ABSTRACT

Title of Document: EVALUATION AND MODELING OF FOOD SAFETY RISK FACTORS ASSOCIATED WITH *TOXOPLASMA GONDII* INFECTION IN THE FARM-TO-FORK FRAMEWORK

Surabhi Rani, Doctor of Philosophy, 2020

Directed By: Abani K. Pradhan, Associate Professor, Department of Nutrition and Food Science

Toxoplasma gondii is a widespread zoonotic parasite with a high seroprevalence in the human population and the ability to infect almost all warm blooded animals. Animal meat may contain viable *T. gondii* tissue cysts that can potentially cause infection if undercooked meat is consumed. The goal of this research is to estimate *T. gondii* distribution in animal meats by integrating experimental data with predictive modeling and statistical analyses to better understand the ecology of *T. gondii* infection and further evaluate mitigation methods to reduce the public health burden of toxoplasmosis.

To understand the infectivity and transmission of *T. gondii* from the environment to animals and thereafter humans, the formation and distribution of *T. gondii* tissue cysts was estimated in varying sizes (5 g, 10 g and 50 g) of animal muscle tissues. Experimentally and

naturally infected pigs, lambs and goats were evaluated. The sensitivity and specificity of different diagnostic tests for detecting *T. gondii* were also evaluated using logistic regression modeling and meta-analysis. Bootstrap and Gibbs statistical sampling techniques were used to assess the complete inactivation of *T. gondii* in pork through cooking and freezing. Dynamic compartmental modeling was used to simulate one year on a hypothetical pig farm to understand *T. gondii* infection transmission via the environment and in multiple hosts such as cats, rats, pigs and humans. Further, this modeled analyzed some of the dynamical behaviors of the *T. gondii* infection in the definite (cat) and intermediate (e.g. rat, pig and human) host populations.

The results suggested that *T. gondii* tissue cysts can develop as early as 7 days after infection in experimentally infected pigs and are unevenly distributed in the muscle tissues of naturally infected lambs and goats based on bioassay in mice. Meat samples as small as 5 g have the potential to cause *T. gondii* infection if consumed raw or undercooked. The regression model predicted varying specificity and sensitivity for different sized meat samples with the highest sensitivity and lowest specificity for the largest samples (50 g). *T. gondii* tissue cysts in fresh pork were completely inactivated at or above 64°C (147.2°F) and below -18°C (0°F). Tissue cysts can remain viable in fresh meat for up to 30 days stored at 4°C (39°F). With the calculated predation rate of the hosts and the transmission rate of infection from environment, the *T. gondii* infection is expected to persist ($R_0 > 1$) in all hosts over the simulation run of one year. This dissertation evaluated the *T. gondii* infection flow in different hosts, assessed mitigation strategies for food safety risks and estimated the distribution of the parasite in fresh cut meats of food animals.

Evaluation and Modeling of Food Safety Risk Factors Associated with *Toxoplasma gondii* Infection in the Farm-to-Fork Framework

By

Surabhi Rani

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2020

Advisory committee:

Dr. Abani K. Pradhan, Chair

Dr. Jitender P. Dubey

Dr. Byung-Eun Kim

Dr. Seong-Ho Lee

Dr. Rohan Tikekar

Acknowledgements

Many people have provided their valuable contribution, support and guidance to successfully finishing this dissertation. Firstly, I would like to thank my advisor, Dr. Abani K. Pradhan who kindly accepted me for this project, supervised and mentored my work and gave me advice to overcome obstacles throughout my thesis work. His advice was very useful and much appreciated. He nurtured me, recommended me to great research opportunities and constantly guided me to be a good researcher.

I would like to thank Dr. Jitender P. Dubey for his kindness and assistance during my laboratory experiments at the Animal Parasitic Diseases Laboratory (APDL) at US Department of Agriculture, Agriculture Research Services (USDA, ARS) and also for serving as a member on my PhD advisory committee. I learned a lot while working in the lab. My thanks go to Camila Ceizer, Fernando Murata, Oliver Kowk and Meghan Sadler for their constant support and help in my experiments. Especially for their generosity, kindness and their welcoming smiles. I would also extend my sincere thanks to other members of my PhD committee, Dr. Rohan Tikekar, Dr. Seong-Ho Lee and Dr. Byung-Eun Kim for always being ready to extend their support and scientific advice on my research.

I am grateful to all the current and past members of Dr. Pradhan's lab for their continuous help and guidance over the last 5 years: Dr. Abhinav Mishra, Dr. Hao Pang, Shraddha Karanth, Collins Tanui, Taryn Horr, Edmund Benefo, Dr. Elisabetta Lambertini, Yuqing Ying, Yinzhi Qu and Dr. Miao Guo. I would also like to acknowledge the support from the Department of Nutrition and Food Science faculty and staff for all these years.

Most importantly, none of this would have been possible without the love and patience of my family and friends. Many friends have helped me stay sane through these years. I greatly value their friendships and I deeply appreciate their belief in me. Thank you for everything and especially for the sincere encouragements. My family has been a constant source of love, concern, support and strength all these years. I would like to express my heart-felt gratitude to my father Mr. Ranjit Singh, mother Mrs. Shashikala, brother Piyiush Raj, and sisters Rashmi Rani and Puja Singh who were always supporting me unconditionally and encouraging me with their best wishes. Finally, special thanks to my best friend, Sagar Singh for encouragement in the worst and best moments during this PhD even with a long distance.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
APHIS	Animal and Plant Health Inspection Service
APDL	Animal Parasitic Diseases Laboratory
ARS	Agricultural Research Service
AUC	Area Under the Curve
BAACUC	Beltsville Area Animal Care and Use Committee
bp	Base Pair
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ERS	Economic Research Service
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
IFAT	Indirect Fluorescent Antibody Test
IHAT	Indirect Haemagglutination Antibody Test
IP	Intraperitoneal
JAGS	Just Another Gibbs Sampler
LAMP	Loop-mediated Isothermal Amplification
LAT	Latex Agglutination Test

MAT	Modified Agglutination Test
MCMC	Markov Chain Monte Carlo
NHANES	National Health and Nutrition Examination Survey
NCHS	National Center for Health Statistics
ODE	Ordinary Differential Equation
OIE	World Organization for Animal Health
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PI	Post inoculation
PSRF	Potential Scale Reduction Factor
QALY	Quality-adjusted Life Year
QMRA	Quantitative Microbial Risk Assessment
RE	Random Effects
RNG	Random Number Generator
ROC	Receiver Operating Characteristic
SC	Subcutaneous
SD	Standard Deviation
SE	Standard Error
SIR	Susceptible-Infected-Recovered
SW	Swiss Webster
USDA	United States Department of Agriculture
UV	Ultra Violet
WHO	World Health Organization

Chapter 1. Introduction and literature Review

1.1 History of *Toxoplasma gondii*

Toxoplasma gondii, discovered in 1908 by Nicolle and Manceaux in tissues of a hamster like rodent called the gundi (*Ctenodactylus gundi*) (Nicole & Manceaux, 1908), is a protozoan parasite, named after its morphology (mod. L. *toxo* = arc or bow, *plasma* = life) and the host (Dubey, 2010). *T. gondii* belongs to the phylum Apicomplexa, subclass Coccidiasina, order Eimeriorina, and there is only one known species in the genus *Toxoplasma* (Dubey, 2010). After the discovery of its life cycle in 1970, the definition of coccidia was changed from being only single-host-specific parasites to also include multi-host pathogens with significant public health and biological importance (Dubey, 2009). *T. gondii* is a coccidian parasite with cats as the definitive host and warm-blooded animals as intermediate hosts (Dubey, 2010). Originally, *T. gondii* was the parasite of cats with a fecal-oral cycle, but later with domestication, it adapted transmission by several other modes.

1.2 Ecology of *T. gondii* infection

1.2.1 Life cycle and infectious stages

The life cycle of *T. gondii* is unique in the sense that the organism is capable of having indefinite replications using either sexual or asexual cycles (**Figure 1.1**) (Dubey et al., 1998). Three infectious stages (tachyzoites, bradyzoites (tissue cysts) or sporozoites (oocycsts)) form the complex life cycle of *T. gondii* with sexual or asexual reproduction phases (Dubey et al., 1998). Sporozoites or oocysts are extracellular encysted stage and are the most infectious form of this

parasite, tachyzoites are rapidly multiplying stage and are associated with acute toxoplasmosis and bardyzoites or tissue cysts are intracellular encysted stage and are associated with the chronic stage of the infection. *T. gondii* is able to survive and circulate in the nature either in the form of oocysts in soil and water or as tissue cysts in a host's body (Tenter et al., 2000). Cats can become infected after the ingestion of any infectious form of the parasite (Dabritz & Conrad, 2010). The apparent optimal way of infection for shedding oocysts in cats is infection with bradyzoites or tissue cysts (Dubey, 2006). The time of shedding oocysts after the initial infection (prepatent period) in cat vary between 3-10 days after ingesting tissue cysts and is over 18 days after ingestion of oocysts or tachyzoites (Dubey, 2010). During shedding, oocysts are unsporulated and not infectious to hosts. Sporulation occurs in the environment upon exposure to various climatic conditions (air, humidity, temperature) within 1-5 days (Dubey, 2010). During sporulation, the unsporulated oocyst (10-12 µm in diameter) develops two sporocysts both containing four sporozoites (Dubey et al., 1998). This structural change helps the oocysts survive for several months in environment as the parasite's most infectious form(Dubey et al., 1998). Moist soil, sand, or surface water offer favorable conditions for the sporulation and survival of *T. gondii* oocyst (Frenkel et al., 1970). Sporulation is inhibited by dryness and temperatures above 50 °C or below than 4 °C as well as ultraviolet rays (Dubey, 2010; Lélu et al., 2012).

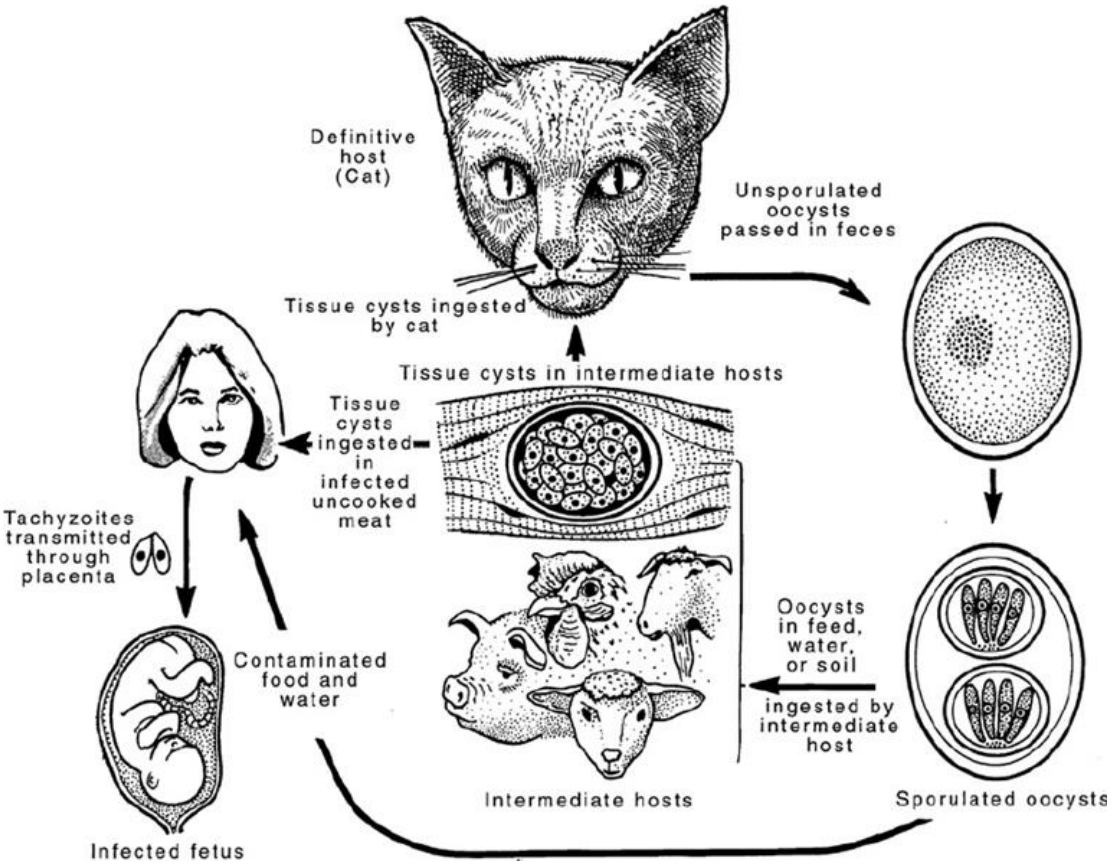

Figure 1.1 The life cycle of *Toxoplasma gondii* (source: Dubey & Beattie, 1988).

The asexual cycle occurs in all hosts and usually ends with the formation of tissue cysts. This cycle is induced either by oocysts, bradyzoites in tissue cysts or tachyzoites (Dubey, 2010). After the ingestion of sporulated oocysts, sporozoites excyst and penetrate cells of the intestinal epithelium (Dubey, 2010). Sporozoites released from oocysts, divide into tachyzoites which are responsible for the primary infection, and undergo rapid asexual multiplication by repeated endodyogeny. Tachyzoites is the predominating form of the parasite in acute toxoplasmosis. They start to transform into bradyzoites with the increase of the hosts' immune response. Bradyzoites

are structurally similar to tachyzoites, but have a nucleus in the posterior end of the structure rather than centrally located. Bradyzoites multiply intracellularly in a variety of host tissues through repeated asexual endodyogeny. This represents the chronic state of toxoplasmosis (Dubey, 2010). A tissue cyst can contain thousands of bradyzoites. The tissue cysts persist indefinitely for the life of the host, and are unevenly distributed among organs with high affinities for brain, heart, muscle and viscera (Dubey et al., 1998).

1.2.2 Routes of transmission

T. gondii has developed a wide range of transmission patterns. It has adapted to three main routes (**Figure 1.1**) in relation to its three forms: oocyst-oral route in all hosts; tissue cyst-oral route in carnivores; and tachyzoite route in all hosts (Hill & Dubey, 2002). The transmission can be horizontal or vertical. The horizontal transmission between different host species or from the environmental reservoirs can be foodborne, waterborne, milk-borne, soil-transmitted or fecally transmitted by cats (CDC, 2018). The most likely source of foodborne *T. gondii* infection occurs through consumption of raw or undercooked meat contaminated with tissue cysts (CDC, 2018; Tenter et al., 2000). Oocyst contaminated seafood have also been identified as potential sources of infection (Arkush et al., 2003; Lindsay et al., 2004). Many epidemiological studies have identified the poor handling of cat litter as an important risk factor (Baril et al., 1999). The vertical transmission of the parasite is blood-borne and can result in congenital toxoplasmosis (Dubey, 2010; Jones et al., 2001). Even though all the three infectious forms of the parasite can be zoonotic, the tissue cysts in undercooked or raw meat from infected food animals are considered to be the main risk factor for humans (Boyer et al., 2005; Cook et al., 2000).

Tissue cysts have been found in many animals, but especially in the farm animals, such as pigs, sheep, and goats, are considered epidemiologically important infection sources to humans (Hill & Dubey, 2002). Studies showed that the number of cysts in meat from livestock is low, only one cyst may be present in 100 g of meat (Dubey, 1998). The size of tissue cysts and the number of bradyzoites in them is dependent on the age of the cyst, the type of host cell, and the cytological method used for measurement (Dubey, 2010). Tissue cysts are more prevalent in neural and muscle tissues, although they may also be located in visceral organs, such as liver, lungs or kidneys (Dubey et al., 1998). In most livestock, viable tissue cysts may persist for several years or even for the life of the host (Dubey, 2010). Tachyzoites can be transmitted transplacentally, by blood transfusion or by organ transplantation, as well as by accidents in laboratories (Dubey, 2010). Oocysts are distributed widely in the nature and can contaminate food and water intended for farm animals or humans. Humans can also be infected through the handling of farm animals. After the sporulation, oocysts may remain infective for 18 months in soil and several years in 4^{o}C water and marine water (Dubey, 2010).

1.2.3 Pathogenicity of *T. gondii*

The predominant genotypes of *T. gondii* found in North America and Europe can be classified as clonal genotypes I, II, and III (Howe & Sibley, 1995). Pathogenicity of *T. gondii* is determined by two factors: virulence of the strain and susceptibility of the host species (Hunter & Sibley, 2012). It has been observed that certain strains of mice are more susceptible than others, and the severity of infection in individual mice within the same strain may vary as well. Type I lineages are uniformly lethal in mice (LD_{100} = 1) whereas the Type II and III lineages are significantly less virulent (LD_{100} >= 10^3) (Sibley & Boothroyd, 1992). Mice of any age are

susceptible to clinical *T. gondii* infection (Dubey, 2010). In humans, disease manifestations vary widely, ranging from asymptomatic to severe acute toxoplasmosis causing lymphadenopathy (enlargement of lymph nodes) and ocular lesions (Bossi & Bricaire, 2004) (Montoya & Liesenfeld, 2004). Type II is the predominant lineage causing human toxoplasmosis (Howe & Sibley, 1995). However, there have been biases reported between disease presentations and parasite genotypes. For examples, Type I or Type I-like atypical isolates are more likely to cause severe toxoplasmic retinochoroiditis in human patients (Grigg et al., 2001) and the atypical isolates are often involved in severe acute, disseminated toxoplasmosis in immunocompetent patients (Bossi & Bricaire, 2004).

1.3 Presence of *T. gondii* in nature

1.3.1 *T. gondii* in food animals

In most farm animals, such as pigs and sheep, detection of *T. gondii* specific antibodies and the presence of tissue cysts have a strong correlation (Dubey, 2010). The different observations reported in studies with naturally infected animals are documented in **Table 1.1**. The lack of tissue cyst detection does not mean that the meat is free of *T. gondii*, as the parasite may be present in unexamined parts of tissues (Dubey, 1992; Esteban-Redondo et al., 1999). Furthermore, seropositive animals may have parasites present at levels below the detection limits. In the United States (U.S.), poultry, pork, and beef are the main meats consumed with lamb and goat meat being popular among some ethnic groups. In a U.S. nationwide survey of 6,282 samples, *T. gondii* was not detected in all 2,094 samples of beef, all 2,094 samples of chickens, and was found in only 7 of 2,094 pork samples from 698 retail meat stores from 28 major geographic areas (Dubey et al., 2005). In a more recent study, viable *T. gondii* was isolated from 2 out of 750 lambs and 1 out of

750 pork samples of fresh, unfrozen, American pasture raised animals obtained from general grocery stores nationwide (Dubey et al., 2020).

Table 1.1 Reports of isolation of *T. gondii* in naturally infected animals in United States

Location	Host	Tissue*	Serology	Bioassay	Number	Isolated	Reference
Nationwide	Pig	M	No	Mice	750	1	(Dubey et al., 2020)
Michigan	Pig	H	Yes (MAT#, 1:25)	Mice	33	17	(Dubey et al., 2012)
Nationwide	Lamb	M	No	Mice	750	2	(Dubey et al., 2020)
Maryland, Virginia	Lamb	H	Yes (MAT, 1:25)	Mice, cats	68	53	(Dubey et al., 2008)
Maryland, Virginia	Goat	H	Yes (MAT, 1:25)	Mice, cats	112	29	(Dubey et al., 2011)
Maryland, Ohio, Massachusetts	Chicken	H	Yes (MAT, 1:25)	Mice, cats	230	0	(Ying et al., 2017)
Nationwide	Beef	M	No	Mice	2043	0	(Dubey et al., 2005)

*(H = Heart, M = Skeletal muscle; MAT: modified agglutination test

1.3.2 Human toxoplasmosis

T. gondii infections in humans are present globally, but the prevalence varies geographically between populations (Dubey, 2010). This is thought to be due to different cooking habits, the level of hygiene, the environment and implemented preventive measures such as confinement farming and screening of cats on farms. Higher prevalence is found in people who are in contact with soil and animals (Dubey, 2010). Seroprevalence usually increases with age (Montoya & Liesenfeld, 2004). The U.S. National Health and Nutrition Examination Survey (NHANES) report estimates of the seroprevalence of *T. gondii* in the population older than 12 years are in **Table 1.2**. The seroprevalence of toxoplasmosis among women of childbearing age (15–44 years) has declined from 14.9% in a 1988–1994 survey to 9.1% in a 2009–2010 survey (Peyron et al., 2017). Despite the reduced prevalence of *T. gondii* in the U.S., the 2014 Food and Agriculture Organization (FAO) and the World Health Organization (WHO) report ranks *T. gondii* as fourth most prevalent foodborne parasite out of 24 (FAO, 2014). The cost of toxoplasmosis in the U.S. has been estimated to be nearly 3 billion dollars and an 11,000 quality-adjusted life year (QALY) loss annually (Batz et al., 2012; Hoffmann et al., 2012).

T. gondii is a well-adapted parasite and once inside a host, may persist dormant in tissues for up to a lifetime – or cause a severe disease. Human toxoplasmosis symptoms depend on the parasite infectious stage, virulence of the parasite strain, and age and physical condition of the infected human (Montoya & Liesenfeld, 2004). The infection can be acquired postnatally or congenitally. The infection is usually followed by non-specific symptoms such as fatigue, weakness, listlessness, malaise, headache, excessive sweating, muscle and joints pains, fever, lymphadenopathy, and ocular signs (Dubey, 2010). Most of the signs resolve after one to several

weeks, while lymphadenopathy, weakness and malaise may persist for months. As most of the mild symptoms are similar to those seen in other diseases, suspicion of toxoplasmosis is often based on the long-lasting enlargement of lymph glands (Dubey, 2010; Montoya & Liesenfeld, 2004). Immunity does not eradicate infection. *T. gondii* tissue cysts persist for years after acute infection (Montoya & Remington, 1996). Severe cases of toxoplasmosis may lead to life-threatening complications, especially in individuals with immunosuppression, including those with acquired immune deficiency syndrome (AIDS) and organ transplant recipients (Nissapatorn et al., 2004; Wang et al., 2017),

T. gondii can be transplacentally passed to the fetus, which can cause abortion or congenital toxoplasmosis. The severity of disease depends on the timing of the infection (Montoya & Liesenfeld, 2004). The infections acquired during the first trimester are more severe than those acquired in the second and third trimesters (Desmonts & Couvreur, 1974; Remington et al., 1995). The clinical toxoplasmosis (retinochoroidal lesions or intellectual disability) may also occur later in life (Dunn et al., 1999).

