder is
thoroughly cleaned [61]. Insufficient cleaning of processor can also lead to cross contamination [62].

**Zoonotic Evidence**

The most common source of *T. gondii* infection is currently unknown. To date, a method to clearly establish which stage of the parasite leads to a particular infection does not exist [23]. This makes it difficult to ascertain and compare information pertaining to the source of infection and its pertinence to human infection [23]. Although the exact amount of oocysts necessary to cause human infection is unknown, it is believed that oocysts are very infectious and induce more severe disease in humans when compared to tissue cyst induced infection [6, 55]. Also, the incidence of toxoplasmosis due to varied organ transplants and blood transfusions is uncertain and there is no system of record keeping for such cases [43].

Despite these limitations, some scientists believe that the most popular mode of transmission is via oocysts contamination [15, 62-64], while others contest that consumption of undercooked meat is [33, 58]. However, the overall consensus seems to be that the ingestion of food and water contaminated with oocysts from cat faeces as well as the ingestion of tissue cyst in undercooked infected meat are the main ways that infection is acquired [26, 31, 32, 39, 46, 57].

**Environmental**

Supporting the data from the reported outbreaks, oocysts can be disseminated in the environment via wind, rain, surface water, or harvested feeds [2]. Studies involving the collection of environmental samples from places where humans are likely to be exposed to oocysts have been conducted and reported. These reports provide insight on the role environmental contamination plays in *T. gondii* route transmission. From September 2008- March 2009, 150 soil samples were collected in the vicinity of garbage dumps, children’s playgrounds, parks, and other public areas
in Tehran, Iran [15]. Thirteen of these samples were contaminated with *T. gondii* oocysts. A study was conducted in Egypt to assess the risk of raw vegetables (RW), irrigation water (IW), and other items as sources of *T. gondii* infections during meal preparation [39]. Carrots, cucumbers and lettuce were collected along with 54 corresponding IW samples from irrigation river canals. Seven of the RW and nine of the IW were contaminated with *T. gondii* [39]. These results suggest that irrigation water not only has the potential to contaminate soil, but also the surface of the raw vegetables in the fields that it irrigates. Scientists in Northern Poland conducted a study to evaluate the presence of *T. gondii* oocysts amongst fruit and vegetables in that area. The fruits and vegetables, which consisted of strawberries, carrots, radish and lettuce, were collected from 175 supermarkets and 41 kitchen-gardens [46]. Twenty-one of the 216 samples were found positive. However, none of the contaminated samples were from large stores. The samples that tested positive were collected from small grocers, stands/bazaars, and home gardens [46]. Fresh produce sold in large stores are usually grown in isolated areas, distant from residents, which decreases risk of exposure to cat faeces. There is also a probability that the fruits and vegetables cultivated by large stores are washed before being sold. The process of washing the potentially contaminated produce, could wash the oocyst away [46]. Contrarily, produce from small grocers and farm home gardens are grown close to residents, increasing the risk of exposure with contaminated cat faeces. The presence of felines was actually noted on the premises where samples were collected from farm home gardens [46]. A study directed to observe the occupational risk of *Toxoplasma* infection among workers exposed to unwashed raw fruits and vegetables demonstrated that 15 of the 200 workers were positive for anti-*Toxoplasma* IgG antibodies [65]. Further analysis revealed that seropositivity was related to reflex impairments and being sick; consumption of raw meat, unwashed floors, and inhabiting a home
with soil floors was associated with *Toxoplasma* infection [65]. It was also discovered that those manipulating contaminated soil may accumulate 10-100 oocysts under their fingernails [66]. It is possible that cool and moist fruits and vegetables create perfect conditions for *T. gondii* oocysts to thrive [46].

Marine animals can also be infected by oocysts present in the marine environment [18]. In Inuit, Canada women that tested serologically-positive for *T. gondii* were four times more likely to have eaten seal meat and six times more likely to have eaten seal liver when compared to seronegative women [67]. Consumption of raw oysters, clams, or mussels have been linked to increased risks of infection [68]. Various species of walruses, sea lions, seals, and dolphins in the U.S. and Spain have been found to be serologically-positive for the parasite [69-72]. Congenital transmission of the parasite amongst dolphins has been documented as well [73]. It is likely that oocysts contamination of the water is the cause of a lot of the infections found in marine animals because their diets do not consist of warm-blooded animals [21, 73]. Studies have shown that some herbivores living in the wild have had very elevated seroprevalence due to oocysts ingestion [62]. Oocysts can be spread via earthworms and invertebrates [2]. Roaches can also act as a vehicle of transmission as oocysts can survive in their guts and remain infectious for a maximum of 19 days [2]. Animals raised in confinement for human consumption that are reared on a vegetarian diet, like birds and pigs, can provide insight on the prevalence of oocysts contamination in a particular area if infected [62]. Lastly, the faeces of animal raised for human consumption can also be a source of environmental infection. It has been reported that majority of the approximately 3.6 billion, 228 million, and 408 million mg of faeces excreted from cattle/buffalo, pigs, and sheep/goats, respectively, are used in pasture with scarce treatment to kill or eliminate the presence of pathogens [73]. Even though *T. gondii* oocysts can only be shed
by felines, if cats shed near or in the faeces of field animals whose waste is used to fertilize soil, further contamination can occur. Another fact to keep in perspective is that there are an estimated 60 million domesticated and 40 million feral cats living in the U.S, with other unaccounted felid species present globally [73]. The ability of a minimum of 17 species of feral felines to shed oocysts have been reported [2]. Lastly, it has been noted that the seroprevalence in feral cats is higher than domesticated cats [2].