Table 1.2 Reports of seroprevalence of *T. gondii* in humans in the United States from 2000 – 2020

Year Sampled	Age Group	Source of sera	Number Tested	Result	References
1999-2000	Age-adjusted 12 - 49 yrs	NHANES	4,234	15.8%	(Jones et al., 2003)
1999-2004	Age-adjusted 12 - 49 yrs	NHANES	15,960	10.8%	(Jones et al., 2007)
2009-2010	Age-adjusted 12 - 49 yrs	NHANES	11,357	9.6%	(Price et al., 2014)
2011-2014	Age-adjusted 12 - 49 yrs	NHANES	13,507	7.5%	(Jones et al., 2018)

1.3.3 Reported outbreaks

Outbreaks of toxoplasmosis involving more than a family or small group are infrequently reported and rarely documented. There are no special monitoring system or routine screening of toxoplasmosis, thus the surveillance is mainly based on detecting symptomatic toxoplasmosis (Andreoletti et al., 2007). The reported cases have been mainly associated with drinking water and/or undercooked meat (**Table1.3**). There has been no reports worldwide of major outbreaks since 2013. The last outbreak reported in the U.S. was in 1982 including nine individuals getting sick after coming in contact with sick cats on Illinois farm (Shenep et al., 1984)

Table 1.3 Major outbreaks of human toxoplasmosis from 2000 - 2020

Year	Country	Number of cases	Transmission medium	Parasitic form of *T. gondii*	Affected group	References
2001	Brazil	426	Water	Oocyst	Municipality residents	(de Moura et al., 2006)
2002	Turkey	171	Sand or soil	Oocyst	Boarding school students	(Doganci et al., 2006)
2003	Suriname	11	Water	Oocyst	Community neighbors	(Demar et al., 2007)
2004	Brazil	40	Sand or soil	Oocyst	Municipality residents	(do Carmo et al., 2010)
2004	India	248	Water	Oocyst	Municipality residents	(Balasundaram et al., 2010)
2004	India	213	Water	Oocyst	Municipality residents	(Palanisamy et al., 2006)
2005	Brazil	10	Pork sausage	Tissue cyst	Intra-family	(Almeida et al., 2005)
2006	Brazil	6	Beef	Tissue cyst	Party participants	(Eduardo et al., 2007)
2006	Brazil	61	Raw lamb	Tissue cyst	Party participants	(Renoiner et al., 2007)
2008	Colombia	18	Water	Oocyst	Soldiers	(Pino et al., 2009)
2009	Brazil	11	Raw vegetables	Oocyst	Industrial restaurant employees	(Ekman et al., 2012)
2013	Brazil	73	Açaí berry	Oocyst	Municipality residents	(Morais et al., 2016)

1.4 Detection methods

1.4.1 Bioassay in mice and cats

During the acute phase of toxoplasmosis, tachyzoites may be detected by microscopic examination in body fluids and tissues particularly from lungs (Dubey, 2010). For the isolation of *T. gondii* from infected animals, bioassays of the tissues in mice and cats have been used (Dubey, 2010). Bioassay method is considered as the gold standard for detecting and isolating viable *T. gondii* in animal samples. This method shows insufficient reliability if the parasite load is low, therefore, it may be necessary to concentrate *T. gondii* in the inoculum (Dubey, Lunney, et al., 1996). Therefore, a pepsin (which destroys tachyzoites) or trypsin digestion step is used in the bioassay method to destroy the tissue cysts wall and release bradyzoites into the suspension (Dubey, 2010). The suspension is then inoculated by subcutaneous (SC) or intraperitoneal (IP) route into mice and mice are kept under observation for 45-60 days allowing enough time for the development of *T. gondii* antibodies (Dubey, 2010).

For larger volumes of tissue samples, *T. gondii* free cats have been used, by feeding them the tissues (Dubey, 2010). To perform bioassay in cats, a portion of tissues that might contain cysts, typically brain, heart, muscle, tongue and diaphragm, are fed to cats orally. After 5-7 days, the cat shed *T. gondii* oocysts in its feces for approximately 14 days and an observation of an oocyst under microscope can confirm the sample positive (Dubey, 1995). For diagnosis, smears of mice brains are examined for *T. gondii* tissue cysts and cat feces are examined for oocysts under microscope (Dubey, 2010).

1.4.2 PCR-based techniques

Since bioassay in mice and cats are time consuming and ethically questionable, molecular methods, such as detection of parasitic deoxyribonucleic acid (DNA) with polymerase chain reaction (PCR) is recommended and gaining popularity (Luptakova et al., 2012). For PCR, amniotic fluid, blood, samples of tissues and cerebrospinal fluid can be used as specimens. By detecting specific and unique genes such as B1 primary gene or 529 base-pair (bp) sequence, *T. gondii* can be quickly characterized using specific biomarkers and the number of *T. gondii* can even be quantified by real-time PCR (Schares et al., 2008; Bezerra et al., 2012). PCR is known to be highly sensitive, specific and fast for the diagnosis of *T. gondii* infection but often there is non-specific interaction, poor binding efficiency of primers and sometimes, the parasitic DNA is shadowed by host's DNA (Erlich, 2015). PCR methods are not suitable for determining viable *T. gondii* as they detect both viable and dead parasite in the samples, which may lead to overestimation of the *T. gondii* infectivity in infected meat sample. Since PCR does not include a step to concentrate the *T. gondii* in the sample before testing, it may lack sensitivity (Dubey, 2010).

In order to resolve low sensitivity problems, new or modified PCR techniques have been developed in recent years. A sequence-specific magnetic capture method for isolation of *T. gondii* DNA was developed (Opsteegh et al., 2010). By using this method, DNA could be extracted from 100 g samples and the likelihood of detection of *T. gondii* DNA was increased. Another novel technique, loop-mediated isothermal amplification (LAMP) was also used to detect *T. gondii* infection in meat animals with higher sensitivity as compared to regular PCR (Zhang et al., 2009). Prevention of contamination while performing these highly sensitive techniques is a major issue.

1.4.2 Serological assays

It is often easier and faster to use serologic tests instead of direct parasite detection methods. Many serologic tests are available for detecting antibodies to *T. gondii* infection. The indirect hemagglutination antibody test (IHAT), the Sabin-Feldman dye test (DT), the latex agglutination test (LAT), the indirect fluorescent antibody test (IFAT), the modified agglutination test (MAT), and the enzyme linked immunosorbent assay (ELISA) have all been used to detect anti-*T. gondii* IgM and IgG antibodies in the serum and tissue fluid from meat animals (Dubey, 2010). However, none of these methods alone are suitable for confirming the diagnosis of acute toxoplasmosis, as the antibodies may persist for several years. In many hosts, the first exposure to *T. gondii* triggers the increase in IgM levels and this is followed by increase in IgG levels (Dubey, 2010). The Sabin-Feldman dye test is considered to be the definitive test for human toxoplasmosis, due to its high sensitivity and specificity. It is based on a complement-mediated neutralizing type of antigen-antibody reaction where live tachyzoites are used as antigen (Wallace, 1969).

Agglutination tests have been used widely for the diagnosis of toxoplasmosis in humans and animals. These tests are species independent, easy to perform with commercial kits, and safe (Dubey, 2010). In MAT, sera are treated with 2-mercaptoethanol to eliminate non-specific IgM or IgM-like substances (Dubey et al., 1995). Apart from serum, this test works well with blood plasma and even with whole blood as hemolysis does not interfere (Dubey & Desmonts, 1987). In LAT, the soluble antigen is coated on latex particles and in IHAT, the soluble antigen is coated on tanned red blood cells. Positive results are observed when the agglutination with antibody occurs (Balfour et al., 1982). In IFAT, killed tachyzoites and fluorescent-labeled antispecies IgG are used to detect

antibodies under fluorescent microscope (Liu et al., 2015). ELISA tests are widely used to detect anti-*T. gondii* antibodies (Sudan et al., 2013). The principal of ELISA is the antigen-antibody reaction that is visualized by the addition of a secondary enzyme-linked antibody-antigen system. The results in antibody identification are seen as colors. However, serological assays also have certain limitations. The performance of serological assays may differ with regard to sensitivity and specificity and the results of the serological assay are not always consistent with the bioassay results (Balfour et al., 1982).

1.5 Control measures

1.5.1 Prevention

The infection with *T. gondii* and toxoplasmosis can be prevented by various methods in all its hosts. The sources of human *T. gondii* infections are parasites in the tissues of food animals or in the environment. Control measures are needed for both (Dubey, 2010). Since the transmission of oocysts to humans can occur by ingesting contaminated food or water, it is important to wash all vegetables and fruits before consumption. As oocysts can also be found in contaminated soil, gardening and soil handling should be done with gloves. To prevent infection in domestic cats, avoid feeding them with uncooked meat, viscera, or bones, and keep cats indoors to prevent hunting (Aguirre et al., 2019). Cat litter boxes should to be cleaned daily, before oocyst sporulation (Dubey, 2010). Pregnant women, especially, should avoid contact with cats, soil, and raw meat. Hands should be washed thoroughly with soap and water after handling meat to avoid potential infection via bradyzoites (tissue cysts) in meat. Water is effective in killing the stages of *T. gondii* found in meat, so all cutting boards, sink tops, knives, and other materials coming in contact with

uncooked meat should be washed with soap and water after and between uses. The meat should be cooked thoroughly to an internal temperature above 66°C (Dubey et al., 1990). Cooking times will vary with the thickness and the type of the cut of meat. Freezing meat below -12°C also kills *T. gondii* in tissues (Kotula et al., 1991). Tachyzoites in milk can be efficiently killed by pasteurization (Dubey, 2010). When looking at the epidemiological picture from a larger scale, the best method in preventing human toxoplasmosis would be to keep animals (particularly domestic cats and livestock) free of *T. gondii*.

For farm animals, the most important source of *T. gondii* are the oocysts, therefore preventing feline fecal contamination of feed, bedding, water and pasture is highly recommended. Oocysts may be found in all places where cats have been allowed to defecate (Afonso et al., 2008). Epidemiological studies have concluded that pastures may be the most common source of infection due to field treatments with manure and beddings from farms where infected cats have lived (San Miguel et al., 2016). Moreover, appropriate rodent control helps to prevent the infection of the farm animals (and cats) via ingestion of infected rodents (Robert-Gangneux & Dardé, 2012). It would be desirable to have a killed vaccine to prevent cats from shedding *T. gondii* oocyst. Such a vaccine is not yet available and its widespread use is highly unlikely due to the huge number of feral cats and the difficulty in catching them (Dubey, 2010). Vaccination of cats against intestinal *T. gondii* infection has been successfully achieved using a mutant strain (T-263) of the parasite (Frenkel et al., 1991; Freyre et al., 1993). Oral administration of strain T-263 bradyzoites results in intestinal infection but does not result in oocyst production in cats.

1.5.2 Treatment

The commonly used sulfonamides, sulfadiazine, sulfamethazine, and sulfamerazine, are all effective against clinical toxoplasmosis (Dubey, 2010). Generally, any sulfonamide which can diffuse across the host cell membrane is useful in anti-*T. gondii* therapy. While these drugs have beneficial action during the acute stage of the disease process, they do not usually eradicate the infection (Beverley, 1958). It is believed that the growth of tissue cysts in mice has been restrained with sulfonamides (Beverley, 1958). Certain other drugs, spiramycin, piritrexin, roxithromycin, clindamycin, cyclosporin A, atovaquone, ponazuril and a novel triazine, have been found effective in experimentally induced *T. gondii* infection in animals or cell cultures (McCabe, 2001). Spiramycin thickens tissue concentrations, particularly in the placenta, which acts as placental barrier to prevent *T. gondii* infection (Dubey, 2010). Clindamycin is reported to give good results, but may cause ulcerative colitis (Dubey, 2010).

1.6 Project overview

T. gondii is a widespread zoonotic parasite with a high seroprevalence in the human population and the ability to infect almost all warm blooded animals. Humans can acquire toxoplasmosis from different transmission routes and food plays a critical role. The research goals are to estimate *T. gondii* concentration in meats of food animals, build predictive and risk assessment models to better understand the ecology of *T. gondii* infection and evaluate mitigation methods to reduce the public health burden of toxoplasmosis. To understand the complete infectivity and transmission of *T. gondii* from environment to farm animals and thereafter humans, the following objectives have been set for this doctoral research and their relationship in farm-to-fork framework is shown in **Figure 1.2**.

1) **Formation and distribution of *Toxoplasma gondii* tissue cysts in muscle tissues of food animals.** Within the food category, meat is of utmost importance, as it may contain viable *T. gondii* tissue cysts which can potentially cause infection if the meat is under-cooked and consumed. Currently, there is no published information on how soon after infection, *T. gondii* tissue cysts are formed in pork nor information on the distribution of viable *T. gondii* in muscle tissues of naturally infected meat animals such as lambs and goats.
2) **Evaluation of test accuracy of direct PCR and bioassay methods for detecting *Toxoplasma gondii* in animal samples.** Although bioassay methods are considered the gold standard to isolate viable *T. gondii*, most of recent studies rely on PCR techniques to estimate the prevalence of *T. gondii*. PCR cannot provide an accurate representation of true

prevalence. A re-evaluation of diagnostic methods to calculate *T. gondii* prevalence in naturally infected farm animals needs to be performed.

3) **Evaluating uncertainty and variability associated with *Toxoplasma gondii* survival during cooking and low temperature storage of fresh cut meats.** The current available data are not sufficient to suggest safe cooking or storage temperatures or holding time for fresh cut meats. Available cooking and storage temperatures data has a wide range of variability and uncertainty due to varying consumer preferences. Given the potential risk of *T. gondii* presence in freshly slaughtered meat animals undergoing minimal processing, it is crucial to evaluate possible intervention strategies such as cooking and freezing to reduce the risk to human consumers.
4) **Modeling the transmission of *Toxoplasma gondii* infection in a hypothetical farm set up.** *T. gondii* has a complex life cycle and has drawn attention of researchers in disciplines from epidemiology, immunology, human behavior, cell biology and parasitology. Although different aspects of the *T. gondii* life cycle have been intensively investigated, the overall transmission dynamics of this parasite and its infection has not been well studied in a farm-to-fork scenario.

As it is evident that raw or undercooked meat and meat products can be contaminated or associated with *T. gondii* cysts resulting in foodborne illnesses and deaths, it is necessary to identify the research gaps in understanding the behavior and ecology of *T. gondii* infection. This would greatly assist in future risk assessments and help risk managers take necessary actions to control and reduce the public burden of toxoplasmosis in future.

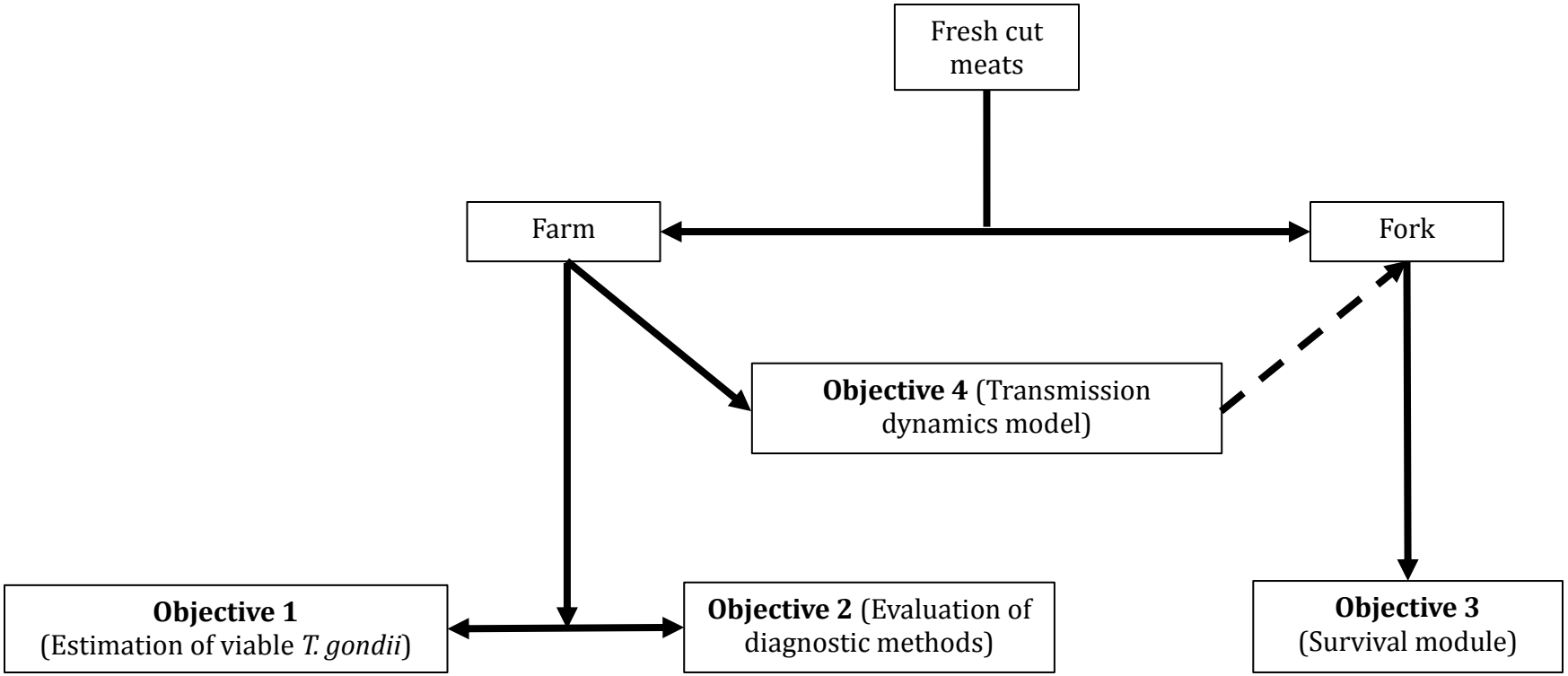

Figure 1.2 Diagram demonstrating relationships among four objectives.

Chapter 2: Formation and distribution of *Toxoplasma gondii* tissue cysts in muscle tissues of food animals

Part – I: *T. gondii* tissue cysts formation and density of tissue cysts in shoulders of pigs 7 and 14 days after feeding infected mice tissues

This work has been published in *Veterinary Parasitology*, 2019, 269, 13–15.

2.1.1 Abstract

Among the meat sources of *Toxoplasma gondii*, pork is considered important in the epidemiology of toxoplasmosis in the U.S. How soon after infection, *T. gondii* forms tissue cysts in pork is unknown. In the present study eight serologically negative ~3 months old pigs were fed mouse tissues infected with VEG (Type III) strain of *T. gondii* and euthanized 7 (4 pigs) and 14 days (4 pigs) post-inoculation (p.i.). Meat from the right shoulder of each pig was bioassayed for *T. gondii* tissue cysts by peptic digestion and fed to *T. gondii* free cats. Feces of cats fed pork were tested for oocyst excretion. From each pig, the shoulder muscle part was cut at 6 random spots into 5 g, 10 g and 50 g portions. Extreme care was taken to use different scalpels and forceps to minimize cross contamination among 17 samples (6 replicates of 5 g and 10 g portions and 5 replicates of 50 g). From the four pigs euthanized at 7 days p.i., a composite of ~200 g of leftover meat from each shoulder was collected for bioassay in cats. All eight pigs developed *T. gondii* antibodies (modified agglutination test, MAT, 1: 80 or higher) and viable *T. gondii* was isolated from shoulder meat of each pig. The four cats fed pork from the pigs euthanized 7 days p.i., excreted many oocysts. The density of *T. gondii*, based on mouse infectivity, varied within 5-50 g

samples from each pig, and between pigs within the same group, day 7 versus day 14 p.i.. Overall, the rate of isolation of *T. gondii* increased with sample size of meat bioassayed. There were no significant differences in mouse bioassay results obtained with day 7 versus day 14 infected pigs. Results demonstrate that tissue cysts are formed early in infection and are unevenly distributed.

2.1.2 Introduction

Toxoplasma gondii infection is widely prevalent in humans and animals, and toxoplasmosis continues to be a public health concern worldwide, including the U.S. (Dubey, 2010). Humans become infected postnatally by ingesting infected uncooked/undercooked meat or food and water contaminated with oocysts excreted by cats (Dubey & Jones, 2008). Among the meat sources of *T. gondii*, pork is considered important in the epidemiology of toxoplasmosis in the U.S. as they are highly susceptible to *T. gondii* infection with a minimum infective dose of 1 oocyst (Dubey et al., 1996). Beef and commercially raised poultry are rarely infected with *T. gondii* (Dubey et al., 2005). Oocyst-induced infections are considered more severe clinically than tissue cyst-induced infections (Dubey, 2010).

After the ingestion of *T. gondii* (oocysts or tissue cysts), *T. gondii* invades, multiplies, and persists in many tissues of pigs. Tissue cysts have been demonstrated in muscle of pigs, up to 875 days after feeding oocysts (Dubey, 1988). The parasite has been isolated from all commercial cuts of pork, including bacon, ham, spare ribs, tenderloin, Boston butt, and shoulder picnic of both naturally infected and experimentally-infected pigs (Dubey et al., 1986). However, there are no quantitative data on distribution of *T. gondii* in pork necessary for risk assessment (Guo et al., 2017). How soon after infection, *T. gondii* forms tissue cysts in pork is also unknown. The

objective of the present study was to qualitatively assess formation and distribution of *T. gondii* tissue cysts in pigs 7 and 14 days p.i. Of the pigs euthanized on days 7 and 14 p.i., whole right shoulder of eight pigs was used for the present report. This report is confined to only results of *T. gondii* tissue cysts in shoulder picnic.

2.1.3 Materials and methods

2.1.3.1 *T. gondii* infection in pigs

Eight mixed breed, 31-35 kg, 12 weeks old mixed-sex pigs were each fed one ground mouse carcass in cookie dough to enhance palatability. Pigs were housed in a climate-controlled facility as per procedures approved by the Institutional (Beltsville) Animal Care and Use Committee Protocol #18-012. The mice were inoculated 4 weeks previously with 100 VEG strain oocysts (Dubey et al., 1996). The mice were euthanized, skinned, eviscerated, and their brains were examined microscopically by removing a small snip (1-2 mm). The rest of the brain and carcass were ground in a blender, mixed in a ball of raw cookie dough (~50 g total weight of cookie and carcass) that was placed on the floor of the pen where each pig was observed for consumption of the preparation within 2 hours.

2.1.3.2 Serological examination

Pigs were bled a day before planned euthanasia (7 and 14 days p.i.) and their sera were tested for antibodies to *T. gondii* using the MAT as described previously (Dubey & Desmonts, 1987). For this, sera were diluted 2-fold starting a 1:10 dilution.

2.1.3.3 Testing of meat for *T. gondii*

Four pigs were euthanized at each time point (7 and 14 days) with a lethal injection of Euthasol (Virbac AH, Inc., Fort Worth, TX, USA) and necropsied. For this study, the right shoulder from each of eight pigs was removed and stored at 4°C for processing within 48 hours. The shoulders were washed with cold water to remove surface debris and wiped with clean tissue papers. The shoulder muscle was cut at 6 random locations into 5 g, 10 g, and 50 g portions. Extreme care was taken to use different scalpels and forceps to minimize cross contamination among 17 samples (6 replicates of 5 g and 10 g portions and 5 replicates of 50 g samples for mouse bioassay). From the 4 pigs euthanized at 7 days p.i., a composite of ~200 g of leftover meat from each shoulder was collected for bioassay in cats.

All 17 samples from each pig shoulder were bio-assayed in mice. For this, 5 g, 10 g and 50 g portions of muscles were homogenized in saline, and incubated in acidic pepsin solution (final muscle to pepsin ratio 1:10) at 37°C for 1 hour in a shaker water-bath as described previously (Dubey, 2010). The digested samples were filtered through 2 layers of gauze, centrifuged, suspended in saline, neutralized with 1.2% sodium bicarbonate, and the sediment suspended 1-5 ml of antibiotic saline solution (1,000 units of penicillin and 100 µl of streptomycin/ml of saline solution), and after 1 hour inoculated subcutaneously into Swiss Webster (SW) mice (2 mice each for 5 g and 10 g meat samples and 5 mice for 50 g samples - in total 49 mice per shoulder).

The mice inoculated with pig tissues were observed for 7-8 weeks and tested for *T. gondii* infection as described previously (Dubey, 2010). Mice were bled after 6 weeks p.i. and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies by MAT. All mice were euthanized 7-8 weeks p.i. and their brain smears were examined microscopically for tissue cysts, irrespective of serological results.

For bioassay in cats, 200 g of meat from the 4 pigs euthanized 7 days p.i. were fed to 4 cats (1 cat per pig), for 1-2 days. Cats were ~3 months old and raised in captivity at the Beltsville Agricultural Research center as reported in detail previously (Dubey, 1995). The cats had no detectible antibodies to *T. gondii* in a 1:10 serum dilution tested by MAT prior to feeding of pig meat. All feces from each cat were collected daily, 3 through 14 days after feeding pork and examined for *T. gondii* oocysts as described (Dubey, 2010).

2.1.3.4 Statistical analysis

Differences among test groups were compared by chi-square ($\chi 2$) test with Yates correction, using EpiInfo 7.2 statistical package. A *P* value of < 0.05 was considered as significant.

2.1.3.5 Ethics

Infected pigs, mice, and cats were housed at the USDA's Beltsville Agricultural Research Center and cared for in accordance with the Animal Welfare Act, Guide for the Care and Use of Laboratory Animals (https://www.nap.edu/search/?term=Guide+for+the+Care+and+Use+of+Laboratory+ Animals)

and with the approval of the USDA/ARS Beltsville Area Institutional Animal Care and Use Committee (BAACUC Approval #18-012, #18-013, #18-014).

2.1.4 Results

All eight pigs developed *T. gondii* antibodies (MAT, 1: 80 or higher) and viable *T. gondii* was isolated from shoulder meat of each pig. All four cats fed pork from the pigs euthanized 7 days p.i. excreted many oocysts (data not shown). Mouse bioassay data are provided in detail in **Table 2.1** for day 7 and **Table 2.2** for day 14 and summarized in **Table 2.3**. All mice inoculated with pig tissues survived. The mouse infectivity data in **Table 2.3** are based on finding tissue cysts in the brains of mice considered infected with *T. gondii*; all parasitologically-positive mice were serologically positive and tissue cysts were not found in the brains of serologically negative mice.

Table 2.1 Details of mouse bioassay of pigs euthanized on day 7 post inoculation

DAY 7			
	Sample size (g)	Replicates	Mouse bioassay (number of mice *T. gondii* positive/total mice inoculated)
Pig #1	5	1	0/2
		2	0/2
		3	0/2
		4	0/2
		5	2/2
		6	0/2
	10	1	0/2
		2	0/2
		3	1/2
		4	0/2
		5	0/2
		6	0/2

	50	1	4/5
		2	2/5
		3	3/5
		4	5/5
		5	4/5
Pig #2	5	1	0/2
		2	0/2
		3	0/2
		4	0/2
		5	0/2
		6	0/2
	10	1	0/2
		2	0/2
		3	0/2
		4	0/2
		5	0/2
		6	1/2
	50	1	0/5
		2	0/5
		3	0/5
		4	0/5
		5	0/5
Pig #3	5	1	1/2
		2	0/2
		3	2/2
		4	0/2
		5	0/2
		6	0/2
	10	1	0/2
		2	2/2
		3	2/2
		4	2/2
		5	2/2
		6	0/2
	50	1	4/5
		2	5/5
		3	5/5
		4	5/5
		5	5/5

Pig #4	5	1	1/2
		2	1/2
		3	0/2
		4	0/2
		5	0/2
		6	2/2
	10	1	0/2
		2	0/2
		3	1/2
		4	1/2
		5	2/2
		6	0/2
	50	1	4/5
		2	4/5
		3	4/5
		4	5/5
		5	4/5

Table 2.2 Details of mouse bioassay of pigs euthanized on day 14 post inoculation

DAY 14			
	Sample size (g)	Replicates	Mouse bioassay (number of mice *T. gondii* positive/total mice inoculated)
Pig #5	5	1	0/2
		2	2/2
		3	2/2
		4	2/2
		5	0/2
		6	2/2
	10	1	2/2
		2	2/2
		3	2/2
		4	2/2
		5	2/2
		6	2/2
	50	1	5/5
		2	5/5
		3	5/5
		4	5/5
		5	4/5
Pig #6	5	1	0/2
		2	1/2
		3	0/2
		4	0/2
		5	0/2
		6	0/2
	10	1	0/2
		2	0/2
		3	0/2
		4	0/2
		5	0/2
		6	0/2
	50	1	0/5
		2	2/5
		3	0/5
		4	1/5
		5	0/5

Pig #7	5	1	2/2
		2	2/2
		3	1/2
		4	2/2
		5	0/2
		6	1/2
	10	1	2/2
		2	2/2
		3	2/2
		4	1/2
		5	2/2
		6	2/2
	50	1	5/5
		2	5/5
		3	5/5
		4	5/5
		5	5/5
Pig #8	5	1	0/2
		2	0/2
		3	0/2
		4	0/2
		5	0/2
		6	0/2
	10	1	0/2
		2	0/2
		3	0/2
		4	2/2
		5	0/2
		6	0/2
	50	1	0/5
		2	3/5
		3	4/5
		4	0/5
		5	1/5

The rate of infection of *T. gondii*, based on mouse infectivity, varied within 5-50 g samples from each pig, and between pigs within the same group, day 7 versus day 14 p.i. Overall, the rate of isolation of *T. gondii* increased with sample size of meat bio-assayed (**Table 2.3**). No statistical differences ($p > 0.05$) were observed in the detection of *T. gondii* from all infected pigs of day 7 and day 14 p.i. by bioassay. However, when the sample sizes were compared within the same group, differences were observed between 5 g and 50 g as well as 10 g and 50 g samples in day 7 p.i. group ($p < 0.05$) (**Table 2.3**).

Table 2.3 Comparison of mouse bioassay results of pig euthanized day 7 and 14 post-inoculation

Day pig euthanized	**Mouse bioassay**		
	Sample size (g)	**No. of pork samples positive/No. tested (%)**	**No. of mice *T. gondii* positive/ No. inoculated (%)**
7	5	6/24 (25.0)[#a]	9/48 (18.7)[c]
	10	9/24 (37.5)[b]	14/48 (29.1)[d]
	50	15/20 (75.0) [a,b]	63/100 (63.0)[c,d]
14	5	10/24 (41.6)	17/48 (35.4)
	10	13/24 (54.1)	25/48 (52.0)
	50	15/20 (75.0)	60/100 (60.0)

[#]Same letter = Groups differ significantly (chi-square (χ^2) test with Yates correction, $P < 0.05$).

2.1.5 Discussion

Results of this investigation confirm findings by others that pigs can be readily infected by feeding *T. gondii* tissue cysts (Boch et al., 1964; de Meuter et al., 1978; Janitschke & Wormuth, 1970; Wingstrand et al., 1997; Work et al., 2009). In these studies, pigs were inoculated orally with homogenized mouse brains containing tissue cysts. In the present study, pigs were fed mouse carcasses mixed in cookie dough to simulate natural ingestion, avoid spillage, and prevent inadvertent infection of the research staff. Natural ingestion has the advantage that *T. gondii* are released slowly from infected mouse tissues as the digestion proceeds, thus allowing more time for bradyzoites to infect host tissue. Previous studies have revealed that the density of *T. gondii* in pork and other meats is low (Dubey, 2010). Therefore, bioassay methods were used to detect low numbers of *T. gondii* in meat. Of all the methods available for the detection of *T. gondii* in meat, the cat and the mouse bioassay are the most efficient for the detection of *T. gondii* in meat (Dubey, 2010) but these methods are qualitative. Therefore, only qualitative data were obtained here.

Results of the present study revealed that *T. gondii* can encyst in muscles of pigs as early as 7 days p.i. and overall the detection of tissue cysts by mouse bioassay was not different than at day 14 p.i. In the U.S., around 100 million pigs are slaughtered annually. Although the prevalence of *T. gondii* in pigs raised in bio-secure facilities has declined drastically, even a 0.1% prevalence of *T. gondii* amounts to 1000 infected pigs. Market pigs (feeder pigs) weigh around 100 kg and thus resulting in more than 600 servings of infected meat. Results of the present study demonstrate that even 5 g samples can be infected and that even recently infected pigs harbor tissue cysts and can be source of infection for humans.

Part – II: Distribution of *Toxoplasma gondii* tissue cysts in shoulder muscles of naturally infected goats and lambs

This work has been accepted in *Journal of Food Protection* and is currently in press.

2.2.1 Abstract

Toxoplasmosis has been recognized as a major public health problem worldwide. The consumption of uncooked/undercooked meat infected with *Toxoplasma gondii* (*T. gondii*) tissue cysts is one of the main sources for the transmission of this parasite. Although sheep, goats, and pigs are commonly infected with *T. gondii*, there is little information available concerning the distribution of *T. gondii* tissue cysts in naturally infected meat. In this study, we investigated the distribution of viable *T. gondii* tissue cysts in shoulder muscles of naturally infected lambs and goats. Hearts and shoulders of 46 lambs and 39 goats (total 85 animals) from a local grocery store were tested for *T. gondii* infection. First, the animals were evaluated for the presence of anti- *T. gondii* antibodies in heart blood/clot by the modified agglutination test (MAT). A total of 14 out of the 85 animals (7/46 lambs and 7/39 goats) were seropositive. A total of 6 to 12 samples weighting 5g, 10g, and 50g were obtained from shoulder muscles of each seropositive animal and bioassayed in mice. The distribution of viable *T. gondii* varied according to the size of the sample analyzed, but in general larger sample sizes resulted in higher isolation rates ($p < 0.05$). Results of the study revealed uneven distribution of *T. gondii* in muscle samples of lambs and goats and that even small serving sizes (5 g and 10 g) of meat have the potential for the transmission of *T. gondii* if consumed raw or under-cooked.

2.2.2 Introduction

Infection by the protozoan parasite, *Toxoplasma gondii*, is widespread in humans and in many other warm-blooded animal species (Dubey, 2010). *T. gondii* is 1 of 3 pathogens (along with *Salmonella* and *Listeria*) that account for 75% of all deaths due to foodborne diseases in the U.S. (Scallan et al., 2011). Although most infections in humans are asymptomatic, severe toxoplasmosis can cause intellectual disability, loss of vision, and mortality in infants (congenital toxoplasmosis) (Dubey, 2010). It is estimated that congenital toxoplasmosis affects 500 to 5000 newborns in the U.S. each year (Jones et al., 2001; Torgerson & Mastroiacovo, 2013). Severe toxoplasmosis may also develop in individuals with immunosuppression, including those with AIDS and organ transplant recipients (Nissapatorn et al., 2004; Peyron et al., 2017; Robert-Gangneux & Dardé, 2012; Wang et al., 2017).

The most likely source of foodborne *T. gondii* infection occurs through consumption of raw or undercooked meat contaminated with tissue cysts (CDC, 2018; Tenter et al., 2000) , although oocyst contaminated seafood as also been identified as potential sources (Arkush et al., 2003; Lindsay et al., 2004). Sporulated *T. gondii* oocysts, from the feces of infected cats, present in the environment in water and soil are a potential source of infection as well (Bowie et al., 1997). Many epidemiological studies have also identified poor handling of cat litter as important risk factors (Baril et al., 1999). However, it is not well known which of the various routes of transmission could be more relevant epidemiologically (Tenter et al., 2000). In Europe, ingestion of inadequately cooked meat (lamb, beef, game) was identified as the main risk but these studies are more than 15 years old (Baril et al., 1999; Cook et al., 2000; Kapperud et al., 1996). In the U.S., of 131 mothers who had given birth to children infected with *T. gondii*, 50% recalled having

some exposure to undercooked or uncooked meat (Boyer et al., 2005). More recently, in a case control study, consumption of meat (eating rare lamb, raw ground beef) was one of the risk factors associated with *T. gondii* infection (Jones et al., 2009).

There is no predilection site for *T. gondii* in meat animals; virtually all edible portions of an animal can harbor viable *T. gondii* tissue cysts, and tissue cysts can persist for the life of the host (Hill & Dubey, 2016). Little is known about the distribution of *T. gondii* tissue cysts in naturally infected meat in grocery stores destined for human consumption and there are no quantitative data on the distribution of *T. gondii* for risk assessment (Guo et al., 2017; Opsteegh et al., 2011). Determination of distribution of *T. gondii* in meat is technically difficult to do with beef, chicken and pork sold in grocery stores because the level of infection in these meats is very low. In a U.S. nationwide survey of 6,282 samples, *T. gondii* was undetected in 2,094 samples of beef and 2,094 samples of chickens and was found in only 7 of 2,094 pork samples from 698 retail meat stores from 28 major geographic areas (Dubey et al., 2005). In a more recent study, viable *T. gondii* was isolated from 2 of 750 lambs and 1 of 750 pork samples of fresh, unfrozen, American pasture raised animals (Dubey et al., 2020); these samples were obtained from general grocery stores nationwide. Thus, the present study was conducted on muscles of naturally infected lambs and goats obtained from a local ethnic grocery store and the objective was to evaluate the distribution of *T. gondii* tissue cysts in varying portions of meat samples.

2.2.3 Materials and methods

2.2.3.1 Lamb and goat samples

Between September 2016 and March 2019, shoulders and hearts of 46 lambs and 39 goats (total 85 animals) were obtained from a local ethnic butcher shop in Maryland. Specific information about the animals and farm practices could not be obtained. All animals were reported to be freshly slaughtered, without any carcass treatment. The animals were presumably under 1 year of age and raised in farms located in Maryland and Virginia region, however no confirmation was available. The animal samples were transported in plastic bags (separate plastic bags in case of more than 1 sample) within 30 min to the Animal Parasitic Diseases Laboratory (APDL), Beltsville, Maryland and immediately stored at 4°C until *T. gondii* testing was performed. The experimental design is shown in **Figure 2.1**.

Blood from the heart was collected, centrifuged at 2,500 × g for 3 min (Model: Labnet Spectrafuge 24D), and serum was separated. Heart fluid collected in the bag (when blood serum was not available for testing) was selected for serological assay as the fluid has shown comparable testing results for *T. gondii* as serum (Villena et al., 2012). Sera of lambs and goats were tested for *T. gondii* antibodies with the MAT as described in (Dubey & Desmonts, 1987). Briefly, 2-fold serial dilutions of sera were performed in 50 µl of 0.2 M 2-mercaptoethanol in phosphate buffered saline (PBS, pH 7.2, 0.01 M) in a 96 well plate. Intact formalin-treated tachyzoites of *T. gondii* RH strain were used as the antigen suspension and the concentration was adjusted at 2×10^4 tachyzoites/µl. A complete carpet of agglutinated parasites was considered as a positive result and clear button-shaped deposit of parasite suspension as negative.

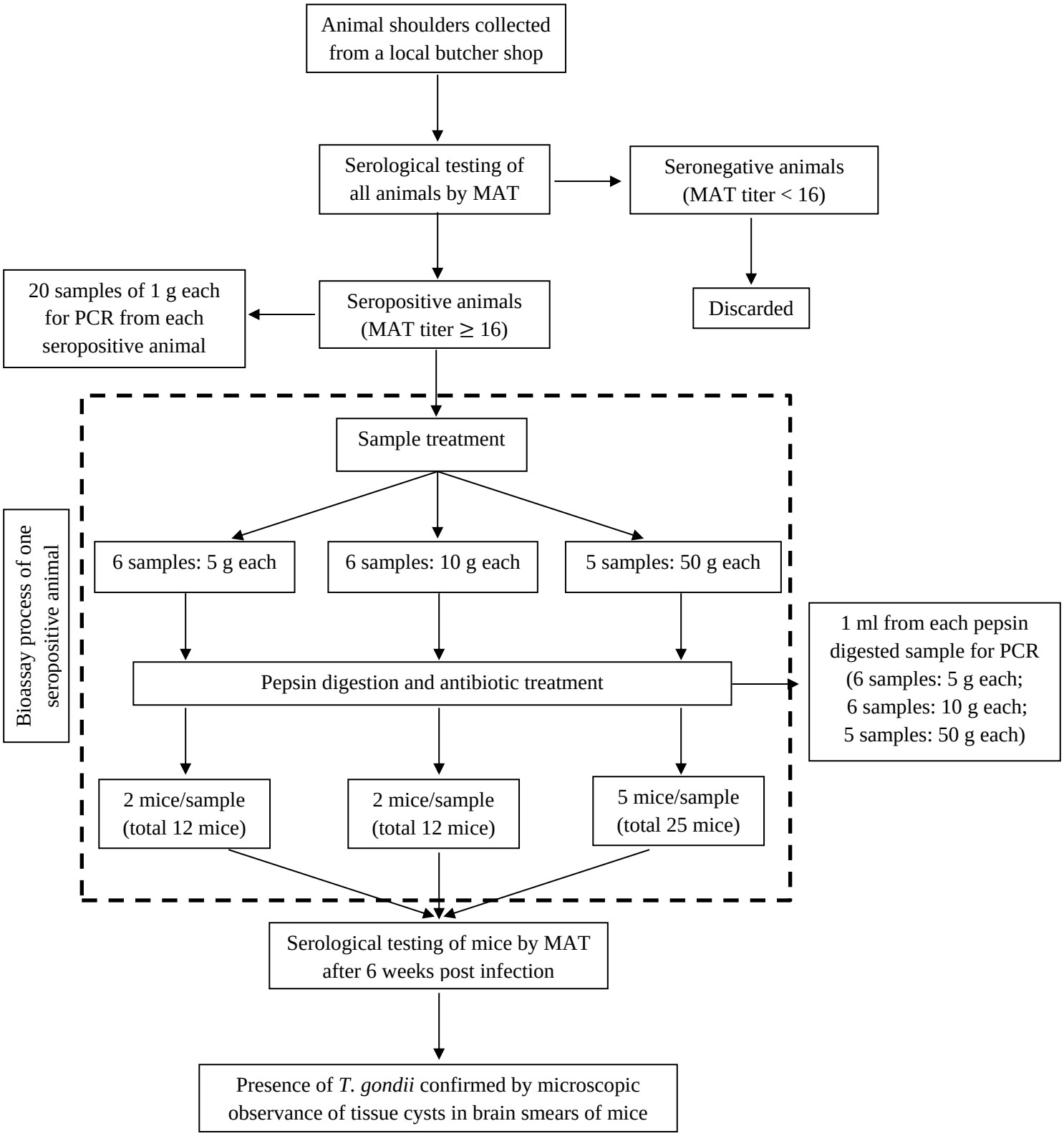

Figure 2.1 Experimental design for detecting viable *Toxoplasma gondii* in muscle tissues of naturally infected lambs and goats.

2.2.3.2 Sample treatment

The shoulders of seropositive animals were washed with tap water to remove dirt and blood and wiped with clean tissue papers. The muscle part was cut at random spots and weighed into 6 samples of 5 g and 10 g each and 5 samples of 50 g portions for bioassay procedure. Each cut was done with separate scalpel and gloves were changed after each sample. From each seropositive animal, 20 samples of 1 g portion were stored at -80°C for DNA isolation and PCR (**Figure 2.1**).

2.2.3.3 Mouse bioassay

To estimate the distribution of viable *T. gondii* based on mouse infectivity, all samples of 5 g, 10 g and 50 g portions of muscles of seropositive lambs and goats (MAT positive) were digested in acidic pepsin and bioassayed in Swiss Webster (SW) albino mice as described in (Dubey, 2010) (**Figure 2.1**). Briefly, samples were homogenized in 5 volumes of 0.85% NaCl (saline) in a blender at high speed for 1 min and digested in equal volume of pre-warmed and freshly prepared pepsin (pepsin, 1: 10,000, 2.6 g; NaCl, 5.0 g; 6 N HCl, 14.0 ml; and distilled water to make final volume 500 ml). After incubation in a shaker water bath for 1 hour at 37°C, the digests were filtered and centrifuged at 1,200 × g for 10 min (Model: Sorvall RC 3B Plus).

The sediment was suspended in saline and neutralized with sodium bicarbonate ($NaHCO_3$ solution, pH 8.3) with phenol red as a pH indicator and centrifuged again. The sediment was suspended in 1 - 5 ml of antibiotics solution (1,000 units of penicillin and 100 µl of streptomycin/ml of saline solution) to make 1:1 dilution and to reduce bacterial contamination in the digest (Dubey, 2010). The sediment was incubated at room temperature for 1 hour and vortexed

before inoculation. About 80 - 90% of the sediment from 5 and 10 g samples were inoculated subcutaneously (SC) into 2 outbred SW mice (1 ml per mouse) each and 50 g samples were inoculated SC into 5 SW mice (1 ml per mouse) (**Figure 2.1**). A total of 49 SW mice were inoculated per seropositive animal. The leftover digest (~1 ml) from each sample was stored at -80°C for DNA isolation. The animals with high MAT titer (≥ 400) were bioassayed twice to increase the chance of detecting *T. gondii*, therefore, 12 samples of 5 g and 10 g each and 10 samples of 50 g and a total of 98 SW mice were inoculated (**Figure 2.1**).

Most *T. gondii* strains in livestock in the U.S. are not mouse virulent and cause only asymptomatic infection (Dubey, 2010). Previous studies have established that mice should be kept for at least 6 weeks post inoculation (p.i.) with animal tissues, allowing enough time for the development of *T. gondii* antibodies (Dubey, 2010). Tissue impression smears of lungs of sick and dead mice were examined for *T. gondii* parasites (Dubey, 2010). The IgG *T. gondii* antibodies assessed here, persist for life in the host, therefore, the survivors were bled at convenience after 6 weeks and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies by MAT. All mice were euthanized after serological testing and smears of brain of each mouse were examined microscopically for tissue cysts, irrespective of serological test results (Dubey, 2010). All mice were observed daily during the entire process from inoculation to euthanasia. Mice were considered infected with *T. gondii* when the parasites were microscopically demonstrable in tissues (lungs or brains).

2.2.3.4 Molecular tests for *T. gondii*

The secondary objective of the present study was the detection of *T. gondii* DNA in animal tissues. For DNA extraction, 1 g undigested muscle samples were ground in a mortar with pestle after adding a few drops of nuclease-free water. The 1 ml samples saved from 5 g, 10 g and 50 g digests were centrifuged at 13,000 × g for 2 min (Model: Eppendorf Centrifuge 5424) and supernatant was discarded. *T. gondii* DNA was extracted from both ground muscle and digest samples by DNeasy blood & tissue kit (Qiagen Inc., Valencia, CA, USA) following the steps in the manual. The kit contained ready to use master mix solution, wash buffers and elution buffer. The isolated DNA samples were identified by conventional PCR with specific primers (Tox5 with sequence (5'-3') as CGCTGCAGACACAGTGCATCTGGATT and Tox8 with sequence (5'-3') as CCCAGCTGCGTCTGTCGGGAT) for a 529 bp (base pair) repetitive DNA fragment called TOX element (Schares et al., 2008).

T. gondii VEG strain DNA isolated from the brains infected SW mice was used as positive control. Negative control contained nuclease-free water instead of template DNA. The cycling conditions for the PCR were: 95^{o}C for 5 min as initial denaturation, followed by 35 cycles of 94°C for 1 min (denaturation), 60°C for 1 min (annealing) and 72°C for 1 min (extension), 72°C for 10 min for final extension and 6°C for 1 min (Schares et al., 2008). The DNA extraction and PCR were performed in a biosafety cabinet with a specialized vertical laminar air flow. Eight -10 µL of 50 bp ladder (Minisizer DNA ladder (25 - 625 bp) from Norgen Biotex Corp.) were used in each gel, followed by 25 µL of amplified DNA added to each well. All PCR products were resolved on a 2.5% agarose gel through electrophoresis and were observed under ultra violet (UV) trans-illumination.

2.2.3.5 Statistical analysis

The differences among test groups of goat and lamb samples were compared by chi-square ($\chi 2$) test with Yates correction, using EpiInfo 7.2 statistical package. A *P* value of < 0.05 was considered as significant.

2.2.3.6 Ethics

All infected mice were housed at the United States Department of Agriculture's (USDA) Beltsville Agricultural Research Center (BARC) and cared for in accordance with the Animal Welfare Act, Guide for the Care and Use of Laboratory Animals (https://www.nap.edu/search/?term=Guide+for+the+Care+and+Use+of+Laboratory+ Animals) and with the approval of the USDA/ARS Beltsville Area Institutional Animal Care and Use Committee (BAACUC Approval #15-017).

2.2.4 Results

Antibodies to *T. gondii* (MAT $\geq$ 16) were detected in 14 out of 85 animal shoulders (16.5%), (7 out of 46 lambs (15.2%) and 7 out of 39 goats (17.9%)) (**Table 2.4**). For animal shoulders with MAT titer < 400 (10 out of 14), a total of 6 samples of 5 g, 6 samples of 10 g and 5 samples of 50 g portions were bioassayed in mice, inoculating a total of 49 SW mice per animal (see **Figure 2.1** for mice distribution among samples). For animal shoulders with MAT titer $\geq$ 400, the bioassay process was performed twice to increase chances of detecting *T. gondii*.