Meat

The most dominant types of meat consumed in the U.S. are beef, chicken, and pork [62]. When compared to the aforementioned food animals, *T. gondii* was found to be more prevalent in goat, sheep, and swine [6]. The annual per capita consumption of pork and chicken in the U.S. is 22.4kg and 37.2kg, respectively [26, 62]. Surveys have been conducted to determine the prevalence of *T. gondii* infection amongst these animals reared for human consumption. In a survey involving the collection of 2,094 beef, chicken, and pork samples each from 698 retail outlets in 28 cosmopolitan statistical areas that covered 80% of the U.S. population, seven samples of pork containing viable *T. gondii* tissue cysts were found [74]. None of the beef samples were detected positive from bioassay or ELISA, while only 1.4% of the chicken samples were positively detected via ELISA [74]. The survey did show that there was a higher prevalence of positive pork samples in the northeastern region of the U.S. compared to other parts of the country [62]. It is important to note that surveys using retail meats to identify the presence of viable parasites have limitations that hinder them from providing accurate information. A lot of pork and chicken are injected with brine and frozen, which can inactivate tissue cysts [26, 62].

Pigs
A nationwide survey started in 1984 observed 23% of market pigs and 42% of sows seropositive for *Toxoplasma* [62]. A study conducted in a slaughter plant in Iowa, U.S. noted that viable *T. gondii* tissue cysts were sequestered from 17% of 1,000 sow [75]. From market weight pigs in the U.S. area of New England, 92.7% viable tissue cysts were isolated [76]. Age may play an important role in the acquisition of *T. gondii* since it was observed in an Illinois farm, that the infection rate is higher in breeding pigs than in market weight pigs [62]. The United States Department of Agriculture (USDA) developed an initiative, in the form of the National Animal Health Monitoring System (NAHMS), to collect, interpret, and share data pertaining to animal health, and management and productivity in the U.S. domestic livestock populations [62]. In 1990, NAHMS included *T. gondii* in its on-farm serological testing, which was used to monitor the seroprevalence of various pathogens [62]. Based on this survey, there has been a decrease in *T. gondii* seroprevalence amongst sows. In 1990 the seroprevalence amongst sows was 20%, in 1995 it was 15% and it decreased even further to 6% in 2000 [62]. A similar trend was observed among grower/finisher hogs as well. In 1984 the seroprevalence of these hogs was 23%, 3.2% in 1995, 9% in 2000 and 2.6% in 2006 [62]. These drastic declines are indicative of the success of pigs being reared in complete confinement systems. Even with such a low seroprevalence, it is feasible that 2.8 million *T. gondii* infected hogs can enter the U.S. food chain every year [62].

Also, the demand for organically reared pork may jeopardize this trend by increasing the risk of exposure to *T. gondii*. According to the National Organic Program (NOP) requirements, all organically reared animals must have access to pasture, and the same standard applies for produce classified as free-range [62]. A few studies support this theory. Viable *T. gondii* tissue cysts were sequestered from 51/55 free range pigs reared in a farm in New England [76]. A similar study was conducted in a farm in Maryland where 14/48 viable cysts were isolated from
the free range reared pigs [77]. In a comparative study conducted on a farm in North Carolina, 22/34 free-range reared pigs were seropositive while only 3/292 conventionally raised pigs were seropositive [78]. Feral pigs hunted for food are also at increased risk for T. gondii exposure [26]. Hence, for humans that consume them, the chances of infection increase particularly when ingesting them cured [62]. In a cross-sectional study designed to evaluate the prevalence of T. gondii among feral pigs in the U.S., 3,247 pigs were tested from 32 states. Seroprevalence was monitored at 50% in Hawaii [62]. Studies conducted in Georgia and North Carolina revealed 18% and 34% seropositivity, respectively [62, 79]. Another study that tested 621 conventionally raised pigs found no seropositive animals compared to the 38 of 1295 pigs that were exposed to pasture via “unconventional” management systems [26].

Chickens

Although no viable tissue cysts were isolated from the previously mentioned survey of 2094 chicken samples collected from various retail stores in the U.S., antibodies were found in 1.3% of the juice extracted from the breast meat [74]. Although, chicken meat is often filled with enhancing solutions and cooled to almost freezing or completely frozen, which should kill viable tissue cysts, the aforementioned results reveals the potential risks of infected commercial chickens entering the food chain in the U.S. [26, 62]. With 8.5 billion chickens being killed for human consumption, the risk of human infection could be high [26, 62]. It is highly unlikely that the source of human infection via ingestion of chickens would come from those raised indoors. There is not a lot of data available on the prevalence of T. gondii infection in indoor reared chickens in the U.S. However, a few studies have shown low levels of infection among this group [62]. Free- range chickens have been documented to be infected at high rates. In the U.S., the prevalence of infected free-range chickens vary from 17-100% [62, 80]. Even though T.
*gondii* has been identified from the ovaries and oviducts of naturally infected hens, it is improbable that raw hens’ eggs will transfer the infection as tachyzoites are easily affected by heating and salt concentrations [2, 62, 80]. Lastly, shelled eggs have yet to be infected, and are not a likely source of infection for humans [62].

**Beef**

Beef and/or dairy ingestion is not considered a threat to humans in transmitting the infection because cattle are not a suitable host for the parasite [6]. They are resistant to infection and majority of serological test are not accurate when using cattle sera [62]. Information pertaining to the sensitivity and specificity of the serological diagnosis of *T. gondii* in cattle is very scarce [62]. While cattle can become infected with *T. gondii*, the tissue phases are minimized or reduced to untraceable levels in a matter of weeks or months [62]. Cross-contamination, either via contaminated processing equipment or mixing other meat products that are more prone to infection may cause beef to become infected [61, 62].