Table 2.4 Presence of *Toxoplasma gondii* antibodies in naturally infected lambs and goats

	MAT titer							Seropositive/Total (%)
	< 16	16	32	64	400	800	1280	
Lamb	39	2	2	1	1		1	7/46 (15.22)
Goat	32		2	3		1	1	7/39 (17.95)
Total	71	2	4	4	1	1	2	14/85 (16.47)

Therefore, a total of 12 samples of 5 g, 12 samples of 10 g and 10 samples of 50 g portions were bioassayed in mice, inoculating a total of 98 SW mice per animal. *T. gondii* was isolated from shoulders of 4 animals (2 lambs and 2 goats) which had MAT titer ≥ 400 and the details of the bioassay are shown in **Table 2.7**. Bioassay results were negative for the rest 10 seropositive animals (MAT titer < 400). The rate of infection of *T. gondii*, based on mouse infectivity, varied within 5 g, 10 g and 50 g samples for both naturally infected lambs and goats (**Table 2.7**).

Overall, the rate of isolation of *T. gondii* increased with portion size of meat bioassayed, total 17/20 (85%) of 50 g, 12/24 (50%) of 10 g and 8/24 (33.3%) of 5 g muscle samples of lambs and 20/20 (100%) of 50 g, 20/24 (83.3%) of 10 g and 16/24 (66.7%) of 5 g samples of goats contained viable *T. gondii* (**Table 2.8**). There were significant differences in the detection of *T. gondii* by mice bioassay in lambs and goats for all the 3 portion sizes compared ($p < 0.05$) (**Table 2.8**). However, when the portion sizes were compared within the same animal, no statistical difference ($p > 0.05$) was observed in the detection of *T. gondii* from 5 g and 10 g samples but significant differences were observed between 5 g and 50 g as well as 10 g and 50 g samples in both lambs and goats ($p < 0.05$) (**Table 2.8**).

T. gondii DNA was detected in pepsin-digested muscle homogenates (**Table 2.7**) but the rate of detection did not correlate with the amount of muscle digested; no statistical difference was observed (**Table 2.8**). *T. gondii* DNA was not detected in any of the 280 (140 of lamb and 140 of goat) samples of 1 g portion collected from 14 seropositive animals. Full details of results of samples are provided in **Table 2.5** for lambs and **Table 2.6** for goats.

Table 2.5 Details of mice bioassay and PCR results of lambs naturally infected with *T. gondii*

Portion size (g)	Sample number	Lamb #6		Lamb #41	
		Mice bioassay (Number of mice *T. gondii* positive/Total mice inoculated)	PCR (1 ml pepsin digested muscle sample)	Mice bioassay (Number of mice *T. gondii* positive/Total mice inoculated)	PCR (1 ml pepsin digested muscle sample)
5	1	- (0/2)	+	- (0/2)	+
	2	- (0/2)	+	- (0/2)	-
	3	+ (1/2)	+	- (0/2)	-
	4	- (0/2)	+	- (0/2)	-
	5	- (0/2)	-	- (0/2)	-
	6	+ (2/2)	+	- (0/2)	-
	7	+ (1/2)	+	- (0/2)	-
	8	+ (2/2)	+	- (0/2)	-
	9	+ (2/2)	-	- (0/2)	-
	10	+ (1/2)	-	- (0/2)	-
	11	+ (2/2)	-	- (0/2)	-
	12	+ (2/2)	+	- (0/2)	-
	Total	(13/24)	8/12	(0/24)	1/12
10	1	- (0/2)	-	+ (2/2)	+
	2	+ (2/2)	-	- (0/2)	-
	3	+ (2/2)	+	- (0/2)	-
	4	+ (1/2)	+	+ (1/2)	-
	5	+ (1/2)	+	- (0/2)	-
	6	+ (2/2)	+	- (0/2)	-
	7	- (0/2)	-	- (0/2)	-
	8	+ (1/2)	+	- (0/2)	-
	9	+ (2/2)	+	- (0/2)	-
	10	+ (2/2)	-	- (0/2)	-
	11	- (0/2)	-	- (0/2)	-
	12	+ (2/2)	+	- (0/2)	-

	Total	(15/24)	7/12	(3/24)	1/12
50	1	+ (5/5)	+	+ (5/5)	+
	2	+ (5/5)	+	+ (3/5)	+
	3	+ (5/5)	+	- (0/5)	+
	4	+ (5/5)	-	+ (1/5)	-
	5	+ (5/5)	+	- (0/5)	-
	6	+ (5/5)	+	+ (1/5)	+
	7	+ (4/5)	+	+ (1/5)	-
	8	+ (5/5)	-	+ (1/5)	-
	9	+ (5/5)	+	+ (2/5)	-
	10	+ (5/5)	+	- (0/5)	-
	Total	(49/50)	8/10	(14/50)	4/10

Table 2.6 Details of mice bioassay and PCR results of goats naturally infected with *T. gondii*

Portion size (g)	Sample number	Goat #10		Goat #38	
		Mice bioassay (Number of mice *T. gondii* positive/Total mice inoculated)	PCR (1 ml pepsin digested muscle sample)	Mice bioassay (Number of mice *T. gondii* positive/Total mice inoculated)	PCR (1 ml pepsin digested muscle sample)
5	1	+ (2/2)	+	- (0/2)	-
	2	+ (2/2)	+	+ (2/2)	+
	3	+ (2/2)	-	- (0/2)	-
	4	+ (1/2)	+	- (0/2)	-
	5	+ (2/2)	-	- (0/2)	-
	6	+ (2/2)	+	- (0/2)	-
	7	+ (1/2)	-	- (0/2)	+
	8	+ (2/2)	+	+ (2/2)	+
	9	+ (2/2)	+	+ (1/2)	-
	10	+ (2/2)	-	- (0/2)	-
	11	+ (2/2)	-	- (0/2)	-
	12	+ (2/2)	+	+ (1/2)	+
	Total	(22/24)	7/12	(6/24)	4/12
10	1	+ (1/2)	+	+ (2/2)	-
	2	+ (1/2)	-	+ (1/2)	-
	3	+ (2/2)	+	+ (1/2)	-
	4	+ (2/2)	-	+ (2/2)	+
	5	+ (2/2)	-	+ (1/2)	-
	6	+ (2/2)	+	- (0/2)	+
	7	+ (2/2)	+	+ (1/2)	-
	8	+ (2/2)	+	- (0/2)	-
	9	+ (1/2)	-	+ (2/2)	+
	10	+ (2/2)	-	- (0/2)	-
	11	+ (1/2)	-	+ (2/2)	+
	12	+ (1/2)	+	- (0/2)	-

	Total	(19/24)	6/12	(12/24)	4/12
50	1	+ (5/5)	+	+ (5/5)	+
	2	+ (5/5)	+	+ (5/5)	+
	3	+ (4/5)	+	+ (5/5)	-
	4	+ (5/5)	+	+ (5/5)	-
	5	+ (5/5)	-	+ (5/5)	+
	6	+ (5/5)	+	+ (5/5)	+
	7	+ (5/5)	+	+ (5/5)	+
	8	+ (5/5)	-	+ (5/5)	+
	9	+ (5/5)	+	+ (5/5)	+
	10	+ (5/5)	-	+ (5/5)	+
	Total	(49/50)	7/10	(50/50)	8/10

Table 2.7 Presence of *Toxoplasma gondii* in samples of naturally infected lambs and goats

Animal ID	MAT titer	Portion size (g)	Mice bioassay		PCR* Number of samples *T. gondii* DNA positive/Total samples tested (%)
			Number of samples *T. gondii* positive/Total samples tested (%)	Number of mice *T. gondii* positive/Total mice inoculated (%)	
Lamb #6	400	5	8/12 (66.7)	13/24 (54.2)	8/12 (66.7)
		10	9/12 (75)	15/24 (62.5)	7/12 (58.3)
		50	10/10 (100)	49/50 (98)	8/10 (80)
Lamb #41	1280	5	0/12 (0)	0/24 (0)	1/12 (8.3)
		10	2/12 (16.7)	3/24 (12.5)	1/12 (8.3)
		50	7/10 (70)	14/50 (28)	4/10 (40)
Goat #10	800	5	12/12 (100)	22/24 (91.7)	7/12 (58.3)
		10	12/12 (100)	19/24 (79.2)	6/12 (50)
		50	10/10 (100)	49/50 (98)	7/10 (70)
Goat #38	1280	5	4/12 (33.3)	6/24 (25)	4/12 (33.3)
		10	8/12 (66.7)	12/24 (50)	4/12 (33.3)
		50	10/10 (100)	50/50 (100)	8/10 (80)

*PCR of pepsin digested muscle samples (1 ml). No *T. gondii* DNA was detected from undigested muscle (1 g) samples.

Table 2.8 Comparison of overall detection of *Toxoplasma gondii* by mice bioassay and PCR methods in different portion sizes of lamb and goat shoulder meat

Animal tested	Portion size (g)	Mice bioassay: Number of samples *T. gondii* positive/Total samples tested (%)	PCR: Number of samples *T. gondii* DNA positive/total samples tested (%)
Lamb	5	8/24 (33.3)^{*a,d}	9/24 (37.5)
	10	12/24 (50.0)^{b,e}	8/24 (33.3)
	50	17/20 (85.0)^{c,d,e}	12/20 (60.0)
Goat	5	16/24 (66.7)^{a,f}	11/24 (45.8)
	10	20/24 (83.3)^{b,g}	10/24 (41.7)
	50	20/20 (100.0)^{c,f,g}	15/20 (75.0)

*Same letter = Groups differ statistically (chi-square (χ^2) test with Yates correction, $P < 0.05$).

2.2.5 Discussion

In the present study, we attempted to detect and estimate the distribution of viable *T. gondii* in muscle tissues of naturally infected lambs and goats using two methods, bioassay in mice and direct PCR. The number of *T. gondii* organisms in meat from naturally infected animals is low, making the parasite difficult to detect (Hill & Dubey, 2013). Thus, the pepsin digestion step in the bioassay method was used to concentrate *T. gondii* in the inoculum. A *T. gondii* tissue cyst may contain hundreds of bradyzoites (Dubey, 2010). The tissue cyst wall is digested immediately after contact with pepsin and released organisms (bradyzoites) can survive in it for more than 1 hr (Dubey, 2010). Therefore, muscle samples were digested for 1 hour and the digests were centrifuged and acid neutralized before inoculating into mice. The percentage of inoculated mice that become infected reflects the presence of viable *T. gondii* in the digested meat samples (**Table 2.7** and **Table 2.8**). Thus, the bioassay method was qualitative and not quantitative. However, the distribution of tissue cysts in a meat sample is uneven and random, therefore we inoculated more than 1 mouse for each digest (2 mice in case of 5 g and 10 g and 5 mice for 50 g).

The detection of *T. gondii* DNA through PCR does not differentiate between dead or live parasite. Thus, PCR results do not provide data for risk assessment. The method was not able to correctly detect true positives as detected in bioassay method and resulted in many false negative and false positive results. Hence, there was not 100% correlation between PCR and bioassay results with muscle digests (**Table 2.5** and **Table 2.6**). There could be several reasons for these inconsistencies including low sensitivity of PCR technique, over-shadowing from host animal DNA, and binding efficiency of primers (Erlich, 2015). Additionally, *T. gondii* DNA was not detected by PCR in any of the 1 g undigested muscle sample.

To our knowledge, this is the first study to estimate the distribution of *T. gondii* in meat obtained from a grocery store. Three previous attempts to study distribution of *T. gondii* tissue cysts in various organs/tissues were made with infected animals obtained directly from the farm. In the first study, 50 g samples of various tissues of 5 pregnant goats naturally infected with *T. gondii* were bioassayed in mice and viable *T. gondii* was isolated from livers of 1, kidneys of 1, hearts of 4, diaphragms of 4, rear leg muscles of 5, and brain of 1 goat (Dubey, 1980). In the second study, 50 g samples of tissues of 4 naturally infected pigs were bioassayed in mice and viable *T. gondii* was isolated from arm picnic of 3, Boston butt of 2, ham of 1, spare ribs of 2, Bacon of 1, tongue of 3, diaphragm of 2, hearts of 2, brain of 1, and none from kidneys and livers (Dubey et al., 1986). In the third study, 100 g samples of various muscles of eight 7-months old lambs infected with *T. gondii* were bioassayed in mice and viable *T. gondii* was isolated from tongue and lamb chops of 7, hearts of 3, and rear leg muscles of all 8 lambs (Dubey & Kirkbride, 1989).

None of the studies mentioned earlier, bioassayed meat samples smaller than 50 g for *T. gondii* isolation. Therefore, to evaluate the distribution of *T. gondii* in muscle tissues, we incorporated smaller sample sizes (5 g, 10 g and 50 g) of lamb and goat meat naturally infected with *T. gondii* (**Table 2.7**). More than 33% of 5 g and 50% of 10 g samples of lamb meat were positive for *T. gondii* and contained viable tissue cysts (**Table 2.8**). The percentages were 66.7% in case of 5 g and 83.3% of 10 g goat meat showing that *T. gondii* tissue cysts are denser in an infected goat (**Table 2.8**). This was also evident from our overall results where isolation of *T. gondii* from goat was higher (the difference was significant at $p < 0.05$, **Table 2.8**). This predilection is difficult to

explain as it completely depends on the physiology of *T. gondii* infection in different hosts and host immune system (Dubey, 2010).

One of the limitations of the study was not able to gather information about the animals collected such as how they were raised and what farm practices and regulations were followed. As the animal samples were simply purchased from a local butcher shop, such information was not possible to obtain. The availability of specific information about the animals could help in the assessment of risk factors associated with the presence of *T. gondii* and evaluation of the public health burden in a broader way. However, our primary objective was to estimate the distribution of viable parasite in the muscle tissues of the seropositive animals, which could pose a potential risk of toxoplasmosis if consumed raw or undercooked. The Center of Disease Control and Prevention (CDC) has recommended proper hygienic measures while handling all raw meat and meat should be cooked thoroughly before human consumption, especially by pregnant women (CDC, 2018). Because the amount of meat consumed per person depends on several factors including age, preference, ethnicity etc., our study provides an insight on the presence of viable *T. gondii* tissue cysts in a serving size as small as 5 g, if consumed raw or undercooked (**Table 2.7** and **Table 2.8**). Collectively, the results indicated that the distribution of *T. gondii* tissue cysts in muscle tissues of animals is irregular and random based on the mouse infectivity.

Chapter 3: Evaluation and meta-analysis of test accuracy of direct PCR and bioassay methods for detecting *Toxoplasma gondii* in meat samples

This work has been submitted to *LWT - Food Science and Technology* and is currently under review.

3.1 Abstract

Toxoplasma gondii infects humans from consumption of raw or undercooked contaminated meat or ingestion of oocysts present in water, soil or on vegetables. Several molecular techniques like PCR have been developed to detect *T. gondii* in animal samples but bioassay method is still considered as the 'gold standard'. The aim of the study was to assess the relationship of detection accuracy of direct PCR in varying amounts of meat samples. Data were collected from peer-reviewed literature. The relationship was fitted in a logistic regression model for different sample sizes with 75% of diagnostic accuracy between predicted and calculated PCR results. We estimated the pooled odds ratio of direct PCR detecting *T. gondii* over bioassay using a random-effects model which suggested that there is a 1.96 times (OR 1.96, 95% CI [1.33-2.86]) higher chance of false positives results as compared to bioassay method. Heterogeneity was reported significant (Q, $p < 0.05$ and $I^2 > 50\%$). The results showed that direct PCR has different accuracies in detecting *T. gondii* in different sample sizes irrespective of animal type and despite of its limited reliability in detecting the parasite, it is a preferred method over bioassay method worldwide.

3.2 Introduction

Toxoplasma gondii is a ubiquitous parasite that infects a large variety of mammals and is considered the most prevalent parasite zoonosis in the world (Gajadhar et al., 2006). In 2012, the FAO/WHO declared *T. gondii* as a foodborne parasite of global concern (Robertson, van der Giessen, Batz, Kojima, & Cahill, 2013; FAO, 2014). Most warm-blooded animals are intermediate hosts, while only members of the Felidae family such as cats, are definitive hosts (Tenter et al., 2000). Humans are infected with *T. gondii* through different routes: by the consumption of raw or undercooked meat from infected animals, by ingesting sporulated oocysts present in water, soil or on vegetables, and by vertical transmission (congenital toxoplasmosis) (Hide, 2016). Toxoplasmosis can result in severe diseases in immunocompromised people and eye disease (ocular toxoplasmosis) in otherwise healthy individuals (Demar et al., 2012; Ozgonul & Besirli, 2017). *T. gondii* has been estimated to cause 8% of hospitalizations and 24% of deaths in the U.S. annually resulting from foodborne illnesses (Scallan et al., 2011). Risk factors related to *T. gondii* exposure for livestock include farm type, feed source, water quality, methods of rodent and bird control, presence of cats and methods of carcass handling (Guo et al., 2015).

Several direct and indirect methods have been developed for the detection of *T. gondii*. While bioassay in mice and cats are considered as the gold standard to detect viable *T. gondii* in meat products, they are laborious, expensive, time-consuming and involve a large number of animals (Dubey, 2010). Other techniques such as serological and molecular assays have been developed as faster and simpler alternatives. Although serology is preferred over other methods

for screening purposes, a positive serological result only indicates that contact has occurred with the parasite and not its actual presence in the tissues (Bezerra et al., 2012). Molecular methods such as PCR assays do not depend on an immune response, and allow direct detection of the parasite DNA in biological samples, thus they can be used to establish a diagnosis when serological tests are not definitive (Switaj et al., 2005).

Along with these inherent complicating issues in detection methods, the number of *T. gondii* organisms in meat from naturally infected animals is very low, making the parasite difficult to detect in the first place (Dubey et al., 1986). Researchers around the world are using methods other than bioassays to detect *T. gondii* in animals with varying diagnostic accuracies, making it extremely difficult to select a robust detection method (Kim et al., 2015). Meta-analysis offers a convenient way to get better insight on what types of detection methods are prevalent in literature and explore their underlying heterogeneities (Irwig et al., 1994). In this study, we attempted to analyze the accuracy of direct PCR method of detecting *T. gondii* as compared to bioassay method and establish a relationship of the PCR accuracy in different sizes of meat samples. Further comparisons were made through meta-analysis of studies incorporating direct PCR and bioassay methods for detecting *T. gondii* in different animal samples.

3.3 Materials and methods

3.3.1 Data sources

To analyze the accuracy of detecting *T. gondii* in meat samples, we relied on our experimental data (Rani et al., 2020). Shoulder parts of 85 naturally infected animals (46 lambs and 39 goats) were collected from local butcher shop and were serologically tested by modified

agglutination test (MAT). It detects *T. gondii* antibodies with the help of antigen-antibody interaction in serum or muscle fluid samples (please refer Dubey & Desmonts, 1987 for detailed MAT procedure). The positive serological cut-off was set at 1:16 titer for MAT test and we used the same cut-off in our analysis. Out of 85 animals, 71 animals had titer lower than 16 and pronounced seronegative, rest 14 were seropositive with MAT titer equal to or greater than 16 (2 animals had titer of 16, 4 had titer of 32, 4 had titer of 64, 1 had 400, 1 had 800 and 2 had titer of 1280). All seropositive animals were bioassayed in mice in varying portion sizes (5 g, 10 g and 50 g) to estimate the density of viable *T. gondii* tissue cysts and their infection rate (please refer Dubey, 2010 for detailed bioassay method). *T. gondii* DNA was also extracted from the pepsin digested solution of each sample and amplified by direct PCR to detect 529 bp band. We selected this study (Rani et al., in press) as the data needed were relevant and suitable for our analysis and there has been no study available in the literature that detected *T. gondii* in smaller amounts of meat such as 5 g and 10 g with the help of both bioassay and PCR methods. Additionally, the presence of *T. gondii* infection in beef, chicken and pork sold in grocery stores is generally very low (Dubey et al., 2005).Thus, we chose the study on lambs and goats, given their comparatively high *T. gondii* seroprevalence (Guo et al., 2016).

3.3.2 Descriptive statistics

All descriptive statistics with corresponding 95% confidence intervals that describe the accuracy of a detection test such as sensitivity (true positive rate) and specificity (true negative rate) were calculated. To measure the agreement between the test results we used kappa statistics (κ), a measure of the discordant pairs or the two cells in which the tests do not agree. A κ-value of 0 indicates that observed agreement is the same as that expected by chance, and a κ-value of 1

indicates perfect agreement. According to convention, a κ-value of 0 to 0.2 indicates slight agreement; 0.2 to 0.4 fair agreement; 0.4 to 0.6 moderate agreement; 0.6 to 0.8 substantial agreement; and 0.8 to 1.0 almost perfect agreement (Landis & Koch, 1977).

3.3.3 Regression model

The associations between different groups of samples (5 g, 10 g and 50 g) of the two types of animals tested (lambs and goats) were checked with the help of Fisher's exact test. The null hypothesis was that the probability of detecting *T. gondii* by PCR is the same in both naturally infected lambs and goats i.e. no variation in accuracy over animal types. The similar or associated groups were combined together and the relationship was fitted by a logistic regression model (Eq.(1)) with PCR results as response variable and bioassay results and sample sizes as two explanatory variables as described in (Coughlin et al., 1992).

$$Logit\ P(Y) = ln\left(\frac{P(Y)}{1-P(Y)}\right) = \beta_0 + \beta_1 X + \beta_2 W \quad (1)$$

Where P(Y) is the probability of positive (Y = 1) or negative (Y = 0) PCR results; X is a dichotomous outcome variable representing bioassay results (X = 0 and X = 1 denote negative and positive bioassay results, respectively) and W is a factor variable defining different sample sizes, 1 = 5 g, 2 = 10 g and 3 = 50 g.

The relationship between sensitivity and specificity for direct PCR method was graphically represented and validated by a Receiver Operating Characteristic (ROC) curve. We used the inference of the relationship between the area under the curve (AUC) and diagnostic test accuracy

as described in (Šimundić, 2009). Additionally, the model was further validated by Wald test and likelihood ratio test. Wald test evaluates the statistical significance of each coefficient in the model and likelihood test detects the goodness of fit of the model (Bewick et al., 2005).

3.3.4 Meta-analysis process

Meta-analysis is a method to integrate the results of multiple studies by using different statistical methods (Lee et al., 2015). We performed meta-analysis to obtain more valid, generalizable estimates of the chances/odds of detecting *T. gondii* by direct PCR as compared with bioassay for different farm animals available in the literature.

3.3.4.1 Literature search of eligible studies

To identify studies eligible for our meta-analysis, we searched PubMed, Web of Science and Google Scholar electronic databases from 1964 to 2019. We conducted a comprehensive search using combinations of the following keywords: "*Toxoplasma gondii*"; "toxoplasmosis"; "detection"; "bioassay"; "direct PCR"; "farm animals"; "tissue samples"; "accuracy". Inclusion criteria were primary research studies with original data either published, studies with a comparative study design and studies including detection of *T. gondii* in animal tissue samples using both direct PCR and bioassay methods. The reference sections of the studies were also screened for eligible studies. We excluded prevalence studies, studies including human subjects, studies of animals which had been experimentally infected, reviews, patents and letters. All data were recorded using Microsoft Excel software. The following information was collected for each study the first author's last name, publication year, country, animal type, sample number, tissue

sample type, serological test and its cut-off, sample size tested, samples detected positive and negative for direct PCR method and samples detected positive and negative for bioassay method.

3.3.4.2 Assessment of heterogeneity and bias

We generated matched pairs marginal odds ratios with 95% confidence intervals for detecting *T. gondii* in animal samples by direct PCR as compared with the bioassay method (gold standard). A forest plot in the random effects model was used to compare the pooled odds ratio of direct PCR and bioassay results from all selected studies. It describes how many times higher the odds are of obtaining a positive test result in a contaminated sample than in a non-contaminated sample (Šimundić, 2009). Heterogeneity between studies was examined by Cochran's Q, and the I^2 statistics tests. I^2 statistic ranges between 0 and 100%, and values of 50% or more was considered heterogeneous. Publication bias was evaluated statistically by Egger's test and was graphically represented as a funnel plot (Egger et al., 1997).