**Lambs**

Around 3-3.6 million lambs are killed in the U.S. annually for food, and the per capita consumption of lamb is approximately 0.5kg per year [26, 62]. Compared to other countries, lamb meat is not a prime product in the U.S. Despite this fact, sheep and goat meat are popular among some ethnic groups [62]. The USDA classifies sheep without permanent teeth, usually, less than one year of age, as lamb. Once their teeth are permanent, the lambs are classified as sheep and their meat is either exported as mutton or used to make pet food [62]. Sheep have been observed to have high levels of *T. gondii* infection, which can be due to their continuous exposure to contaminated soil as they are usually reared outdoors [62]. Many studies were conducted to identify the presence of *T. gondii* in sheep in the U.S. Lamb chops collected from
markets in California and isolated for the parasite revealed that two of the 50 samples contained viable tissue cysts [81]. The diaphragms of 86 butchered sheep from a slaughterhouse in Maryland were tested and 8 viable tissue cyst were analyzed [82]. A total of 383 lamb hearts were collected from lamb slaughterhouses located in the mid-Atlantic states of the U.S. Viable tissue cysts were present in 104 of these samples that were available for sale to consumers via retail markets [83]. In a California abattoir, indirect haemagglutination antibodies to *T. gondii* revealed that 8% of 1,056 lambs were positive for the parasite [84]. Five slaughterhouses surveyed in the northern U.S. resulted in the findings of seropositivity among 42% of lambs and 80% of adult sheep [62]. Modified Agglutination Test (MAT) testing of 1,564 mature female sheep from thirty-three farms located in the northwest area of the U.S. showed 65.5% seropositivity [62].

Goats

With more than 850,000 of these animals being slaughtered annually, goats are a staple diet for many immigrant populations in the United States [62]. The hearts of goats reared from farms in Maryland, Virginia, and Pennsylvania were collected from a retail store and analyzed via mice bioassay and MAT [85]. *T. gondii* antibodies were present in 53.4% of the goats [85]. From the 112 hearts that were bioassayed via mice, *Toxoplasma* was sequestered from 29 [85]. As previously reported, raw milk and cheeses made from these animals is a possible source of *T. gondii* infection [26, 60, 62]. It has been proposed that tachyzoites can access the hosts’ circulation or lymphatic system before reaching the stomach via penetration of mucosal tissue [2]. Hence, possibly allowing the transmission of the tachyzoites to milk.

Wild animals hunted for food are also important sources of *T. gondii* infection [26]. A family of hunters became infected while manipulating and removing the viscera of game. In the U.S., bear,
elk, moose, and wild pig are commonly hunted game. Black bears have a very high prevalence of *T. gondii* and venison, such as deer, are not only widely available, but also are common targets for many hunters [26]. Both bioassay and elevated seroprevalence results, ranging from 30-60%, indicate that the ingestion of white-tailed deer pose a risk of transmission of *T. gondii* to humans [26].

**Preventative Measures**

**Cooking**

According to the USDA, slabs of pork, lamb, veal, and beef should be cooked at temperatures between 145°F (62.7°C) and 160°F (71.1°C) with a resting time of 3 minutes [26, 62]. To ensure that these temperatures are reached, it is imperative that a food thermometer be placed in the thickest area of the meat [26, 32]. Poultry, ground meat, and undomesticated game meat should be cooked at 165°F (73.9°C) and 160°F (71.1°C) or higher, respectively [26]. Consumer compliance to these recommendations is crucial. In an experiment involving cooking ground pork infected with bradyzoites, tissue cysts still remained viable after being cooked at 147°F (64°C) for 3 minutes [26, 62]. A survey revealed that 24% of pork samples prepared by consumers were cooked at temperatures below 145°F [62]. Another survey revealed that deep frying pork contaminated with tissue cyst for five minutes did not kill the parasite, while cooking in a frying pan for less time (3 minutes) did [61]. Although salt and sugar solutions can kill tissue cysts, its viability is determined by the concentration of the solution and temperature of storage [2, 32]. A study using 3% table salt on tissue cysts showed it took up to a week for cysts to die [2]. Experimentally, tissue cysts were killed using 6% NaCl solution at 4-20°C but lasted several weeks at lower concentrations [2]. Lastly, microwave cooking is not a reliable source for inactivating *T. gondii* because it heats food unevenly [2, 26].
The way in which consumers prepare and wash foods can also prevent infection and cross-contamination. When handling infected raw meat, it is possible for cross-contamination to occur from hand to mouth or via open lesions on one's hands [39]. Wearing gloves can prevent transmission via this route. A recent study showed that the percentage of women who rarely use gloves when manipulating raw meat had a higher seropositivity when compared to those who wear them often [39]. Washing hands and preliminary cooking appliances with hot water containing detergent before and after use with raw meat, poultry, seafood and unwashed fruits and vegetables can also prevent cross-contamination [2, 26, 32]. A lethal *T. gondii* infection was acquired via a brutal stabbing with a butchers knife [39]. Fruits and vegetables should be washed intensively or peeled before eaten [26]. It was demonstrated that soaking lettuce in water at 45°C for one minute successfully inactivated *T. gondii* sporulated oocysts without affecting sensory characteristics [39]. As long as meats are at an inherent temperature of -12°C or -13°C, *T. gondii* tissue cysts will not survive [26, 32]. In a study aimed at observing the prevalence of *T. gondii* in fresh buffalo meat (FBM) compared to imported frozen buffalo meat (IFBM), the prevalence among FBM was 15.4% and IFM was 0% [39].

**Production Practices**

Cats and rodents should be segregated from animal production regions since contamination of hay, straw, and grain with cat faeces can be a source of infection for livestock [2, 26, 32]. *Toxoplasma gondii* infections were nearly non-existent on small islands where no cats inhabited [2]. Australian native marsupials are very vulnerable to infection because cats were only recently introduced to their habitat a few hundred years ago [2]. The seroprevalence of *T. gondii* in 1,264 pigs living on a remote island in Ossabaw, Georgia was significantly lower than the 170 feral pigs in the mainland of Georgia - 0.9% and 18-20%, respectively [2]. It has also been proposed
that animals raised for human consumption should only be fed sterilized foods and kept confined throughout their lifetime [2]. Also, traps, baits, poison, and extermination of rodents via certified experts can be used to decrease *T. gondii* prevalence in farms [62]. Sheep should be vaccinated with the live cyst-free vaccine which provides immunity for up to 18 months and reduces sheep abortion [18, 26]. Although, more practical for pet owners, there is an oral live vaccine available that prevents cats from shedding oocysts [22, 26, 86]. When cats on an Illinois farm were orally vaccinated, the seroprevalence of pigs and mice drastically decreased [86]. Clean and treated water should be used for all aspects of animal production [26]. Tissue cysts in meat can be inactivated via irradiation and high pressure processing at 0.4- 0.7kGy and 300-400 MPa [2, 18, 26, 32].

**Consumer/Pet Owner Practices**

The previously mentioned live oral vaccine would be advantageous for pet owners to treat their cats as it inhibits the cat from shedding oocysts [2, 26]. However, this vaccine is no longer commercially available because it was expensive, unpopular among pet owners, and has a short shelf-life [26]. Pet owners should change cat litter and litter boxes daily and clean them with a mixture of water above 70°C and detergent using gloves [2, 26]. However, pregnant women and immune-compromised individuals should avoid contact with litter [2, 26]. Pet cats should be fed dry, canned, or cooked food [2]. Contingent upon the types of food eaten and level of confinement, *T. gondii* can be detected, serologically in 74% of adult cat populations [2]. Pet cats, ranging from 9-46%, in Europe, South America, and the U.S. revealed serological evidence of previous exposure [2]. It is also recommended that owners get their pets serologically examined for *T. gondii* infection [2]. Stray and/or feral cats should not be adopted or held [26]. Consumers should refrain from eating raw oyster, mussels, and clams; ingesting unpasteurized
goats’ milk and untreated water, particularly in developing countries, should also be avoided [26]. Pastoral camels in Sudan have a high seroprevalence of *T. gondii* putting nomads who consume raw camel milk at high risk for infection [2].

Hunters should bury the viscera of the animals they prey to decrease the risks of cats ingesting them and becoming infected [2, 26]. Sandboxes should also be covered when not in use [26].

**Government/Organization Regulations**

The World Health Organization (WHO) has urged for strategies for the surveillance of toxoplasmosis in humans and for the gathering of explicit epidemiological information on *T. gondii* [2]. Although not standardized, some states in the U.S. and European countries have implemented screening programs designed for early detection of primary maternal infection with the parasite during pregnancies [2]. These screenings are mandatory in Austria and France [2]. A system of record keeping can be developed for monitoring toxoplasmosis via organ transplants and transfusions. Establishing methods to distinguish oocyst-initiated infections from those initiated by tissue cysts is of grave epidemiological importance [2, 23]. Theoretically, antigens specific only to oocysts paired with their corresponding antibodies should serve as a marker to distinguish/confirm the route of infection [23]. Data from these type of tests could be used to estimate the most dominate route of infection in humans and monitor the differences in the severity of symptoms/disease from each type of infection, if any are present. Further understanding of the genetic factors that cause *T. gondii* virulence and the methods in which genotypes are selected depending on host species could aid in the development of medications, vaccines, and other form of therapies designed to terminate transmission or cure toxoplasmosis [16, 23].
Local and national monitoring programs aimed at preventing the contamination of marine animals, wildlife, and humans via recreational activities could also be developed [73]. Recently, in efforts to prevent contamination of the ocean, a law was passed in California that mandated all cat litters sold had to contain a warning label informing owners not to rid of cat faeces in toilets or outside environments [22]. It would also be beneficial to have a tracking system for pigs entering the food chain and regulations that demand pork be processed extensively to inactivate the parasite needs to be created as well [62]. The government could also aid in disseminating information about *T. gondii* and how to prevent infection to the public [26].

**Methods of Detection**

**Serological**

Various serological methodologies are used to determine the seroprevalence of *T. gondii* in a given population. The Sabin – Feldman dye test, which is still deemed as the gold standard for the identification of antibodies to *T. gondii* in humans, is the first test system created to reveal distinct antibodies to *T. gondii* at abate levels and to distinguish acute from latent infections [2, 87]. Detection of IgM, IgA, and IgG antibodies allows for early and late detection of *T. gondii* infection in human and animal sera [87]. IgG can remain present for decades while IgM usually stays for approximately 6-9 months [32]. The majority of cats with measurable levels of IgG antibodies to *T. gondii* are probably immune and won’t produce oocysts soon [2]. The C2 active factor in human serum allows for detection [88]. This test uses complement-mediated osmotic lysis of antibody-coated live *T. gondii* tachyzoites [87]. These coated tachyzoites are identified by their incapacity to absorb methylene blue [87]. Although this test is very sensitive and specific with no possibility for false results among humans, it is labor intensive and requires a continual supply of live *T. gondii* animals [2, 18]. Test detecting IgM antibodies are important
for diagnosing congenital toxoplasmosis, because unlike IgG antibodies, IgM antibodies do not traverse the placenta [18, 43]. The indirect fluorescent test antibody test and the ELISA were customized to identify IgM in cord blood [18, 43]. Direct agglutination tests (DAT), which uses the entire killed organism and a specific antigen are also used for the detection of *Toxoplasma* [18, 43, 88] The DAT for *Toxoplasma*, although simple and easy to use, lacks sensitivity and specificity [88]. Later, the IgM-ELISA was incorporated with the agglutination test. This modified agglutination test (MAT) removed the requirement of an enzyme conjugate and increased the sensitivity and specificity of the test [18, 43, 88]. MAT is also used frequently for the detection of toxoplasmosis in animals [18, 43]. Latex agglutination tests are also used [32]. It should be noted that although useful, these methods are not standardized and they differ in sensitivity, specificity, and prognosticative values. Hence, it is difficult to replicate results as no two test generate the same data in every case [2].