3.3.5 Data analysis

All analyses were performed in R statistical software, Version 3.6.2 (R Core Team, 2019). In all statistical analyses, the significance level was considered as $p < 0.05$. The package "epiR" was used to calculate all descriptive statistics and "glm" function was used for logistic regression model to fit a relationship of direct PCR results with bioassay results and sample size tested. The package "pROC" was used for plotting ROC curve and packages "survey" and "lmtest" were used for running Wald test and likelihood ratio test, respectively. Meta-analysis was performed by using "rma" function of "metafor" package and functions "forest" and "funnel" were used for generating

forest plot and funnel plot, respectively. For Egger's test, we used "regtest" function of "metafor" package.

3.4 Results

The estimated accuracies for the detection of *T. gondii* by direct PCR and bioassay methods in different sample sizes lamb and goat meat are shown in **Table 3.1**. The corresponding kappa (κ) statistics showed moderate, almost perfect and substantial agreements between the results of direct PCR and bioassay methods in lambs for 5 g, 10 g and 50 g samples, respectively and the agreements between results were substantial, moderate and substantial for 5 g, 10 g and 50 g samples, respectively in goats (**Table 3.1**). The sensitivities and specificities of direct PCR method were higher in 50 g as compared to other smaller samples (5 g and 10 g) in both lambs and goats (**Table 3.1**). The difference of test accuracy among different sample sizes was statistical significant ($p < 0.05$) but there was no statistical difference observed between animal types ($p > 0.05$). The results of the logistic regression model are shown in **Table 3.2**.

Table 3.1 Descriptive statistics for the detection of *T. gondii* by direct PCR and bioassay methods in different sample sizes of lambs and goats

Animal type	**Comparing groups**	**Index test**	**Gold standard**		**Sensitivity (95% CI[#])**	**Specificity (95% CI)**	**κ-value* (±SE)**
			Bioassay (+)	Bioassay (-)			
LAMB	5 g	PCR (+)	5	4	0.62 (0.24, 0.91)	0.91 (0.79, 0.98)	0.51 (±0.16)
		PCR (-)	3	42			
	10 g	PCR (+)	8	0	0.73 (0.39, 0.94)	1 (0.92, 1)	0.81 (±0.11)
		PCR (-)	3	43			
	50 g	PCR (+)	11	1	0.65 (0.38, 0.86)	0.96 (0.82, 1)	0.65 (±0.12)
		PCR (-)	6	27			
GOAT	5 g	PCR (+)	10	1	0.62 (0.35, 0.85)	0.97 (0.86, 1)	0.66 (±0.12)
		PCR (-)	6	37			
	10 g	PCR (+)	9	1	0.45 (0.23, 0.68)	0.97 (0.85, 1)	0.47 (±0.12)
		PCR (-)	11	33			
	50 g	PCR (+)	15	0	0.75 (0.51, 0.91)	1 (0.86, 1)	0.77 (±0.09)
		PCR (-)	5	25			

* κ-value of 0 to 0.2 indicates slight agreement; 0.2 to 0.4 fair agreement; 0.4 to 0.6 moderate agreement; 0.6 to 0.8 substantial agreement; and 0.8 to 1.0 almost perfect agreement; SE: standard error; [#]CI: confidence interval.

Table 3.2 Results of the logistic regression model defining the relationship of the detection accuracy of direct PCR as compared with bioassay method for different sample sizes

Regression coefficient		**Estimate**	**SE[#]**	**95% CI[1]**	***P* value**	**Wald test statistic**
Intercept (β_0)		-1.46	0.41	(-2.18, -0.83)	0.000	
Bioassay (β_1)	negative* (X = 0)					45.41 (*p* value = 0.000)
	positive (X = 1)	4.78	0.71	(3.73, 6.10)	0.000	
W (β_2)	50 g*					3.40 (*p* value = 0.035)
	10 g	-0.06	0.53	(-0.93, 0.82)	0.900	
	5 g	-1.55	0.65	(-2.69, -0.53)	0.017	

* Reference variable, [#]SE: standard error; [1]CI: confidence interval.

Table 3.3 Comparison of sensitivity and specificity of direct PCR in different sample sizes as predicted by the logistic regression model

	Sensitivity		**Specificity**	
	Model predicted (95% CI*)	Observed (95% CI)	Model predicted (95% CI)	Observed (95% CI)
5 g	0.85 (0.24-0.99)	0.62 (0.41-0.81)	0.95 (0.80-0.99)	0.94 (0.87-0.98)
10 g	0.96 (0.65-1.00)	0.55 (0.36-0.73)	0.82 (0.50-0.96)	0.99 (0.93-1.00)
50 g	0.97 (0.82-1.00)	0.70 (0.53- 0.84)	0.81 (0.70-0.90)	0.98 (0.90-1.00)

*CI: confidence interval

The predicted sensitivities of direct PCR were 70.7% for 5 g, 91.2% for 10 g and 91.8% for 50 g samples and predicted specificities were 93.7% for 5 g, 76.9% for 10 g and 75.8% for 50 g samples after applying logistic transformation (**Table 3.3**). The model suggested a good diagnostic accuracy of 74.8% (AUC) for direct PCR in detecting *T. gondii* in animal samples (**Figure 3.1**). Regarding likelihood ratio test and Wald test, both statistics were statistically significant validating the model predictions ($p < 0.05$).

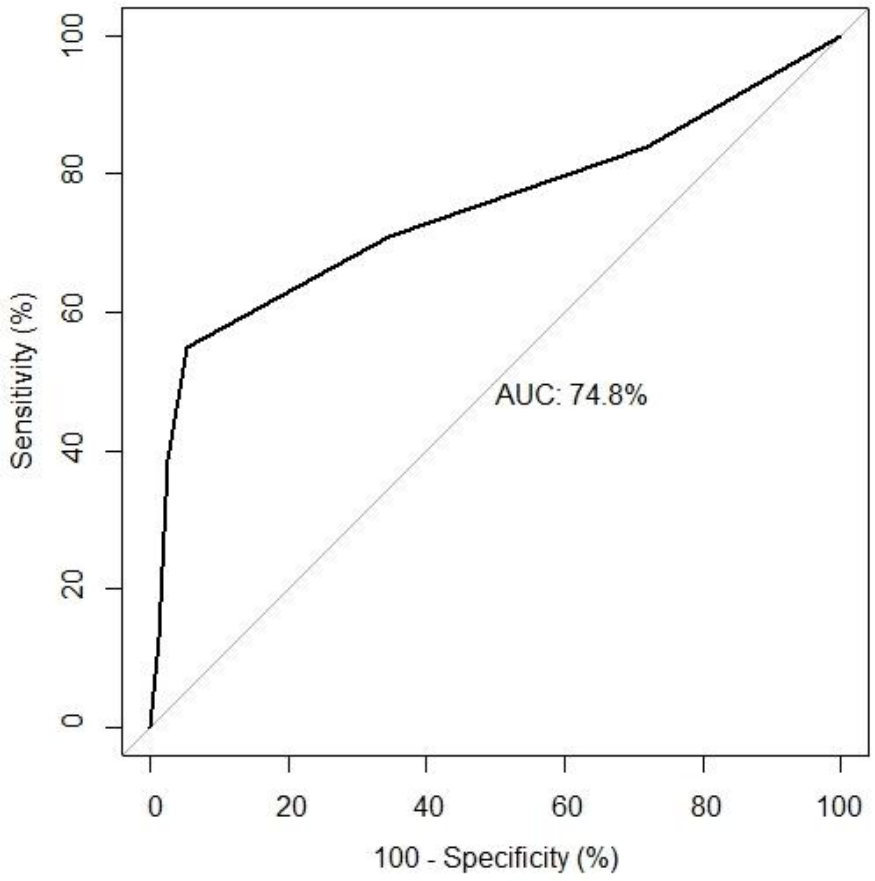

Figure 3.1 Receiver Operating Characteristic (ROC) curve depicting the accuracy of direct PCR as predicted by the logistic regression model. AUC: area under the curve.

The literature search yielded 107 studies from Google scholar search, 19 from PubMed and 9 from Web of Science. After a careful examination of each article's title and abstract based on inclusion and exclusion criteria, a total of 8 publications were included for our meta-analysis (**Table 3.4**). The random effects model estimated pooled odds ratio of detecting *T. gondii* in animal sample by direct PCR as compared with bioassay method was 1.96 (95% CI [1.33-2.86], $p < 0.01$) (**Figure 3.2**). There were1.96 times higher odds of a sample testing positive for *T. gondii* by direct PCR when it tested negative by bioassay method which indicated higher rate of false positive in PCR results in published literature.

The studies passing the vertical line of no effect or with larger 95% confidence interval were not considered statistically significant for overall heterogeneity (**Figure 3.2**). The model showed significant heterogeneity in our meta-analysis ($Q = 18.53$, $I^2 = 63.02\%$, $p < 0.01$). Publication bias was evaluated from the contour-enhanced funnel plot (**Figure 3.3**). The Egger's test was statistically non-significant, giving: $t = 0.0244$, $df = 6$, $p = 0.9813$, suggesting no asymmetry in the funnel plot.

Table 3.4 Details of the studies selected for the meta-analysis

Study#	Author (s)	Year	Location	Animal Type	Serology (cut-off)	Sample type[#]	Bioassay (positive/total)	Direct PCR (positive/total)
1	da Silva & Langoni	2001	Brazil	Lamb/sheep	IFAT (1:16)	B, D	26/39	5/39
2	Hill et al	2006	USA	Pig	MAT (1:25)	H, T	21/25	0/25
3	Silva & Langoni	2009	Brazil	Lamb/sheep	MAT (1:16)	B, M, L2	20/66	22/66
4	Bezerra et al	2012	Brazil	Pig	NA*	B, T	5/20	11/20
5	Holsback et al	2012	Brazil	Chicken	MAT (1:25)	B,H	11/27	16/27
6	Hamidinejat et al	2014	Iran	Chicken	MAT (1:25)	B, H	29/106	49/106
7	Pastiu et al	2015	Romania	Horse	MAT (1:48)	H	2/10	0/10
8	Samico-Fernandes et al	2017	Brazil	Pig	IFAT (1:64)	B, D, L1, L2, H	18/85	12/85

* NA: not available; [#] (B: brain; D: diaphragm; H: heart; L1: liver; L2: lung; M: muscle; T: tongue).

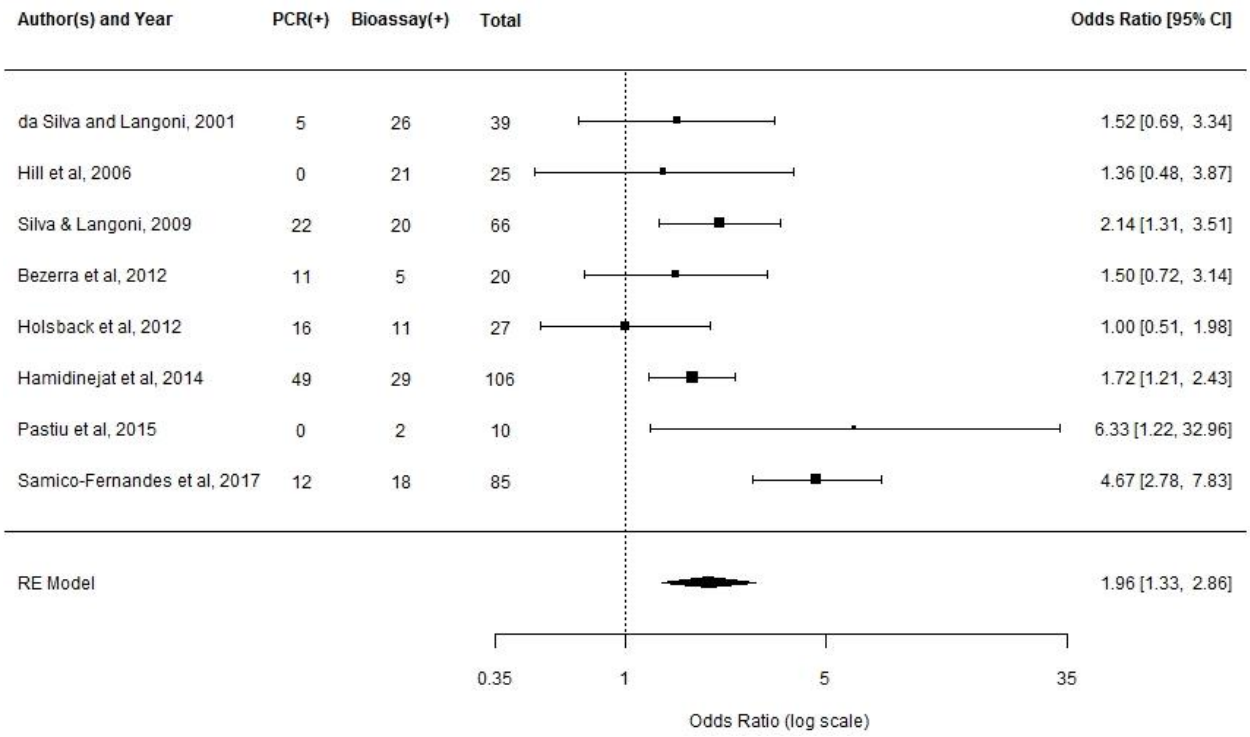

Figure 3.2 Forest plot showing the meta-analysis of the studies detecting *T. gondii* by direct PCR and bioassay in different animals with random-effects analysis. PCR (+): samples detected positive by direct PCR; Bioassay (+): samples detected positive by bioassay method; Total: total sample tested; RE: random-effects; CI: confidence interval; vertical dotted line is the line of no effect.

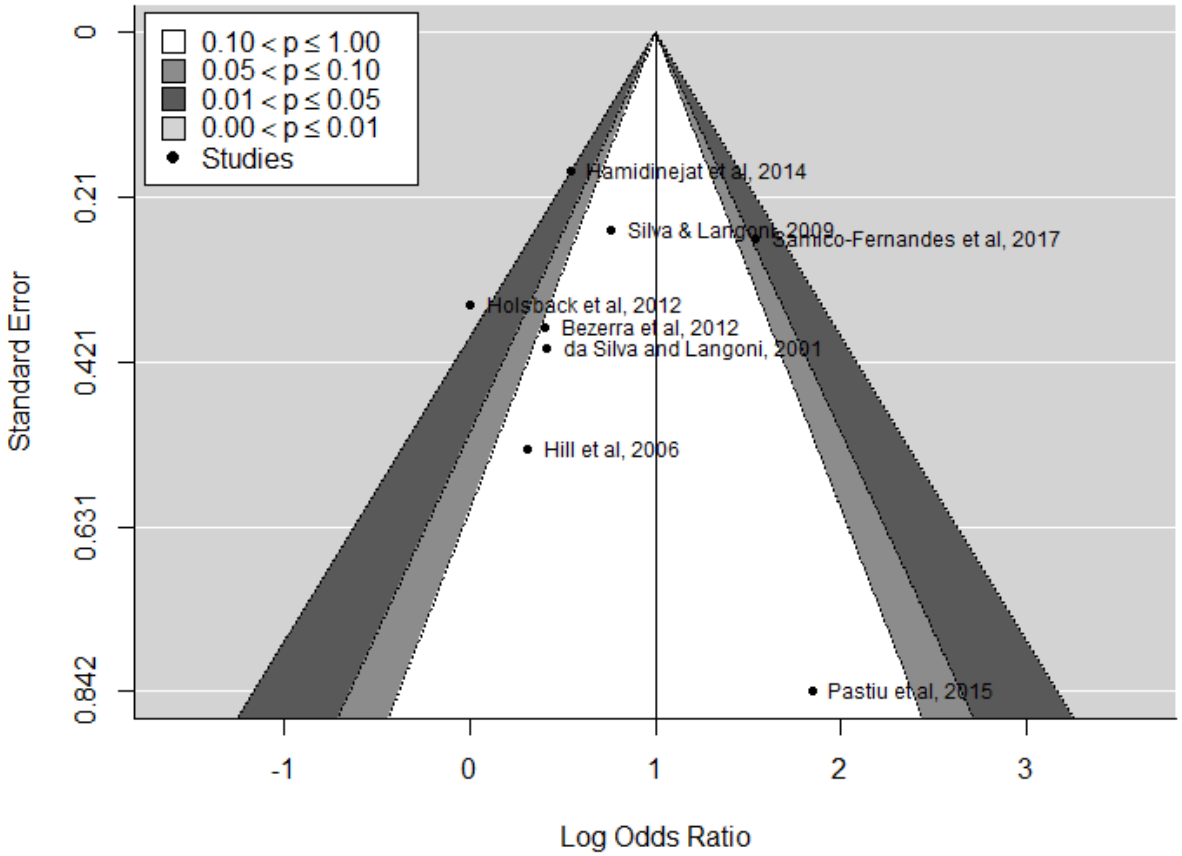

Figure 3.3 Contour-enhanced funnel plot for evaluating bias and heterogeneity in the included studies. The funnel is centered at 1, i.e., at the value of no effect. Various levels of statistical significance of the points (studies with authors and years) are indicated by the shaded regions; the unshaded (i.e., white) region in the middle corresponds to p values greater than 0.10, the light gray-shaded region corresponds to p values between 0.10 and 0.05, the dark gray-shaded region corresponds to p values between 0.05 and 0.01, and the region outside of the funnel corresponds to p values below 0.01.

3.5 Discussion

In this study, the detection accuracy of direct PCR were statistically significant in different amounts of meat samples (5 g, 10 g and 50 g) as compared with the results of bioassay method (gold standard) and the relationship was fitted in a logistic regression model (**Table 3.2**). The model predictions showed that the direct PCR is more sensitive in detecting *T. gondii* in larger samples as there is higher possibility of finding the parasite in larger contaminated samples as compared to smaller ones (**Table 3.3**). On the other hand, the model predicted lower specificities in larger samples as well, which could be explained due to non-specific interactions of *T. gondii* primers and over-shadowing by host DNA, resulting in high numbers of false positive (**Table 3.3**) (Gisbert Algaba et al., 2017). There was no statistical significant difference in results between lambs and goats ($p > 0.05$) which means the regression model could hold true for other animals as well.

Although the introduction of PCR in the detection of *T. gondii* infection represented a step forward, it is insufficiently accurate in presenting the correct concentration of *T. gondii* in the samples due to a non-homogeneous and low tissue cyst density in meat which often results in high numbers of false negatives (da Silva & Langoni, 2001; Hill et al., 2006) and false positives (Hamidinejat et al., 2014; Bezerra et al., 2012) (**Table 3.4**). None of the selected studies for the meta-analysis mentioned how much sample they used to extract DNA and run PCR, also none of them detected *T. gondii* by bioassay method in samples smaller than 50 g. Therefore, our regression model would create a spectrum in literature for detecting *T. gondii* by PCR and bioassay methods in different sample sizes and estimating their corresponding accuracies.

PCR methods are gaining popularity among researchers around the world for detection purposes. DNA extraction and PCR amplification can be done with any kind of animal sample, let it be muscle tissue, serum, blood, soil or fecal sample, hence it is in high demand due to being cheaper, less time consuming and not ethically questionable as compared to animal bioassay methods (Dubey, 2010). However, these methods are not suitable for determining viable *T. gondii* as they detect both viable and dead parasite in the samples, leading to unreliable results such as overestimation of the *T. gondii* infectivity in meat sample (Dubey, 2010). Evidently, our meta-analysis showed higher chances of getting a sample positive for *T. gondii* by PCR than that by bioassay method (OR 1.96, 95% CI [1.33-2.86], $p < 0.01$) (**Figure 3.2**). This showed the presence of high level of false positive results in literature, asserting that PCR detects parasite DNA which could from either live or dead parasites in the sample often leading to over-estimation of the contamination.

Heterogeneity is almost always presumed in diagnostic test accuracy reviews, and hence, a use of a random-effects model is recommended by default (Huedo-Medina et al., 2006). A random-effects model provides a pooled estimate of the average effect of a diagnostic test accuracy by assuming both the between-and-in study heterogeneities. There was significant heterogeneity in the studies selected for our meta-analysis ($Q = 18.53$, $I^2 = 63.02\%$, $p < 0.01$). Publication bias that is generally presumed to occur if there is heterogeneity among studies and could be assessed by asymmetry in a funnel plot, whereas a symmetric funnel plot suggests that the heterogeneity or bias is due to factors other than publication bias such as variable study quality and methodological design (Song et al., 2002). Contouring a funnel plot aids its interpretation and shows which study significantly contributes to the overall heterogeneity (Peters et al., 2008). In our analysis, only

three studies (Silva & Langoni, 2009; Hamidinejat et al., 2014; Samico-Fernandes et al., 2017) contributed significantly in the overall heterogeneity (**Figure 3.3**). The other five studies either passed through the line of no effect or had exceptionally large 95% CI, proving their non-significance (**Figure 3.2**). Egger's test was statistically non-significant ($p > 0.05$) suggesting a symmetric funnel plot and the existing heterogeneity could be explained by other factors such as serological cut-offs, animals tested, geographical locations as well as sample type tested (**Table 3.4**). Since the results in meta-analysis are combined from different studies, which have not followed a common protocol, heterogeneity is usually a major concern (Huedo-Medina et al., 2006). In conclusion, our results suggest that direct PCR has different accuracies in detecting *T. gondii* in different sample sizes as compared with bioassay irrespective of animal type tested and despite of its limited reliability in detecting the parasite, it is a preferred detection method over bioassay method worldwide.

Chapter 4: Evaluating uncertainty and variability associated with *Toxoplasma gondii* survival during cooking and low temperature storage of fresh cut meats

This work has been submitted to *International Journal of Food Microbiology* and is currently under review.

4.1 Abstract

Toxoplasmosis is an infection caused by the protozoan parasite, *Toxoplasma gondii*. It has been reported as the fourth leading cause of hospitalization and second leading cause of death among 31 major foodborne pathogens in the U.S. Humans are infected through consumption of raw or undercooked meat containing *T. gondii* tissue cysts or ingestion of food, soil, or water contaminated by *T. gondii* oocysts. People often lack knowledge about how to prevent *T. gondii* infection, especially the risks associated with eating or handling raw or undercooked meat. Current available data on cooking or low temperature storage for whole cuts of meat are not sufficient to validate the complete inactivation of *T. gondii*. The objectives of the present study were to estimate the relationship of time and temperature with the survival rate of *T. gondii* during cooking and low temperature storage of fresh cut meats. We used different statistical sampling techniques such as bootstrap resampling and Gibbs sampling to establish those relationships. Monte Carlo simulation was used to estimate the safe temperature for cooking and storing meats. The results showed the complete inactivation of *T. gondii* at or above 64°C (147.2°F) and below -18°C (0°F). The tissue cysts can remain viable up to 30 days at 4°C (39°F) and about 3% of cysts survived at 62.8°C

(145°F). This study can provide helpful information in improving the risk models to further mitigate the public health burden of toxoplasmosis.

4.2 Introduction

Toxoplasmosis is an infection caused by the protozoan parasite, *Toxoplasma gondii*. It has been reported as the fourth leading cause of hospitalization and the second leading cause of death among 31 major foodborne pathogens in the U.S., estimating about 4,428 hospitalizations and 327 deaths annually (Scallan et al., 2011). Although most infections in humans are asymptomatic, toxoplasmosis can cause intellectual disability, loss of vision, and mortality in individuals with immunosuppression, including those with AIDS and organ transplant recipients (Dubey, 1988; Gilbert et al., 2007). The cost of illness caused by *T. gondii* in the U.S. has been estimated to be nearly 3 billion dollars and an 11,000 QALY loss annually (Batz et al., 2012; Hoffmann et al., 2012).

Cats are the definitive host in which the parasite can complete its sexual cycle (Dubey, 2010). Cats usually shed oocyst form of the parasite in their feces for 1 - 2 weeks after infection which upon sporulation become quite resistant to environmental changes; they can remain infective in a moist environment for a year or more (Frenkel et al., 1970). Humans are accidental hosts and can be infected through consumption of raw or undercooked meat containing *T. gondii* tissue cysts or ingestion of food, soil, or water contaminated by *T. gondii* oocysts (Weiss & Dubey, 2009). Risk-factor analysis indicates that 30 - 63% of human infections can be attributed to the consumption of undercooked meat contaminated with *T. gondii* tissue cysts (Balzan et al., 2014; Batz et al., 2012). Congenital toxoplasmosis affects an estimated 500 to 5000 newborn infants in the U.S.

annually and can cause pregnancy loss (miscarriage or stillbirth) or severe disease in the newborn, including developmental delays, blindness, hydrocephalus and epilepsy (Frenkel et al., 1970; Gilbert et al., 2007; Hoffmann et al., 2012; Jones et al., 2007).