**Molecular**

Other useful tests for the identification of *Toxoplasma* in humans, animals, and the environment are Polymerase Chain Reactions (PCR) and PCR-based molecular methods as they tend to be less expensive and more sensitive than older methods [4, 18, 32, 43]. Commonly used PCR assays for the detection of *T. gondii* include traditional PCR, nested PCR (n-PCR), and quantitative real-time PCR of repetitive DNA sequences [4]. Nested PCR allows for a higher sensitivity than the conventional method because it is likely that more of the desired fragment would be produced when a smaller sequence is used [4, 13]. The q-PCR which has a higher sensitivity than n-PCR assays, is favorable because it can approximate the potency of the infection after drug treatment, and detect minimal infection levels [4]. Lastly, a PCR-based molecular method that is quite popular is PCR-Restriction Fragment Length Polymorphism
(PCR-RFLP). Restriction endonucleases are used to identify single nucleotide polymorphisms (SNPs), cleave PCR products, and reveal informative bands via gel electrophoresis. PCR-RFLP aids in genotyping *T. gondii* isolates, which is of major epidemiological importance [4, 46]. These test have been created using various gene targets [18, 43]. There are a variety of gene targets available for use, including B1, REP, SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico, ROP1, 62, 850, and L328 [3, 4, 12, 13, 15, 25, 31, 42, 46, 89]. The B1, SAG1, and SAG2 genes are commonly used for detection [13]. The highly specific, 35-fold repetitive B1 gene is often used in clinical applications as well as environmental [4, 46, 90, 91]. This gene was also found to be present amongst many strains of the parasite as well [91].

Proteins P22 and P30, which are encoded by SAG2 and SAG1, respectively, are essential to the mode of operations among tachyzoites invading the host cell [25]. Surface antigen P22 has been used for serological diagnosis and vaccine formulation [92]. Although the aforementioned markers can be used for the detection of *T. gondii*, SAG2 is unique in that it can be used for detection as well as genotyping [4, 13, 25]. This is possible because it encodes two distinct forms of P22 that are distinguished by strain-specific monoclonal antibodies [13]. In the coding region, type I and III strains are the same, whereas, type II has a different form [13, 25]. Restriction enzymes *Sau3AI*, *HhaI*, and *CfoI* are used to distinguish genotypes with nested PCR products [13, 25, 31, 90]. These products, which are designed to amplify the 5’ (241-bp) and/or 3’ (221-bp) ends of the SAG2 locus, will be cleaved by *Sau3AI* to detect type III strains or cleaved by either *HhaI* or *CfoI* to detect type II [4, 13, 25, 31, 42, 46]. There are several epidemiological studies that have employed this method [13, 15, 31, 42, 46]. Recent studies, designed in Iran and Poland, observed the presence of *T. gondii* in soil samples and fruits and vegetables using the B1 gene for detection and the SAG2 locus for genotyping [15, 46, 90]. The study conducted in Iran
revealed that of the 13 positive soil samples collected, 9 were type III, three were a mixture of type I and type III and one was type one [15]. Researchers in Poland discovered that 18 of the 101 soil samples collected from sand-pits, farming areas, and waste disposals tested positive for *T. gondii*; five of the eighteen samples were identified as type I and two more were identified as type II [90]. The same group of researchers discovered that 21 of the 216 fruit and vegetable samples gathered from household gardens and shops were positive, while eight of them were classified as type II and two of them as type I [46]. Studies designed to observe the prevalence of *T. gondii* in meat products collected from commercial markets overseas have targeted the SAG2 locus for detection and genotyping. Meat samples totaling 164 were collected in East Azerbaijan Province, near Iran. The data revealed that 52 of the samples were amplified by the 5’ flanking region whereas 65 were amplified by the 3’ flanking region, and 40 of which all were genotyped as type I were amplified by both regions [42]. Hence 154 of 157 were positive, with only 40 of them being eligible for genotyping via PCR-RFLP. Collection of 71 meat samples in the UK revealed that 27 of them were contaminated with *T. gondii* – 21 of the samples were identified as type I and 6 were shown to contain both type 1 and type II genotypes [31]. Two disadvantages to this method are that atypical isolates won’t be identified and both 3’ and 5’ fragments have to be amplified in order for restriction analysis to occur [25, 42, 46]. Expressed Sequence Tags (ESTs) can also be used to characterize genes [93].

**Virulence**

*Toxoplasma* virulence has been measured by the LD$_{100}$ in mice. Type I strains are more virulent in mice with an LD$_{100}$ equivalent to 1 compared to types II and III with and LD $\geq 10^3$ [3, 4, 23]. The same outcome may not be the same for humans as virulence is circumstantial and a strain that induces severe disease in one host may have a different effect on another [23]. A study
conducted with free-range chickens revealed that *T. gondii* isolates present in asymptomatic chickens were virulent in mice [18]. However type I strains have been associated with causing severe toxoplasmic retinochoroiditis among patients, and severe ocular and congenital toxoplasmosis and severe acute disseminated toxoplasmosis in immune-competent individuals [3, 4, 16, 23, 33]. It has been observed that most cases of human toxoplasmosis are correlated with strains of the type II genotype [2, 16, 23]. Many studies have noted that there is an overwhelming presence of type II strains in AIDS and congenital infection [16, 23]. Type II strains are frequently affiliated with reactivation of chronic infections, which is responsible for 65% of cases in AIDS patients [33]. The popularity of these type II strains could be an accurate representation of their ability to cause disease in humans or it could be inaccurate and merely a reflection of infection via animals reared for human consumption as studies in France and the USA reveal that isolates from majority of these infected animals are associated with type II strains [23]. As previously mentioned, many people infected with *T. gondii* are asymptomatic. The strain or strains that are accountable for the lack of symptoms is unknown [23]. However, the severity of infection is not only contingent upon parasite strain, but also host-immunity, genetic background, and parasite specifics including inoculum size and parasite life stage [42]. Based on a conglomerate of information from various reports, it has been concluded that the incubation period for systemic symptoms from common-source outbreaks ranges from 7-38 days [60]. Type I strains have also been observed to propagate in human foreskin fibroblasts approximately a third faster than types II or III [23]. Factors such as growth rate and motility can also have an effect on the virulence of the parasite in a host. Some strains may have a shorter doubling time, an advanced resistance to the hosts’ immune system, or may be less prone to bradyzoite conversion than others [16]. It has been postulated that Type I strains of the parasite
may be able to spread more effectively to new cells than types II or III because extracellular type I strains stay infectious for a longer period of time compared to the other two strain types [16]. Lastly, various factors affecting the efficacy of migration may aid in the propagation and entry to immune-privileged areas within the human host in which the parasite is often discovered to be proliferating in [16].
References


Chapter 2

DETECTION OF *TOXOPLASMA GONDII* IN FRESH PRODUCE

*Toxoplasma gondii* is an intracellular obligate protozoan parasite that causes toxoplasmosis in humans [12]. *Toxoplasma* has been identified in various organisms and is likely able to infect all warm-blooded animals and humans however [2-8]. *Toxoplasma gondii* is the only species and felids are its only definitive host [22]. Infections caused by this parasite are a major cause of morbidity and mortality in immunocompromised individuals, particularly AIDS patients and infants who are congenitally infected [26-31, 35]. Approximately one-third of the human population is infected with the parasite [2, 32, 33, 42]. It has three clonal lineages, classified as type I, type II, and type III, with type II strains dominating human infections in the U.S. [4, 12, 16]. *Toxoplasma gondii* is one of the most important foodborne pathogens during pregnancy as infection can be fatal to the fetus or cause long-term disease after birth [32]. Although there are many routes of infection for the parasite - (1) horizontally via oral digestion of sporulated oocysts from the environment, (2) horizontally via oral digestion of tissue cysts present in raw/undercooked meat or viscera of the intermediate host, or (3) vertically, via the transplacental transmission of tachyzoites, the illness this parasite causes is classified as the leading cause of death associated with foodborne illness in the United States [33, 34]. The most common source of *T. gondii* infection is currently unknown. It is believed that oocysts infections are very infective and induce more disease in humans when compared to infections caused by other stages of the parasite [6, 23, 55]. Over the years, there have been a few reported outbreaks and surveys
pertaining to oocysts contamination in the environment and its relation to human infection [15, 39, 46, 50, 52-56, 65].

This study seeks to understand the role *Toxoplasma gondii* plays environmentally in the occurrence of fresh produce in the United States and Peru and to distinguish the genotype of the identified parasites.

**Materials and Methods**

**Oocysts and DNA Isolation**

Collectively, 818 fresh produce samples including basil, black mint (limited to Peru), cilantro, green onion (limited to the U.S), lettuce, mesclun (limited to the US) parsley and spinach (limited to the US) were collected from 16 markets in Atlanta, GA and 5 markets in Lima, Peru. Vegetables were washed with an elution buffer consisting of 10mls of Laureth 12 solution, 10mls of 1M Tris Buffer pH 7.4 and 2ml EDTA pH 8.0, brought to a final volume of 1000 ml and 150µl of Antifoam A was added in individual labeled stomacher bags, 25 grams of each sample was added along with 100ml of elution buffer. These bags were then closed and allowed to rock on a rocking platform for 15 minutes on each side. The liquid was transferred to 50ml conical tubes and centrifuged at 3,000 rpm for 20 minutes. Pellets were disassociated and transferred/combined in corresponding 15ml conical tubes. Once again they were centrifuged for 3,000 rpm for 20 minutes. Most of the supernatant was aspirated using a vacuum flask and transferred to their respective 1.5ml microcentrifuge tubes. DNA was extracted from samples using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Solon, OH) following the manufacturer’s instructions.

**DNA Detection**
Preliminary tests were conducted to distinguish the best marker for *T. gondii* DNA detection amongst samples. A small amount of DNA from each of the vegetable types collected from the U.S. (Table 6) were spiked with *T. gondii* positive control DNA and screened for positivity using the following markers: SAG2, BTuB, GRA6, SAG3, and HOMA (Table 1). These markers were selected and ran based on work published by Su et. al [3]. None of the markers used were specific enough to detect *Toxoplasma* DNA (Figures 1-4). The nested SAG 2 markers (Table 2) were deemed to be the best candidate for detection as it was the most commonly used marker in the detection of *Toxoplasma* DNA in environmental samples [15, 46, 90]. Samples were then pooled according to vegetable type in groups of five, from which the DNA was amplified in triplicate. Pooled samples that amplified by PCR, were then tested individually in triplicate.