Preventing toxoplasmosis of pregnant women through education is a potentially beneficial strategy. A study of pregnant women in the U.S. found that they often lack knowledge about how to prevent *T. gondii* infection, especially the risks associated with eating or handling raw or undercooked meat (Jones et al., 2003). Among the ways to reduce human infection from meat, cooking is the most common process. In case of freshly cut meats, home cooking would be the only inactivation step and may not be effective in reducing the risk if meat is undercooked, especially for pork and lamb. In 2011, USDA recommended internal cooking temperature for whole cuts of meat be reduced from 160°F (71.1°C) to 145°F (62.8°C) with a minimal three-minute rest time due to elimination of the risk of infection from *Trichinella spiralis*, another zoonotic meat-borne parasite (Wilson et al., 2015). The recommended temperature might not be sufficient to kill *T. gondii* in case of fresh cuts of pork and lamb as there were evidences of its survival at 64°C after 3 min (Dubey et al., 1990).

Low temperature storage/freezing fresh cuts of meat before cooking is another method to inactivate *T. gondii* tissue cysts and a good home food safety practice to reduce the risk. Tissue cysts remain infectious in refrigerated carcasses (1 - 6°C) or minced meat for up to three weeks (Kotula et al., 1991). Freezing alone is not a reliable means of rendering all tissue cysts non-infective, the meat should be cooked properly before its consumption. The study on long term persistence of *T. gondii* tissue cysts in pork found that the cysts remained viable for more than 11

days at −7°C. However, the deep-freezing of meat at −12°C or lower for at least three days is usually efficacious to kill cysts, although it may depend on the thickness of the piece of meat (Dubey, 1988). Therefore, the cuts of meat that have not been held at a temperature below freezing point (e.g. fresh meat) or have been stored at refrigerated temperature (4 - 7°C) possess a potential risk of the presence of viable *T. gondii* tissue cysts.

Current available cooking or low temperature storage data for whole cuts of meat have a wide range of variability and uncertainty due to varying consumer preferences. These data are not sufficient to validate complete inactivation of *T. gondii* nor safe consumption and storage of meats. Given the limited experimental data and small sample size, regression analyses through statistical sampling such as bootstrap and Gibbs, can be handy and can provide exemplary insights on evaluating associated uncertainty and variability (Fisher and Hall, 1991; Lee and Song, 2004). Bootstrap resampling is a frequentist inference of determining properties of population distribution via the observation of sample whereas Gibbs sampling is Bayesian inference of describing the probability of an event based on certain prior knowledge of conditions related to the event. The objectives of the present study were to estimate the relationship of time and temperature with the survival rate of *T. gondii* during cooking and low temperature storage of fresh cut meats using both the aforementioned sampling techniques and calculate the number of viable *T. gondii* tissue cysts surviving after both the inactivation processes. Different scenarios were assumed to evaluate the survival pattern of tissue cysts at USDA recommended minimum internal cooking temperature, low temperature storage (refrigerated storage) and deep freezing temperature.

4.3 Materials and methods

4.3.1 Data sources and analyses

To obtain estimates of the effectiveness of inactivation of *T. gondii* after cooking and low temperature storage processes, multiple linear regression models were fitted to data from mice infection experiments described in the literature. Two experiments that had the largest groups of mice and the best defined treatments were selected (Cook et al., 2000; Foerster et al., 2015). In these experiments, *T. gondii* infected meat was processed at different temperatures and durations and were bioassayed in groups of mice to determine the probability of *T. gondii* infection. Heating treatments included temperatures of 49, 52, 55, 58, 61, 64 and 67 °C and durations of heating were 0.01, 3, 6, 12, 24, 48 and 96 min (**Table 4.1**, data adapted from (Dubey et al., 1990)). Freezing treatments included temperatures of 4, −1, −3.9, −6.7, −8.0, −9.4, −12.2 and −15 °C and durations of freezing were 64, 128, 256 min, 8.5, 17 h and 1.4, 2.8, 5.6, 11.2, 16.8, 22.4, 33.6, 44.8 and 67.2 days (**Table 4.2,** data adapted from (Kotula et al., 1991)). While incorporating the experimental data in our study, we assumed that each mouse got infected with single *T. gondii* tissue cyst and the probability of infection in mice was equal to the survival rate of *T. gondii* tissue cysts in the processed meat (**Table 4.1** and **Table 4.2**). Multiple regression models were built with survival rate of *T. gondii* as the dependent variable and time and temperature as independent variables for both the processes.

Table 4.1 Data of the mouse bioassay experiment (Dubey et al., 1990) used to fit a multiple linear regression model to predict the survival rate of *T. gondii* during cooking of meat. Number of mice positive for the presence of *T. gondii* per total number of mice inoculated is given for different time-temperature combination

Time (min)	**Temperature (°C)**						
	49	**52**	**55**	**58**	**61**	**64**	**67**
0.01	45/45	29/45	6/45	2/45	0/45	0/45	0/45
3	42/45	7/45	0/45	5/45	0/45	1/45	0/45
6	37/45	0/45	0/45	0/45	0/45	0/45	0/45
12	21/45	1/45	0/45	0/45	0/45	0/45	0/45
24	0/45	0/45	0/45	0/45	0/45	0/45	0/45
48	0/45	0/45	0/45	0/45	0/45	0/45	0/45
96	0/45	0/45	0/45	0/45	0/45	0/45	0/45

Table 4.2 Data of the mouse bioassay experiment (Kotula et al., 1991) used to fit a multiple linear regression model to predict the survival rate of *T. gondii* in frozen meat. Number of mice positive for the presence of *T. gondii* per total number of mice inoculated is given for different time-temperature combination

Time (hours)	**Temperature (°C)**								
	4	**-1**	**-3.9**	**-6.7**	**-8**	**-9.4**	**-12.2**	**-15**	**-18**
0.05	45/45	NA[a]	NA	NA	NA	NA	NA	NA	NA
0.1	45/45	NA	NA	NA	NA	NA	NA	NA	NA
0.2	45/45	NA	NA	NA	NA	NA	NA	NA	NA
0.4	45/45	NA	NA	NA	NA	NA	NA	NA	NA
0.8	45/45	NA	NA	NA	NA	NA	NA	NA	NA
1.1	15/15	15/15	15/15	14/15	NA	0/15	1/15	0/15	0/15
1.6	15/15	NA	NA	NA	NA	NA	NA	NA	NA
2.1	15/15	15/15	15/15	10/15	NA	0/15	0/15	0/15	0/15
4.3	15/15	15/15	15/15	15/15	NA	0/15	0/15	0/15	0/15
8.5	15/15	15/15	15/15	14/15	NA	0/15	1/15	0/15	0/15
17	15/15	15/15	15/15	15/15	NA	0/15	0/15	0/15	0/15
33.6	15/15	27/30	30/30	26/30	1/15	0/15	0/15	0/15	0/15
67.2	15/15	41/45	42/45	20/45	0/15	0/15	0/15	0/15	0/15
134.4	15/15	45/45	45/45	19/45	0/15	0/15	0/15	0/15	0/15
268.8	15/15	45/45	33/45	4/45	0/15	0/15	0/15	0/15	0/15
403.2	NA	12/30	10/30	0/30	0/15	0/15	0/15	0/15	0/15
545.8	NA	3/30	2/30	0/30	0/15	0/15	0/15	0/15	0/15

[a]NA: not available

The number of *T. gondii* tissue cysts in a naturally infected animal is quite low, approximately 1 tissue cyst in every 50 g or 100 g of tissues. Thus, to get the idea of how many tissue cysts are surviving after cooking and low temperature storage processes, we adapted the initial number of *T. gondii* cysts in fresh cuts of meat from mice experiments described in (Rani et al., 2020). In that study, 14 naturally infected lambs and goats were serologically tested and bioassayed in mice in varying portion sizes (5 g, 10 g and 50 g) to estimate the density of viable *T. gondii* tissue cysts and their infection rate (please refer Dubey, 2010 for detailed bioassay method) (**Table 4.3**). We selected this study (Rani et al., 2020) for two reasons, (1) the data needed were relevant and suitable for our analysis and (2) no study is available in literature on amount of viable *T. gondii* tissue cysts in muscles of food animals. Additionally, the level of *T. gondii* infection in beef, chicken and pork sold in grocery stores is generally very low; therefore, we chose a study on lambs and goats, given their comparatively high *T. gondii* seroprevalence. In our calculation, we assumed that each positive sample contained only one *T. gondii* tissue cyst and total number of tissue cysts per animal was calculated by adding all positive samples (**Table 4.3**). The number of *T. gondii* tissue cysts was modelled through linear regression with total amount of meat (g) bioassayed per animal.

Table 4.3 Data from the mouse bioassay experiment (Rani et al., 2020) used to fit a linear regression model to predict the number of viable *T. gondii* tissue cysts present in fresh cuts of meat

Animal type	Portion size (g)	Number of samples *T. gondii* positive/Total samples tested	Total amount of meat bioassayed[a]	Number of viable *T. gondii* tissue cysts present[b]
Lamb #1	5	8/12	680	27
	10	9/12		
	50	10/10		
Lamb #2	5	0/12	680	9
	10	2/12		
	50	7/10		
Lamb #3	5	0/6	340	0
	10	0/6		
	50	0/5		
Lamb #4	5	0/6	340	0
	10	0/6		
	50	0/5		
Lamb #5	5	0/6	340	0
	10	0/6		
	50	0/5		
Lamb #6	5	0/6	340	0
	10	0/6		
	50	0/5		
Lamb #7	5	0/6	340	0
	10	0/6		
	50	0/5		
Goat #1	5	12/12	680	34
	10	12/12		
	50	10/10		
Goat #2	5	4/12	680	22
	10	8/12		
	50	10/10		
Goat #3	5	0/6	340	0
	10	0/6		
	50	0/5		
Goat #4	5	0/6	340	0
	10	0/6		
	50	0/5		
Goat #5	5	0/6	340	0
	10	0/6		
	50	0/5		
Goat #6	5	0/6	340	0
	10	0/6		
	50	0/5		

Goat #7	5	0/6	340	0
	10	0/6		
	50	0/5		

[a]Total amount of meat bioassayed was calculated by multiplying and adding portion sizes with their respective number of samples tested (for e.g. for Lamb #1, 5*12 + 10*12 + 50*10 = 680 g), [b]number of viable *T. gondii* tissue cysts was calculated based on the assumption that each positive sample contained only one tissue cysts (for e.g. in Lamb #1, number of cysts equals to the total number of positive samples i.e. 27 (8 + 9 + 10 = 27).

4.3.2 Computational approaches

Bootstrap resampling and Gibbs sampling were performed in R statistical software, Version 3.6.1 (R Core Team, 2018). We used "*boot*" and "*car*" (car stands for companion to applied regression) packages for running bootstrap resampling process for 10,000 bootstrap resamples (https://cran.r-project.org/web/packages/boot/boot.pdf). For Gibbs sampling process, we used "*rjags*" and "*coda*" packages (https://cran.r-project.org/web/packages/rjags/rjags.pdf). These packages require JAGS (Just Another Gibbs Sampler) program installed in the system. JAGS uses Markov Chain Monte Carlo (MCMC) to generate a sequence of dependent samples from the posterior distribution of the parameters (http://people.stat.sc.edu/hansont/stat740/jags_user_manual.pdf). This takes place in five steps. All steps are chronologically defined in details in later sections. The "rjags" package provides an interface from R to the JAGS library for Bayesian data analysis and the "coda" package provides functions for summarizing and plotting the output from MCMC simulations, as well as diagnostic tests of convergence to the equilibrium distribution of the Markov chain. Scenario analyses were performed with a Monte Carlo simulation technique for 100,000 iterations using @Risk software, Version 7.6 (Palisade Corp., Ithaca, NY) (Latorre et al., 2011). The '*RiskSimtable*" feature was used to include different time and temperature points for different scenarios (Foerster et al., 2015).

4.3.3 Bootstrap resampling

The bootstrap method is a resampling technique used to estimate statistics on a population by sampling a dataset with replacement. It is useful in cases of limited data or small sample sizes when inferences on population statistics are difficult to state. To obtain bootstrap replicates of the parameters of the regression model, we used non-parametric bootstrap for resampling residuals of regression model. It resampled thee coefficients for 10,000 times and delivered a new regression equation between time, temperature and survival rate of *T. gondii* after cooking and low temperature storage processes. The confidence intervals for each coefficient was obtained using *confint* command with type "bca" (stands for biased-corrected and accelerated). The main advantage to the bca interval over percentile interval is that it corrects for bias and skewness in the distribution of bootstrap estimates.

4.3.4 Gibbs sampling (running JAGS)

Gibbs sampling (also called alternating conditional sampling) is a Bayesian inference of drawing samples from specified probability distributions, called posterior distribution and each sample is dependent on the previous sample specified by prior distribution. The steps involved in running JAGS in R include defining a model, compiling and initializing it, adapting and burn-in and drawing samples for analysis.

Step1: Definition of a model - There are two parts to the definition of a model in JAGS; the first part specifies the likelihood of data defined by the multiple linear regression model. Each value of the survival rate of *T. gondii* tissue cysts is a random draw from a normal distribution whose mean linearly function of time and temperature values. JAGS uses precision rather than

variance to specify normal distributions (precision is reciprocal of variance). The parameters were the intercept and coefficients of the regression equation whose values were to be established. The second part of the model specifies the prior distributions for each parameter and for the precision of the normal distribution. In accordance with the general practice, we used normal distributions with zero mean and high variance (10^4) as prior distributions for all parameters and gamma distribution as prior for the precision of the likelihood function (Plummer, 2003). The model was defined in a similar fashion for both cooking and storing/freezing processes.

Step 2: Compilation - When a model is compiled, a graph representing the model is created in computer memory. This step is a check point whether the model is correctly defined or not. It allocates relations (nodes) and checks for any undeclared variables in the model. The number of parallel chains to be run by JAGS is also defined at compilation time. Each parallel chain produces an independent sequence of samples from the posterior distribution. We used five chains in our compilation for both the processes.

Step 3: Initialization – Before running a model, it must be initialized. There are three steps in the initialization of a model; (1) setting initial values of the model parameters, (2) choosing a Random Number Generator (RNG) for each parallel chain, and setting its seed and (3) choosing samplers for each parameter in the model. This step is included in the compilation step when running JAGS through R using *jags.model* command.

Step 4: Adaptation and Burn-in - In theory, output from an MCMC sampler converges to the posterior distribution of the model parameters in the limit as the number of iterations tends to infinity but in practice, all MCMC runs are finite. By convention, the MCMC output is divided into two parts: an initial “burn-in" period, which is discarded, and the remainder of the run, in which the output is considered to have converged (sufficiently close) to the posterior distribution. Samples from the second part are used to create approximate summary statistics for the posterior distribution, called monitoring. The burn-in period of a JAGS run is the interval between model initialization and the creation of the first monitor.

When a model is initialized, it may be in adaptive mode, meaning that the model is not stable and may modify their behavior for increased efficiency. Therefore, adaptive mode must be turned off at some point during burn-in, and a sufficient number of iterations must take place after the adaptive phase to ensure successful burn-in. By default, adaptive mode is turned off half way through first burn-in of the model. We used 20,000 iterations for burn-in and adaptive mode was switched off after 10,000 iterations using *update* command for both cooking and storing/freezing processes.

Step 5: Monitoring – A monitor in JAGS is an object that records sampled values and monitoring a model refers to generating samples from the posterior distribution of the model parameters. We used *coda.samples* command to generate samples for 20,000 iterations for both the processes and calculate statistics (mean, standard deviation, confidence intervals etc.) for the posterior distributions of all parameters. After the test of convergence (section 2.4.1), we increased the iterations for generating samples to 60,000 for cooking process. Using *plot* command in R, the

trace plots were generated to visualize the mixing of chains, accompanied by probability distribution of the mean value of each parameter.

4.3.5 Test for convergence

The Gelman–Rubin diagnostic evaluates MCMC convergence by analyzing the difference between multiple Markov chains (Cowles & Carlin, 1996). The convergence is assessed by comparing the estimated between-chains and within-chain variances for each model parameter. Large differences between these variances indicate no convergence. The potential scale reduction factor (PSRF) is defined to be the ratio of between-chains and within-chains variances. If the M chains have converged to the target posterior distribution, then PSRF should be close to or equal to 1 whereas PSRF greater than 1 indicates no convergence. The lack of convergence can be diagnosed by either increasing adaption and burn-in iterations or increasing iterations for generating samples or combination of both. We estimated convergence in our model by using *gelman.diag* command at significance level of 0.05. The convergence can be visualized by running mean plots. The y and x axes are mean value of the parameter and iteration number, respectively. We used *rmeanplot* command for this task.

4.3.6 Scenario analyses

In order to validate safe cooking and storing/freezing whole cuts of meat, multiple scenarios were included in this study. In scenario 1, we validated different cooking temperature (49 - 64°C) along with the current USDA recommended minimum internal cooking temperature (62.8°C or 145°F) for the complete inactivation of *T. gondii* tissue cysts. The time measurement in the mice

experiments (Cook et al., 2000; Dubey et al., 1990) started once the designated core temperature was reached and consumer reported temperatures probably represent the maximum temperatures reached (FDA, 2017). For that reason, we set the time to 0.01 min while analyzing results in scenario 1. Consumers reported lack of knowledge on the presence of variable temperature points inside a fridge and about 25 - 50% of them confirmed storing fresh meat at 4 - 8°C for uncertain number of days (preferably depended on the occasion or eating preferences) (Balzan et al., 2014). Hence, scenario 2 dealt with the survival of *T. gondii* in fresh meats stored at refrigerated temperature (4°C). One survey reported 64% consumer stored fresh meat cuts in freezer and thawed for 12 h (average) before cooking (Gilbert et al., 2007). Therefore, we evaluated the survival rates of *T. gondii* at different freezing points for storing meat cuts for 24 h in scenario 3.

The amount of meat purchased from a retail shop was assumed to be a PERT distribution with minimum of 250 g, most likely of 500 g and maximum of 2500 g (2.5 kg) based on consumers' entries (FDA, 2017). The initial number of *T. gondii* tissue cysts was calculated from the linear regression equation with amount of meat as serving size (section 4.3.1). All parameters of the multiple linear regression relationship of time and temperature with the survival rate of *T. gondii* were fitted as normal distribution with mean and standard deviation values obtained from the Gibbs sampling process. The remaining numbers of *T. gondii* tissue cysts in meat cuts after cooking and storing/freezing processes were calculated by multiplying the survival rate with the initial number of tissue cysts.

4.4 Results

4.4.1 Bootstrap resampling

The relationship of time and temperature with the survival rate of *T. gondii* tissue cysts in fresh cuts of meat defined by multiple linear regression model (p value < 0.05) by bootstrap resampling technique for cooking ($R^2 = 0.53$) is shown in **Figure 4.1** and for low temperature storage ($R^2 = 0.82$) in **Figure 4.2**. The descriptive statistics of all parameters of the regression equations are provided in **Table 4.4**. The bootstrap results indicated that the meats should be cooked till internal temperature reaches 62.4°C for the complete inactivation of *T. gondii* tissue cysts (survival rate equals to 0). *T. gondii* cysts can remain viable up to 30 - 32 days if stored at refrigerated temperature (4 - 6°C) and get inactivated instantly at deep freezing storage (-18°C) given the center of the meat reaches the respective temperature.

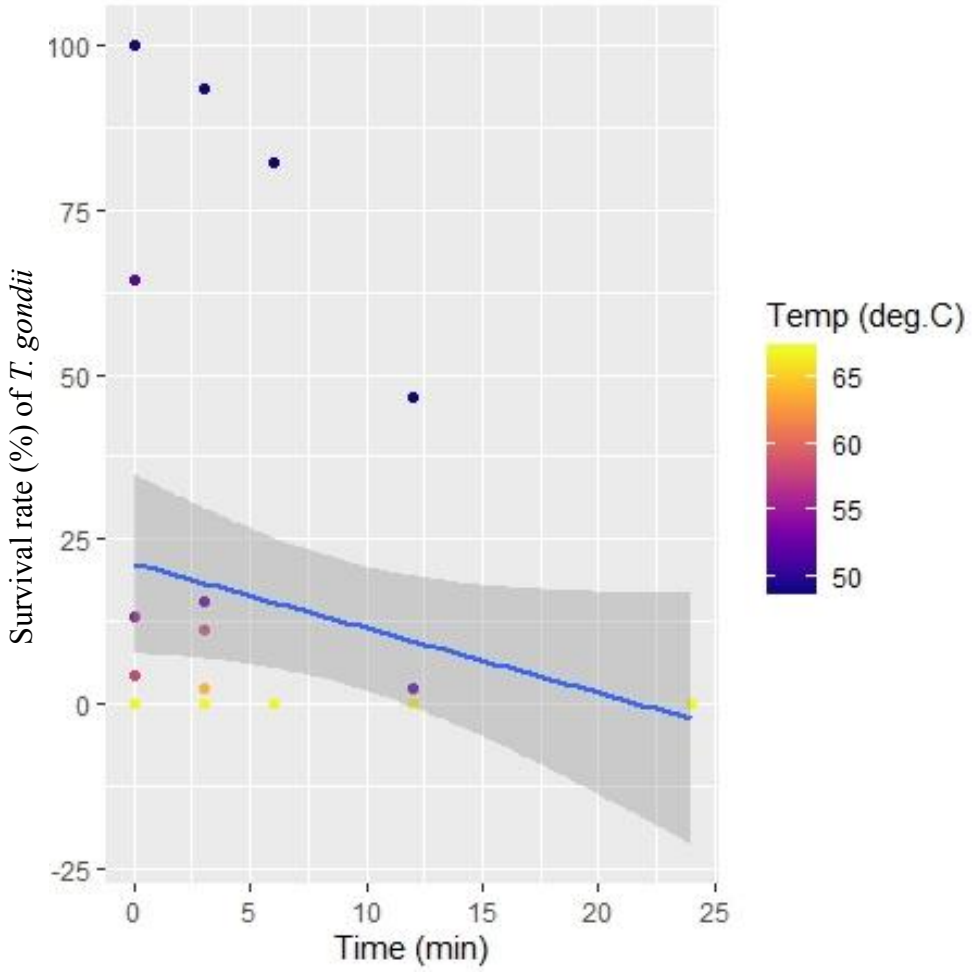

Figure 4.1 Multiple linear regression analysis from bootstrap resampling of time (x-axis) and cooking temperature (dots) with the survival rate (y-axis) of *T. gondii* tissue cysts in fresh meats. The shaded region is the standard error of the regression analysis and side gradient bar is the internal cooking temperature.