PCR amplification using external primers was conducted in a total volume of 15µl containing 5X PCR buffer, 2mM MgCl₂, 200 µM of each of the dNTPs, 30ng of designated primers, 0.15 units of Taq polymerase and 1.00µl of DNA sample. Similarly, internal amplification using nested primers was done with the same reagents with a final volume of 50µl. Samples were amplified using a Master Cycler Gradient 5331 (Eppendorff, Hamburg Germany) under the following cycling conditions: 5min at 94°C, 30sec at 94°C, 30sec at 60°C, 30sec at 72°C and a 7min incubation at 72°C after the 35th cycle. Products were observed in 2% agarose gels. The PCR product from the samples deemed positive based on the amplification of the 241bp SAG2 5’ end or the 221bp SAG2 3’ were purified and extracted from the gels using the QIAquick® Gel Extraction Kit and the QIAquick® PCR Purification Kit from Qiagen (Valencia, CA) and sent to Macrogen USA to be sequenced.

**Results**
Collectively, 818 samples were tested from twelve markets in Georgia, USA and 5 in Lima, Peru. A total of twelve were confirmed positive via sequencing. The sequences obtained from the 12 isolates, all were homologous to *Toxoplasma*. One of them had a 1 base substitution.

**Peru**

Of the 501 samples tested from Peru, thirteen were shown to be positive via nested PCR. Twelve of these samples were sent for sequencing to confirm positivity. From the twelve sent, only four were confirmed positive. Half of the positive samples were derived from black mint, and the other half from lettuce (Table 3). Both black mint samples and one of the lettuce samples were collected from the same market (Market A) while the other positive lettuce sample was derived from a different market (Market C) (Table 4). The majority of the positive samples (n=2) were collected during November with equivalent positive samples being collected in June (n=1) and August (Table 4). Lastly, all samples confirmed positive by sequencing were from the SAG 2 3’ end.

**Georgia, United States**

From the 317 U.S. samples tested, nested PCR revealed 10 of them to be positive. All ten were sent for sequencing and eight were confirmed positive. Of these eight, four were lettuce, 2 were parsley, 1 was green onion, and the last positive sample was spinach (Table 7). The most predominate markets from which positive fresh produce was identified was collected from a Market C and Market N (Table 6). All four of the positive lettuce samples were derived from different markets (Table 6). There was one positive sample collected from each of markets A, F, K, and L (Table 6). The majority of the positive samples were collected in March, with 2 more
collected in December and 1 in June (Table 8). All samples confirmed positive were done so via analysis of the SAG 2 3’ end of the nested PCR.

<table>
<thead>
<tr>
<th>Table 1. Markers Used for Preliminary Screening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>SAG2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>BTUB</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>GRA6</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>SAG3</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Figure 1 Amplification of spiked U.S. vegetable samples targeting the BTUB gene

Non-specific binding was present as well as high background when using the BTUB marker for *Toxoplasma* DNA detection in vegetable samples.
Figure 2. Amplification of spiked U.S. vegetable samples targeting the GRA6 gene.

Although there is no background, non-specific binding is evident.

Figure 3. Amplification of spiked vegetable samples targeting the SAG3 gene.

No *Toxoplasma* DNA was amplified using the SAG 3 marker.
Table 2. PCR Nested SAG 2 primers. This is another PCR system targeting the SAG2 gene. These primers amplify the 3’ and 5’ segments of the SAG2 gene.

<table>
<thead>
<tr>
<th>5’ SAG2 primary</th>
<th>SAG2 F4</th>
<th>5’-GCTACCTCGAACAGGAACAC-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAG2 R4</td>
<td>5’-GCATCAACAGTCTTCTGTTGC-3’</td>
<td></td>
</tr>
<tr>
<td>3’ SAG2 primary</td>
<td>SAG2 F3</td>
<td>5’-TCTGTCTCCGAAGTGACTCC-3’</td>
</tr>
<tr>
<td>SAG2 R3</td>
<td>5’-TCAAAGCGTGATTATCGC-3’</td>
<td></td>
</tr>
<tr>
<td>5’ SAG2 secondary</td>
<td>SAG 2 F</td>
<td>5’-GAAATGTTTCAGGTTTGCTGC-3’</td>
</tr>
<tr>
<td>SAG2 R2</td>
<td>5’-GCAAGAGCGAAGCTGACAC-3’</td>
<td></td>
</tr>
<tr>
<td>3’ SAG2 secondary</td>
<td>SAG2 F2</td>
<td>5’-ATTCTCATGCCTCGCTTC-3’</td>
</tr>
<tr>
<td>SAG2R</td>
<td>5’-AACGTTTCACGAAGGCACAC-3’</td>
<td></td>
</tr>
</tbody>
</table>
This assay was selected as it amplified *Toxoplasma* best with fresh produce. These set of primers were used throughout the rest of the study.

### Table 3. *Toxoplasma* distribution by market – Peru

<table>
<thead>
<tr>
<th>Market</th>
<th># Samples</th>
<th>3'</th>
<th>%3'</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>125</td>
<td>3</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>B</td>
<td>76</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>51</td>
<td>1</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>D</td>
<td>125</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>124</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>501</strong></td>
<td><strong>4</strong></td>
<td><strong>0.79</strong></td>
<td><strong>0.79</strong></td>
</tr>
</tbody>
</table>

Toxoplasma DNA was detected in only 2/5 markets with Market A having the most positive samples followed by Market C.

### Table 4. *Toxoplasma* distribution by vegetable – Peru

<table>
<thead>
<tr>
<th>Vegetable</th>
<th># Samples</th>
<th>3'</th>
<th>%3'</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basil</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cilantro</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>black mint</td>
<td>101</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Lettuce</td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Parsley</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>501</strong></td>
<td><strong>4</strong></td>
<td><strong>0.79</strong></td>
<td><strong>0.79</strong></td>
</tr>
</tbody>
</table>
Equal amount of *Toxoplasma* samples was detected among black mint and lettuce samples. Hence only 2 out of 5 collected vegetable types were contaminated.