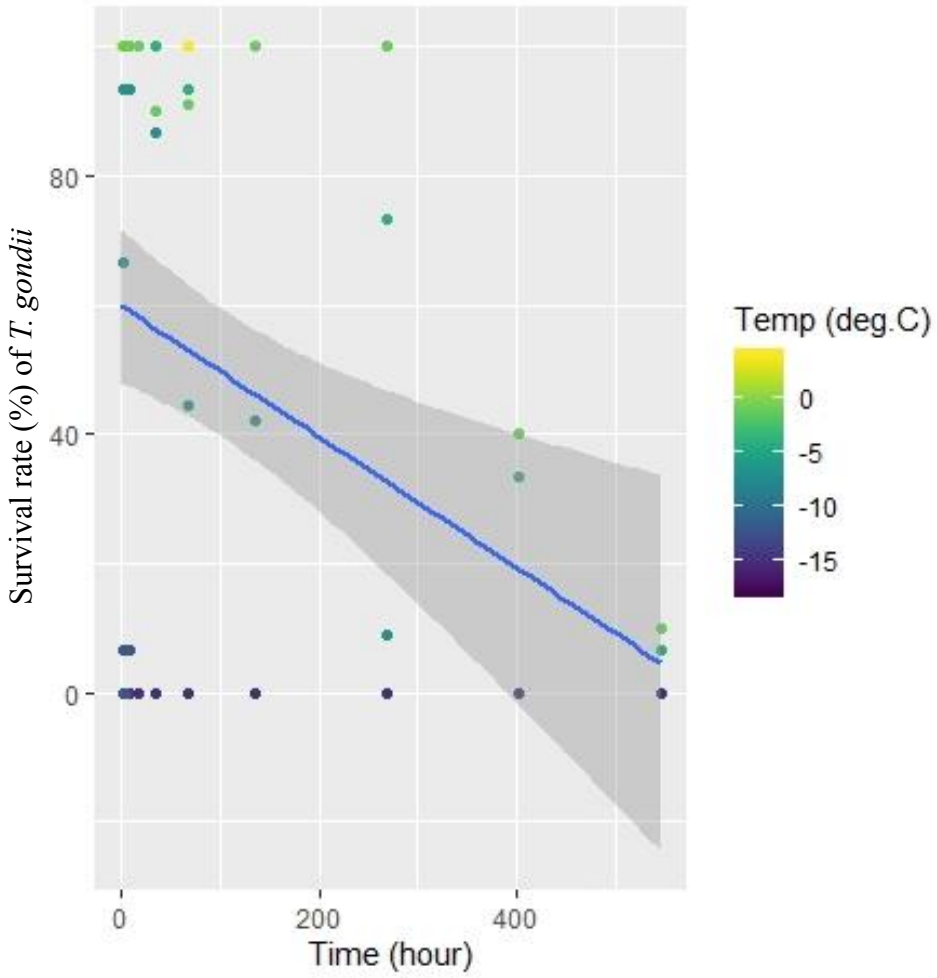

Figure 4.2 Multiple linear regression analysis from bootstrap resampling of time (x-axis) and low storage temperature (dots) with the survival rate (y-axis) of *T. gondii* tissue cysts in fresh meats. The shaded region is the standard error of the regression analysis and side gradient bar is the storage temperature.

Table 4.4 Descriptive statistics of each parameter from bootstrap resampling of the regression analysis of the survival rate of *T. gondii* tissue cysts after cooking and low temperature storage of fresh meats

Coefficients	*Cooking*				*Low temperature storage*			
	Mean	*SE**	*2.5th percentile*	*97.5th percentile*	*Mean*	*SE**	*2.5th percentile*	*97.5th percentile*
β_0 (intercept)	283.72	45.88	192.35	370.76	96.63	3.44	90.13	103.81
β_1 (time coefficient)	-12.73	3.71	-19.58	-5.22	-0.12	0.02	-0.16	-0.07
β_2 (temperature coefficient)	-4.55	0.79	-6.03	-2.96	5.76	0.34	5.06	6.41
β_3 (interaction coefficient)	0.20	0.06	0.07	0.32	-0.01	0.002	-0.01	-0.002

*SE: standard error

4.4.2 Gibbs sampling

The trace plots of the posterior distributions of all parameters of the defined regression model for cooking and low temperature storage processes are shown in **Figure 4.3** and **Figure 4.4**, respectively, and their descriptive statistics are provided in **Table 4.5**. The standard deviation of the posterior distribution of the survival rate of *T. gondii* was estimated to be 20.13% for the cooking process and 20.16% for the low temperature storage process. The Gelman-Rubin diagnostic test assessed the convergence of MCMC chains and the running mean plots of all parameters for the cooking process and low temperature storage process were obtained. The PSRF was equal to 1 for all parameters of the regression equations for both the processes. The mean and standard deviations of all parameters were used to run simulations for scenario analyses.

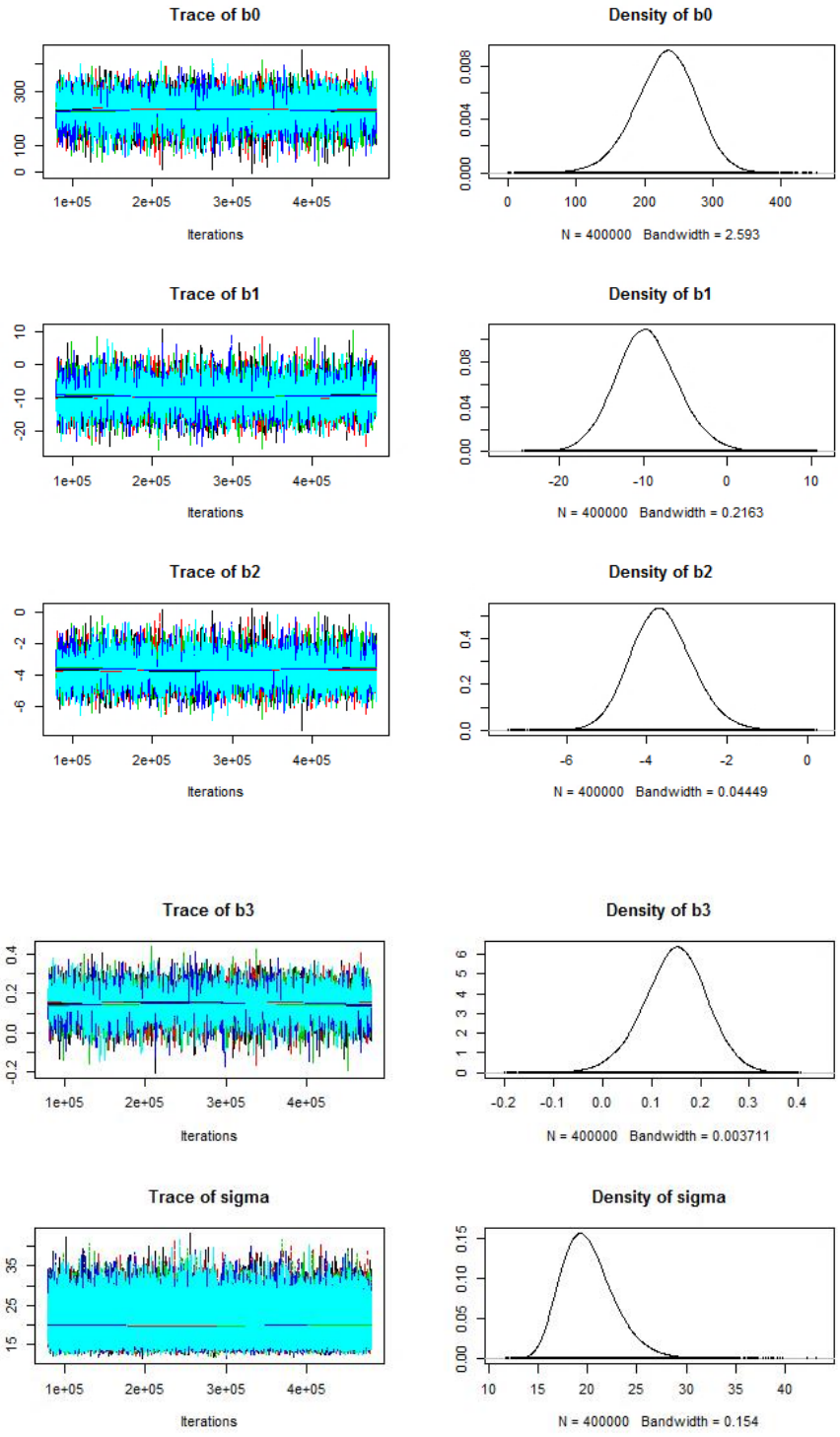

Figure 4.3 Traceplots (left) and posterior distributions (right) of each parameter after Gibbs sampling of multiple linear regression analysis of time and cooking temperature with the survival rate of *T. gondii* in fresh meats. Y-axis: value of each parameter; x-axis: iteration number on the traceplots. Y-axis: probability density; x-axis: value of each parameter on the posterior distribution graphs. Sigma: standard deviation of the regression analysis and N: sampling iterations.

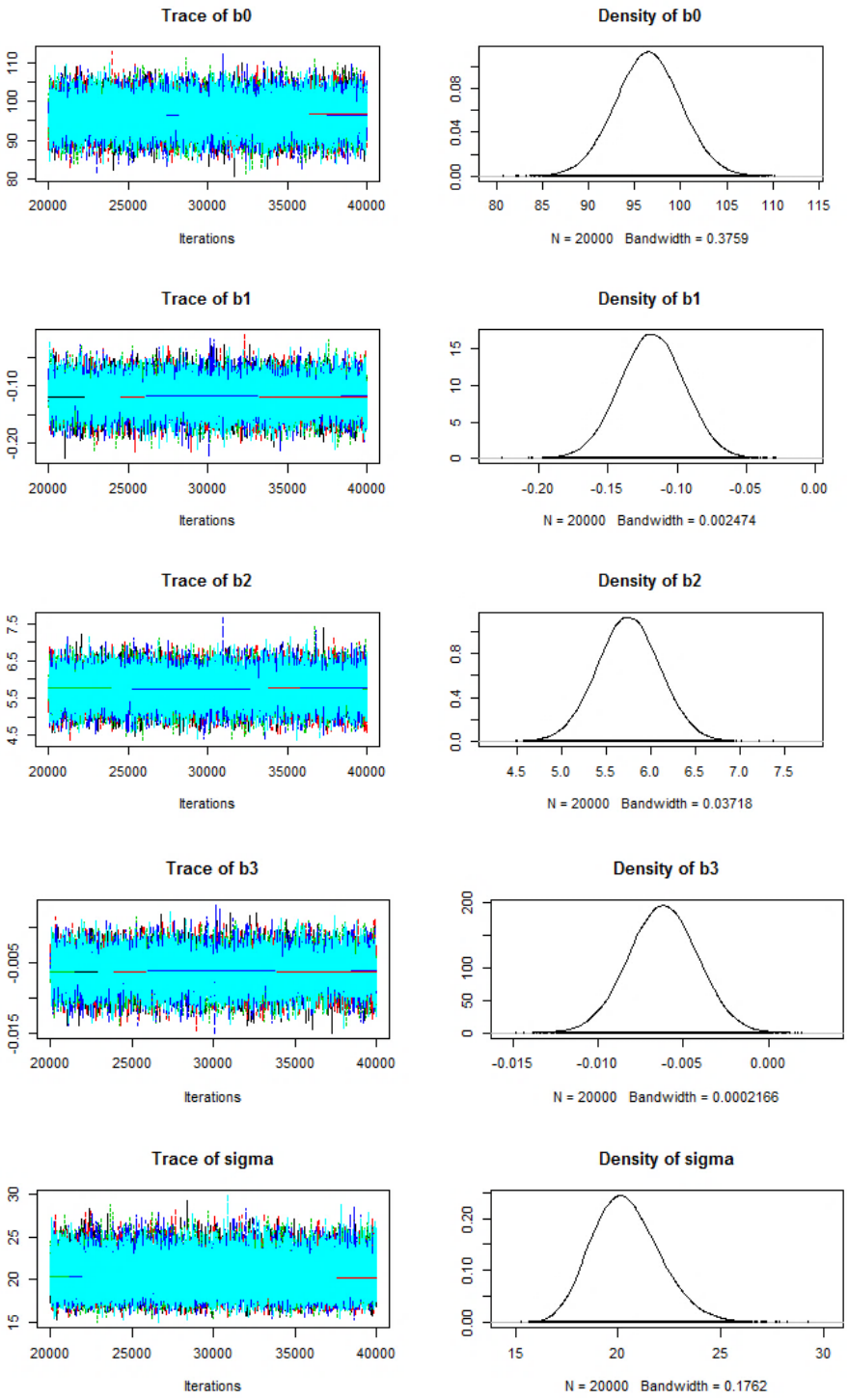

Figure 4.4 Traceplots (left) and posterior distributions (right) of each parameter after Gibbs sampling of the regression analysis of time and low storage temperature with the survival rate of *T. gondii* in fresh meats. Y-axis: value of each parameter; x-axis: iteration number on the traceplots. Y-axis: probability density; x-axis: value of each parameter on the posterior distribution graphs. Sigma: standard deviation of the regression analysis and N: sampling iterations.

Table 4.5 Descriptive statistics of each parameter from Gibbs sampling of the regression analysis of the survival rate of *T. gondii* tissue cysts after cooking and low temperature storage of fresh meats

Coefficients	*Cooking*				*Low temperature storage*			
	Mean	*SD**	*2.5th percentile*	*97.5th percentile*	*Mean*	*SD**	*2.5th percentile*	*97.5th percentile*
β_0 (intercept)	230.60	45.15	138.33	315.88	96.52	3.56	89.45	103.48
β_1 (time coefficient)	-9.48	3.78	-16.71	-1.84	-0.12	0.02	-0.16	-0.07
β_2 (temperature coefficient)	-3.62	0.77	-5.08	-2.03	5.76	0.35	5.06	6.44
β_3 (interaction coefficient)	0.15	0.06	0.02	0.27	-0.01	0.002	-0.01	-0.002

*SD: standard deviation

4.4.3 Scenario Analyses

The number of viable *T. gondii* tissue cysts present in the meat was defined by linear regression model with slope as 0.07 and intercept as -23 (R^2 = 0.80, p value < 0.05). The mean value of the amount of meat purchased was estimated as 792 g from PERT distribution. The initial number of viable *T. gondii* tissue cysts in the meat purchased was calculated to be an average of 29.25 with maximum as 133.5 and minimum as 0. On an average 3.32% *T. gondii* tissue cysts survived at 62.8°C and 2.6% survival rate at 63°C (**Table 4.6**). The complete inactivation of *T. gondii* was achieved when the meat was being cooked at or above 64°C (**Table 4.6**).

Table 4.6 Survival rates and surviving number of *T. gondii* tissue cysts in fresh meats after cooking process estimated from Monte Carlo simulation

Temp. (°C)*	Time (min)	Survival rate of *T. gondii* tissue cysts (%)	Number of viable *T. gondii* tissue cysts survived
49	0.01	53.26	15.65
52	0.01	42.4	12.47
55	0.01	31.55	9.3
58	0.01	20.69	6.12
60	0.01	13.45	4.01
61	0.01	9.84	2.95
62	0.01	6.22	1.89
62.8	0.01	3.32	1.05
63	0.01	2.6	0.84
64	0.01	0	0

*represents the internal cooking temperature.

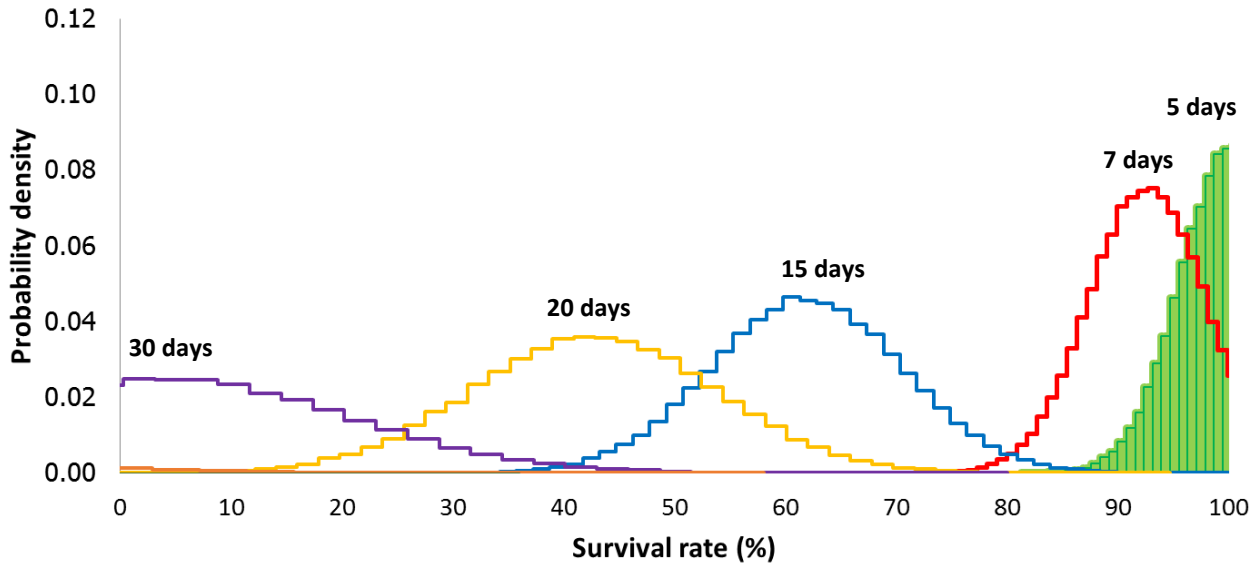

Figure 4.5 Probability distributions of the survival rates of *T. gondii* tissue cysts in meats stored at 4°C for different time periods.

Table 4.7 Survival rates and surviving number of *T. gondii* tissue cysts in fresh meats stored at different low temperature points for different time periods estimated from Monte Carlo simulation

Temp. (°C)	Time (hours)	Survival rate of *T. gondii* tissue cysts (%)	Number of viable *T. gondii* tissue cysts survived
-18	24	0	0
-15	24	12.73	3.72
-12	24	29.00	8.48
-9	24	45.25	13.23
-6	24	61.50	18.00
-4	24	72.35	21.16
-1	24	88.61	25.92
0	24	94.03	27.50
4	24	100	29.25
4	72	100	29.25
4	120	100	29.25
4	168	92.67	27.11
4	360	62.00	18.13
4	480	42.75	12.52
4	720	4.35	1.30
4	1080	0	0

The survival rate pattern of *T. gondii* in meat stored at 4°C for different duration of time is shown in **Figure 4.5**. The cysts can survive up to 30 days with a mean survival rate of 4.35% (**Table 4.7**). All cysts survived (100%; no inactivation) in the meat stored at temperature higher than 4°C. **Figure 4.6** shows the probability distributions of the survival rate of *T. gondii* in meat stored at different deep freezing temperature points for 24 h. *T. gondii* cysts survived up to -15°C at an average survival rate of 12.73% and got completely inactivated at -18°C.

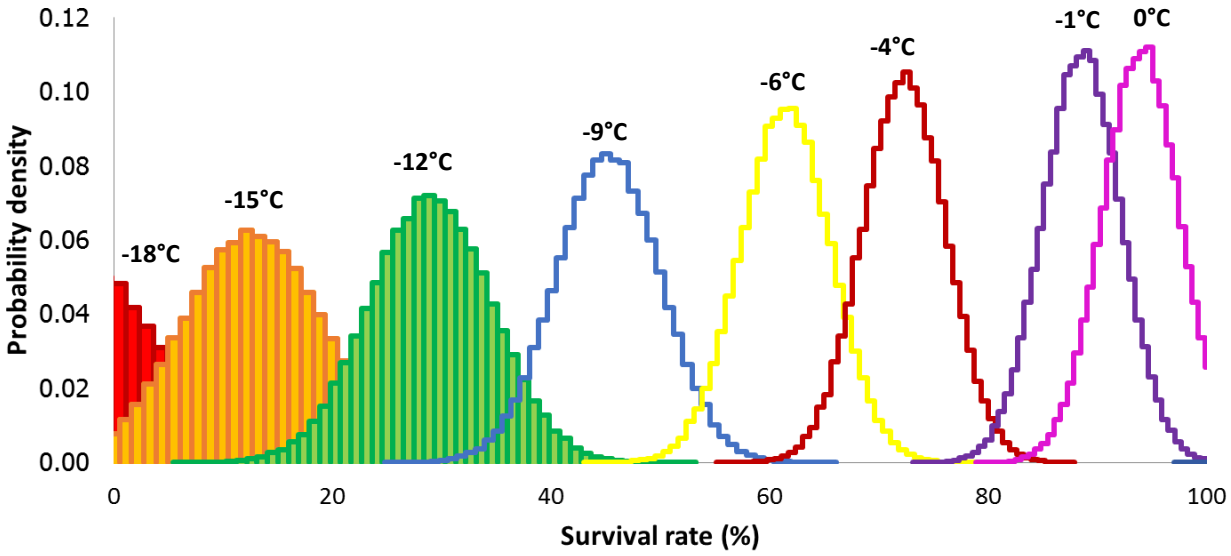

Figure 4.6 Probability distributions of the survival rates of *T. gondii* tissue cysts in meats stored for 24 h at different freezing temperature points.

4.5 Discussion

In this study, we attempted to evaluate the uncertainty and variability associated with the survival rate of *T. gondii* tissue cysts in fresh cuts of meat after cooking and low temperature storage. Our results indicated that cooking of meats above 64°C (**Table 4.6**) would ensure complete inactivation of *T. gondii* as well as storage in deep freezer (-18°C) (**Figure 4.6** and **Table 4.7**). If meat cuts are stored at refrigerated temperature (4 - 6°C), then those should be thoroughly cooked to ensure their safe consumption because *T. gondii* tissue cysts can remain viable up to 30 - 32 days at 4°C (**Figure 4.5** and **Table 4.7**). The Food Safety and Inspection Service (FSIS) of USDA has recommended consumers to cook all whole cuts of meat till the internal temperature reaches 62.8°C (145°F) to ensure safe consumption. In various surveys on consumer knowledge of home food safety practices, only 63% of 1,000 American respondents cooked meat to recommended temperature and 47 of 593 (7.9%) people considered rare or medium cooked pork safe for consumption (Academics of Nutrition and Dietetics, 2011; Bruhn and Schutz, 1999). In our simulation, about 3.32% tissue cysts survived at 62.8°C and one cyst can contain up to 100 - 1000 bradyzoites which is sufficient to cause infection in humans (**Table 4.6**). Furthermore, based on our results, whole cuts of meat should be cooked above 64°C to render all tissue cysts inactive. The U.S. Food and Drug Administration (FDA) has recommended to store fresh meats for 3 - 5 days at refrigerated temperature (4°C) and for 4 – 12 months in deep freezer (-18°C). Studies and surveys reported that 66% of 611 respondents preferred to store meat in freezer at home (Li-Cohen & Bruhn, 2002). Our results are in accordance with the regulations set for storing fresh cuts of meat at low temperature.

The seroprevalence of *T. gondii* antibodies is still relatively common in the U.S. but it has continued to decline. In the NHANES report from 2011 through 2014, the seroprevalence of *T. gondii* antibodies in age adjusted population and women in child bearing age were estimated to be 10.42% and 7.50%, respectively recording almost 2% decline from the 2009 - 2011 survey (Frenkel et al., 1970; Li-Cohen and Bruhn, 2002). With the help of different sampling, we reduced the uncertainty associated with the survival of *T. gondii* in fresh meats at the aforementioned temperature points and with proper dissemination of information to public can help reduce the variability among consumer preferences. A survey designed to assess knowledge of toxoplasmosis among 403 pregnant women in the U.S. revealed that only 30% were aware of the risk of undercooked or raw meat (Jones et al., 2003). Pregnant women need further reinforcement of the risk of handling raw and undercooked meat to lower the risk of congenital toxoplasmosis. Education of women has been shown to be effective in increasing general knowledge of toxoplasmosis and potentially decreasing the incidence of congenital toxoplasmosis (Dubey et al., 1990; Li-Cohen and Bruhn, 2002).

Statistical analyses are time efficient and are not as laborious and expensive as animal experiments. On the other hand, they need computational skills and understanding to interpret the obtained data to make sense. We used bootstrap resampling and Gibbs sampling techniques as there were not many experimental data available on the temperature mediated inactivation of *T. gondii* tissue cysts in fresh cuts of meat. Although theses sampling techniques provided valuable information, they do have some limitations. Bootstrap method doesn't yield reliable results when sample size is too small to make good approximation of the population and when the dataset has multiple outliers (Chernick, 2011). A major limitation towards more widespread implementation

of Bayesian approaches is that obtaining the posterior distribution requires integration of high dimensional functions which is monitored by convergence of Marchov chains (Rasmussen & Ghahramani, 2003). All Gibbs samplers have a built in test to determine whether they have converged to their optimal sampling behavior. To ensure optimal sampling behavior, the model should be run again from scratch using a longer adaptation period. Nevertheless, these techniques are widely used by statisticians to evaluate uncertainty and variability associated with model parameters.