**Table 5. Toxoplasma distribution by month-Peru**

<table>
<thead>
<tr>
<th>Month</th>
<th># Samples</th>
<th>3'</th>
<th>%3'</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>December</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>May</td>
<td>101</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>August</td>
<td>100</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>November</td>
<td>99</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>March</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>June</td>
<td>100</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>501</strong></td>
<td><strong>4</strong></td>
<td><strong>0.79</strong></td>
<td><strong>0.79</strong></td>
</tr>
</tbody>
</table>

All positive samples were collected during the winter months.

**Table 6. Toxoplasma distribution by market-U.S.**

<table>
<thead>
<tr>
<th>Market</th>
<th># Samples</th>
<th>3'</th>
<th>%3'</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22</td>
<td>1</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>28</td>
<td>2</td>
<td>7.1</td>
<td>7.1</td>
</tr>
<tr>
<td>D</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>14</td>
<td>0</td>
<td>7.1</td>
<td>7.1</td>
</tr>
<tr>
<td>G</td>
<td>16</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>J</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K</td>
<td>29</td>
<td>1</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>L</td>
<td>27</td>
<td>1</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>M</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N</td>
<td>23</td>
<td>2</td>
<td>8.7</td>
<td>8.7</td>
</tr>
<tr>
<td>O</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>317</strong></td>
<td><strong>8</strong></td>
<td><strong>2.5</strong></td>
<td><strong>2.5</strong></td>
</tr>
</tbody>
</table>

The most predominant markets from which fresh positive produce was identified was
collected from Market C, a farmer's market and Market N, which is a supermarket

Table 7. *Toxoplasma* distribution by vegetable type- U.S.

<table>
<thead>
<tr>
<th>Vegetable</th>
<th># Samples</th>
<th>3'</th>
<th>%3'</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basil</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cilantro</td>
<td>46</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Green Onion</td>
<td>44</td>
<td>1</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Lettuce</td>
<td>111</td>
<td>4</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Parsley</td>
<td>47</td>
<td>2</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Spinach</td>
<td>36</td>
<td>1</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>317</strong></td>
<td><strong>8</strong></td>
<td><strong>2.5</strong></td>
<td><strong>2.5</strong></td>
</tr>
</tbody>
</table>

Four of six vegetable samples tested positive. In terms of percentage, parsley had the highest positivity, followed by lettuce, spinach, and green onion.

Table 8. *Toxoplasma* distribution by month- U.S.

<table>
<thead>
<tr>
<th>Month</th>
<th># Samples</th>
<th>3'</th>
<th>%3'</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>July</td>
<td>59</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>December</td>
<td>64</td>
<td>2</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>March</td>
<td>94</td>
<td>5</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>June</td>
<td>100</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>317</strong></td>
<td><strong>8</strong></td>
<td><strong>2.5</strong></td>
<td><strong>2.5</strong></td>
</tr>
</tbody>
</table>

Majority of positive samples were collected during Spring months. However two samples were collected during the winter.

**Discussion**

Interestingly, half of the positive samples collected from Georgia, U.S. and Lima, Peru were lettuces. This supports data that chilled vegetables with high water content provide prime conditions for *T. gondii* oocysts survival [46]. The data also supports that seasonality influences oocyst survival [2]. Even though the four lettuce samples found positive in the U.S. were
collected from different markets, they were all gathered during months when rain was frequent and temperatures ranged from 24.5 to 26°C [94]. Furthermore, of the eight positive U.S. samples, a total of five were collected during these months. Positive samples from Peru were all gathered during months in which misty rains and temperatures ranging from 13 to 26°C were common [95]. Climate conditions in Lima, Peru were similar to those of Georgia, U.S. during the time majority of the samples were collected. Samples collected from farmers’ markets in the U.S. consisted of half of the total positive samples collected. The most predominate markets from which positive fresh produce was identified was collected from a farmers market (Market C) and a super market (Market N). Two of the four positive lettuce samples were derived from two different farmers markets and the other half from two different super markets. Even though the number of positive samples collected from farmers market in the U.S. were equivalent to those collected in super markets, the data still supports that there is a risk of exposure to T. gondii when purchasing produce from the farmers market. However, these results do not coincide with findings from Lass et. al. in which no T. gondii DNA was detected in produce collected from supermarkets [46]. Lettuce was tested in their study as well, and 18% (n=50) of the samples collected from shops, bazaars and gardens were positive.

It is also possible that there were more positive samples, but due to inhibition they could not be amplified. Initially, Toxoplasma DNA was amplified from 19 U.S. samples. However, upon re-extraction from 9 samples of the stock, no DNA was amplified. The plant matrices may have interfered with successful recovery of oocysts from the samples. Lass et. al. also noted inhibition among samples [46]. It is also possible that there could have been a very low number of oocysts present on samples upon collection and initial wash.
Chapter 3

CONCLUSION

The detection of *T. gondii* in fresh produce indicates that the consumption of raw unwashed vegetables may play a role in the prevalence of toxoplasmosis in both countries, particularly the U.S. Although fewer samples were tested in Georgia, U.S., there was a greater percentage of positivity among samples in the U.S. The data suggests that the risk of exposure to contaminated fresh produce purchased in farmers’ markets is just as high as those purchased from supermarkets. This emphasizes the importance of segregating cats from where fresh produce are grown and sold. The development of therapies that prevent cats from shedding oocysts may greatly decrease the risk of infection among fresh produce and other environmental elements. Rodent segregation from cats should also help decrease risk of oocysts infection by preventing the cat from ingesting infected prey [26].

Unfortunately, this study was not able to differentiate between the common *Toxoplasma* assemblages. The lack of the restriction site used to differentiate among the three assemblages when using RFLP was absent in these isolates, suggesting that they could either be assemblage I or III. Further tests are needed to further characterize them. Hence, other assays with increased sensitivity and specificity need to be developed for the genotyping of *T. gondii* oocysts from environmental samples.