Our simulations results indicated that *T. gondii* tissue cysts may survive (3.32%) in cooked meat at the internal cooking temperature of 62.8°C. Freezing for at least 1 day at −18°C allowed the killing of all cysts in fresh cuts of meat. Therefore, the purchase of frozen meat (below −18°C) may be suggested to people who may not prefer eating well done pieces of lamb or goat, on the basis that long periods of freezing at low temperatures should kill all *T. gondii* cysts (Jones & Dubey, 2012). The reduction in prevalence of *T. gondii* infection in the U.S. suggests that efforts to improve quality control by meat producers and improved cat-care hygiene, as well as efforts to increase knowledge about toxoplasmosis among physicians and the public, have been successful in reducing toxoplasmosis risk (Jones et al., 2007). Further reductions in infection could be achieved through spreading awareness on consumer level about cooking and handling raw or undercooked meat. Since more than 90% of acute toxoplasmosis infections are asymptomatic, primary prevention is the best way to lower the risk of congenital infection (Kravetz & Federman, 2005). Our study could provide an aid in reinforcing the risk models to disseminate valuable information on handling raw and undercooked meat and to further mitigate the pubic heath burden of toxoplasmosis.

Chapter 5: Modeling the transmission dynamics of *Toxoplasma gondii* infection in a pig farm setting

5.1 Abstract

Toxoplasma gondii is among the most prevalent parasites globally, with around one third of the human population being infected. Cats are the definite hosts and all other warm blooded species are intermediate hosts of *T. gondii*, which can become infected by ingestion of oocysts from environment or by ingestion of tissues cysts by predation or meat consumption. *T. gondii* has a complex life cycle with multiple sexual and asexual replications. In this study, we investigated the transmission of *T. gondii* on a pig farm with different hosts using a deterministic dynamic compartmental model and evaluated different strategic measures to decrease the *T. gondii* contamination of environment such as decreasing the number of cats on farm and vaccination of cats. The host population was divided into different subpopulation, such as susceptible, infected or recovered depending upon the nature of *T. gondii* infection in them and the infection was assessed over one year. The model parameters were collected through literature search. Limiting the number of cats on farm by more than 70% was more effective in decreasing the environmental contamination as well as the number of infections in pigs and humans as compared to vaccination of cats with vaccine efficacy of 48%. Vaccination of cats showed 15% decrease in the number of infected of pigs, 8.6% decrease in the number of humans and 28.8% less proportion of farm environment carrying *T. gondii* as compared with the base model. Greater decline in the number of infected hosts was observed when the initial number of cats on farm was changed from 15 (base model) to 5 cats (18.6% decrease in pigs, 12% in humans and 38% in environmental

contamination). With highest oocysts contamination of the farm in the spring season, all hosts were observed to have higher number of infected individuals in summer season. This model helped understanding the life cycle of *T. gondii* with more focus on evaluation of mitigation measures.

5.2 Introduction

Toxoplasma gondii is a widely spread protozoan that infects almost all warm-blooded mammals including humans and causes a disease condition, known as toxoplasmosis (Dubey, 2010). It has been reported as the fourth leading cause of hospitalization and the second leading cause of death among 31 major foodborne pathogens in the U.S., estimating about 4,428 hospitalizations and 327 deaths annually (Scallan et al., 2011). Human toxoplasmosis can be congenitally and postnatally acquired (Hill & Dubey, 2002). The most common manifestations of congenital toxoplasmosis are ocular disease and neurological damage and it could be fatal as well (Peyron et al., 2017). Postnatally acquired infections are more severe in immune compromised individuals (HIV patients) and in individuals with immune suppressed condition such as during organ transplant causing lymphadenopathy (enlargement of lymph nodes), encephalitis and can also result in deaths (Nissapatorn et al., 2004).

T. gondii has a complex life cycle and has drawn attention of researchers in disciplines from epidemiology, immunology, human behavior, cell biology and parasitology (Ferguson, 2009). The life cycle of *T. gondii* involves multiple hosts and includes sexual and asexual replication (Dubey, 2009). Felids such as cats, are the definitive hosts, where the parasite completes its sexual replication. Cats usually shed the oocyst form of the parasite in their feces for one to two weeks after infection which on sporulation become quite resistant to environmental

changes; they can remain infective in a moist environment for a year or more (Frenkel et al., 1970). All warm-blooded vertebrates including humans serve as intermediate hosts, allowing asexual replication of the parasite during the course of infection (Dubey, 2010). Environmental contamination, like water or soil contaminated with *T. gondii* oocysts, is a major source of *T. gondii* infection for animals and humans (Du et al., 2012). Humans are accidental hosts and are majorly infected by consuming raw or undercooked meat containing *T. gondii* tissue cysts or ingesting food, soil, or water contaminated by *T. gondii* oocysts (Weiss & Dubey, 2009).

Although different aspects of the *T. gondii* life cycle have been intensively investigated, the overall transmission dynamics of this parasite and its infection has not been well studied in a farm-to-fork scenario. Mathematical models allow us to understand the global dynamical behavior of toxoplasmosis. Prevention and control strategies can be modeled using these numerical simulations. A few mathematical models have been built to understand the transmission dynamics of *T. gondii* in different host populations (Aranda et al., 2008; Arenas et al., 2010; Jiang et al., 2012; Mateus-Pinilla et al., 2002). However, these models did not consider the complete life cycle of *T. gondii*. In this study, our attempt was to develop a deterministic model, using the compartmental modeling, for the transmission of *T. gondii* infection in a hypothetical pig farm including different hosts (cats as definite host and rats, pigs and humans as intermediate hosts) with environment as the reservoir of *T. gondii* oocysts. Further, different scenarios were performed to evaluate the common strategic measures to decrease the environmental contamination of *T. gondii*.

5.3 Materials and methods

5.3.1 Routes of *T. gondii* transmission

To describe the complete life cycle of *T. gondii* and study the infection flow in different hosts, we considered the spread of the parasite on a hypothetical pig farm consisting of cats, rats and humans. A schematic representation of the life cycle of *T. gondii* on the farm model and common routes of contamination is shown in **Figure 5.1**.

In the model, an infected cat sheds feces containing *T. gondii* oocysts in the farm environment. The oocysts survive and decay in the farm environment as well as infect all hosts at the same time. When a rat comes into contact with the contaminated environment, it ingests the oocysts and gets infected. The ingested oocysts go under structural modifications inside a host's body and become tissue cysts (Dubey, 2010). The cats were infected either by consuming the tissue cysts in a predated infected rat or by ingesting oocysts from the environment (with a much lower probability) (Dubey, 1995). Pigs in the farm were also considered to acquire the infection when they ingest *T. gondii* oocysts from the contaminated environment. Humans were modeled to get infected when they consume pork containing tissue cysts or food or water contaminated with *T. gondii* oocysts.

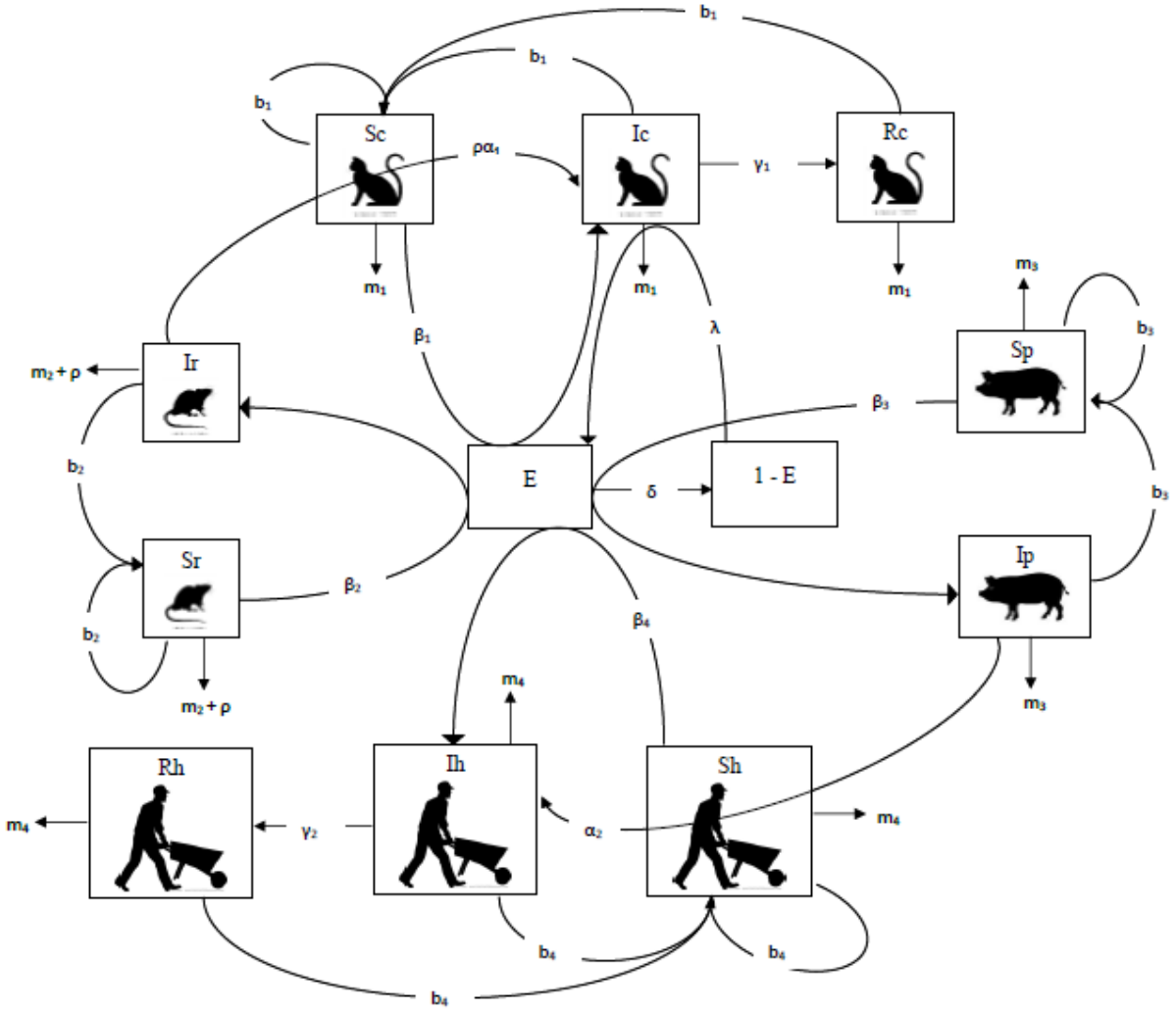

Figure 5.1 A schematic diagram depicting the transmission of *T. gondii* among cats, rats, pigs, humans and farm environment. S_c, I_c and R_c represent the numbers of susceptible, infected and recovered cats, respectively. S_r and I_r stand for the numbers of susceptible and infected rats, respectively. S_p and I_p represent the numbers of susceptible and infected pigs, respectively. S_h, I_h and R_h are the numbers of susceptible, infected and recovered humans, respectively. E and $1 - E$ represent the proportion of contaminated and uncontaminated farm environment, respectively. Definitions of parameters are summarized in **Table 5.1.**

5.3.2 Model description

The model was developed based on the partition of the host population into different subpopulations (compartmental modeling) based on the physiology of *T. gondii* infection in each host. The dynamics of the host subpopulations and environment were described over time by a system of ordinary differential equations (ODEs).

The system of ODEs representing the transmission of *T. gondii* in cats, rats, pigs, humans and farm environment (base model). The definition and values of parameters are given in **Table 5.1**.

($N_c = S_c + I_c + R_c; N_r = S_r + I_r; N_p = S_p + I_p; N_h = S_h + I_h + R_h$)

$$\frac{dS_c}{dt} = b_1 N_c - \beta_1 E S_c I_c - \frac{\rho \alpha_1 I_r S_c}{N_r} - m_1 S_c$$

Eq. (A.1)

$$\frac{dI_c}{dt} = \beta_1 E S_c I_c + \frac{\rho \alpha_1 I_r S_c}{N_r} - \gamma_1 I_c - m_1 I_c$$

Eq. (A.2)

$$\frac{dR_c}{dt} = \gamma_1 I_c - m_1 R_c$$

Eq. (A.3)

$$\frac{dE}{dt} = \lambda I_c (1 - E) - \delta E$$

Eq. (A.4)

$$\frac{dS_r}{dt} = b_2 N_r - \beta_2 E S_r I_r - \frac{\rho S_r N_c}{N_r} - m_2 S_r$$

Eq. (A.5)

$$\frac{dI_r}{dt} = \beta_2 E S_r I_r - \frac{\rho I_r N_c}{N_r} - m_2 I_r$$

Eq. (A.6)

$$\frac{dS_p}{dt} = b_3 N_p - \beta_3 E S_p I_p - m_3 S_p$$

Eq. (A.7)

$$\frac{dI_p}{dt} = \beta_3 E S_p I_p - m_3 I_p$$

Eq. (A.8)

$$\frac{dS_h}{dt} = b_4 N_h - \beta_4 E S_h I_h - \frac{\alpha_2 I_p S_h}{N_p} - m_4 S_h$$

Eq. (A.9)

$$\frac{dI_h}{dt} = \beta_4 E S_h I_h + \frac{\alpha_2 I_p S_h}{N_p} - \gamma_2 I_h - m_4 I_h$$

Eq. (A.10)

$$\frac{dR_h}{dt} = \gamma_2 I_h - m_4 R_h$$

Eq. (A.11)

The system of ODEs representing the transmission of *T. gondii* between cats and environment during vaccination of cats (scenario 2). The rest of the equations representing other hosts like rats, pigs and humans remain same as the base model.

$$\frac{dS_c}{dt} = b_1 N_c - (1-\varphi)\beta_1 E S_c I_c - \frac{(1-\varphi)\rho\alpha_1 I_r S_c}{N_r} - m_1 S_c - \varphi S_c$$

Eq. (A.12)

$$\frac{dI_c}{dt} = (1-\varphi)\beta_1 E S_c I_c + \frac{(1-\varphi)\rho\alpha_1 I_r S_c}{N_r} - \gamma_1 I_c - m_1 I_c$$

Eq. (A.13)

$$\frac{dR_c}{dt} = \varphi S_c + \gamma_1 I_c - m_1 R_c$$

Eq. (A.14)

$$\frac{dE}{dt} = \lambda(1-\varphi) I_c (1-E) - \delta E$$

Eq. (A.15)

For simplicity, we assumed closed population for our model i.e. no net change in the population, and no vertical transmission of *T. gondii* infection i.e. all births were considered to be susceptible. The parameters related to the properties of all hosts and the rules governing various processes, including population dynamics, predation rate, oocyst shedding, infection, recovery etc. were collected from thorough literature search and are listed in **Table 5.1**.

5.3.2.1 Cats

In a field study (Warner, 1985), Warner estimated that the average number of cats on a farm was 6.3 per km². According to the market report published by National Hog Farmer, an average size of pig farm is 1.7 km² (420 acres) (Ketchem & Rix, 2016). We assumed a hypothetical pig farm of 2 km² and accordingly, about 15 cats living on the farm. The cat population was compartmentalized into three divisions which are susceptible (S_c), infected (I_c), or recovered (R_c) (**Figure 5.1**). The ODEs representing the cat dynamics are given in Eq. (A.1) – Eq. (A.3). Susceptible cats were those that were not previously infected and could become infected by ingesting tissue cysts or oocysts.

Table 5.1 Description of parameters used in the dynamics model

Notation	*Parameter*	*Value (unit)*	*Reference*
Cat domain			
b_1	Birth rate	0.081 (litter/ per week)	(Nutter et al., 2004)
m_1	Death rate	Equals to birth rate	Assumption = Closed population
β_1	Rate of transmission from contaminated environment	0.103 (infections/week)	(Dubey, 2006)
α_1	Probability of infection after ingesting infected rat	1	(Dubey, 2010)
γ_1	Rate of recovery	0.5 (per week)	(Dubey, 1995)
φ	Vaccine efficacy	0.48	(Mateus-Pinilla et al., 1999)
Rat domain			
b_2	Birth rate	0.115 (litter/ per week)	(Lélu et al., 2010)
m_2	Death rate	0.038 (rat/week)	(Lélu et al., 2010)
β_2	Rate of transmission from contaminated environment	0.025 (infections/week)	(Lélu et al., 2010)
ρ	Rate of predation	0.69 (rat/cat per week)	Calculation (section 2.2.2)
Pig domain			
m_3	Death rate	0.038 (pig/week)	(USDA APHIS, 2011)
b_3	Birth rate	Equals to death rate	Assumption = Closed population
β_3	Rate of transmission from contaminated environment	0.0196 (infections/week)	(Mateus-Pinilla et al., 2002)
Human domain			
b_4	Birth rate	0.0044 (child/week)	(CDC NCHS, 2017)
m_4	Death rate	Equals to birth rate	Assumption = Closed population
β_4	Rate of transmission from contaminated environment	0.0091 (infections/week)	Calculation (section 2.2.4)
α_2	Probability of infection after consuming contaminated pork	0.0013	(Scallan et al., 2011) (Jones & Holland, 2010)
γ_2	Rate of recovery	0.125 (per week)	(CDC, 2018)

Environmental contamination			
λ	Rate of oocyst shedding by one infected cat	0.0625	(Afonso et al., 2008)
δ	Rate of oocyst decay	0.07 (per week)	(Dumètre & Dardé, 2003)

After infection, the cats shed *T. gondii* oocysts in their feces for 10 – 15 days (Dubey, 2010). Then, the cats were assumed to recover (γ_1) from the infection symptoms after two weeks and attain lifelong immunity to *T. gondii* with no chance of re-infection (Dubey, 1995). A study of 2,332 female cats revealed an average of 1.4 litters/year with litters averaging 3 kittens/litter (Nutter et al., 2004); thus, the birth rate (b_1) was calculated by multiplying average litter per year and litter size (**Table 5.1**). The birth rate was equal to the death rate (m_1) as we assumed no net change in the population. Given an extremely high probability of a cat getting infected and shedding oocysts after predating an infected rat (Dubey, 2010), we assumed the probability (α_1) as 1. The probability of a cat shedding oocysts after ingesting oocysts was 1/29, extracted from a study where total 29 cats were fed oocysts and only one cat shed oocysts (Dubey, 2006). We multiplied this probability with three contacts per week (assumption) to calculate the rate of transmission from contaminated environment (β_1) as 0.103 infections per week (**Table 5.1**). We set susceptible cats as 13, infected as 2 and recovered as 0 to begin the simulation.

5.3.2.2 Rats

We assumed the population of rats to be nine times the population of cats (total 135 rats) based on the average host populations obtained after 100 simulation trials in (Jiang et al., 2012). According to the nature of *T. gondii* infection in rats, the rats were assumed to be either susceptible (S_r) or infected (I_r). The ODEs defining the rat dynamics are listed in Eq. (A.5) and Eq. (A.6). A susceptible rat became infected through ingesting oocysts from the farm environment. A rat would stay in the infected status permanently until it dies (Dubey, 2006). For the birth rate, we chose average parameters of birth rate (b_2) as 0.115 rat/week and death rate (m_2) as 0.038 rat/week in accordance with the fecundity and survival observed for rodents (M. Lélu et al., 2010).

The predation rate (ρ) was assumed to be the difference of birth rate and death rate of rats and multiplying it with the ratio of number of rats and cats present in the farm to satisfy the assumption of closed population (**Figure 5.1**). The predation rate was calculated as 0.69 rat/cat per week, i.e. 36 rat/cat per year which was under the range of 0 to 52 prey/cat per year as estimated in a study on predation rates of domestic cats in suburban areas (Gillies & Clout, 2003). We used the probability of infection from contaminated environment for rats 0.008 (0.4/52) as given in (Lélu et al., 2010) and assuming 3 contacts per week with the contaminated farm environment, we calculated the rate of transmission from environment (β_2) as 0.025 (**Table 5.1**). In our model, we initiated our simulation with 5 infected and 130 susceptible rats.

5.3.2.3 Pigs

According to Swine Management Services database, pig farms in the U.S. have 22+ pigs to maintain the constant supply demand to the meat industry. Thus, we assumed to have 30 pigs in our farm (Ketchem & Rix, 2016). The pig population was assumed to be susceptible (S_p) and infected (I_p). The ODEs representing the pig dynamics are listed in Eq. (A.7) and Eq. (A.8). According to the USDA, the average age of finishing pigs is 26 weeks (USDA APHIS, 2011). Although the life expectancy of domestic pigs are 15-20 years, the farm pigs are slaughtered much early (26 weeks) for human consumption; therefore we used 0.038 per week (1/26) as the death rate (m_3) as well as the birth rate (b_3) of pigs in our model (**Table 5.1**). The seroprevalence of *T. gondii* in market pigs and pig products is low (Dubey et al., 2005; Dubey et al., 2020).

The infection was assumed solely from environment and the weekly probability of infection for the pigs was calculated by dividing seroprevalence of *T. gondii* (0.056 according to Dubey et al., 2005) in market-age finishing pigs by the period of potential exposure to *T. gondii* (20 weeks) (this equation is given in Mateus-Pinilla et al., 2002). We assumed that the pigs were raised in free-range housing system where they were allowed to have access to the outdoors; once a day coming in contact with the *T. gondii* contaminated environment (7 contacts per week). Thus, the rate of transmission from contaminated farm environment (β_3) was calculateed as 0.0196 infections per week (**Table 5.1**). The simulation was started with 29 susceptible pigs and 1 infected.

5.3.2.4 Humans

According to the CDC National Center for Health Statistics (NCHS), the birth rate for humans is 11.6 per 1000 people annually (CDC NCHS, 2017). We calculated the birth rate (b_4) accordingly for group of 50 people working on the farm and this rate was also used as death rate (closed population). The human population was divided into susceptible (S_h), infected (I_h) and recovered (R_h) subpopulation. The ODEs defining the human dynamics are given in Eq. (A.9) - Eq. (A.11). For simplicity, we assumed that the human infections occurred only due to two routes of contamination, by consuming tissue cysts in undercooked pork or by ingesting oocysts from the contaminated farm environment (**Figure 5.1**). The proportion of *T. gondii* infection attributed to food was estimated to be 0.5 (Scallan et al., 2011). We assumed the proportion of infection attributed to the contaminated farm environment as 0.5 as well. The overall infection rate of *T. gondii* for 50 people was 2.6×10^{-3} infections/year as calculated accordingly (Jones & Holland, 2010).

The rate of infection after consuming contaminated pork (α_2) was calculated by multiplying the proportion of infection by overall infection rate (**Table 5.1**). Assuming once a day contact with the contaminated farm environment (7 contacts per week), the rate of transmission from the environment (β_4) was estimated to be 0.0091 infections per week. According to CDC, a healthy adult shows mild flu-like symptoms which usually last for weeks to couple of months (CDC, 2018); therefore, we assumed the recovery rate (γ_2) to be 0.125 per week (**Table 5.1**). The simulation started with 49 susceptible, 1 infected and 0 recovered humans.

5.3.2.5 Farm environment

From a 15-week survey, (Afonso et al., 2008), the authors estimated that the proportion of contaminated defecating sites was 5/16 (0.3125), we used this as the contaminated proportion (E) of the farm in our model. The same study observed that a given cat used on an average 2 of the 16 identified defecating sites. We assumed that, over one week, an infected cat contaminated half of the defecating sites; thus the contaminated rate (λ) was 1/16 (**Table 5.1**). From (Dumètre & Dardé, 2003), we averaged the survival of the parasite in soil at 100 days; the decontamination or decay rate (δ) of *T. gondii* oocysts was used as 0.07 per week (**Table 5.1**).We also assumed a homogeneous presence of the defecating sites by cats and derived an equation for the contamination dynamics of the defecating sites similarly to (Berthier et al., 2000) (Eq (A.4)). Uncontaminated defecating sites are contaminated by infected cats at a rate (λ), and contaminated areas decontaminate over time at a rate (δ).

5.3.3 Basic reproductive number (R_0)

R_0 is defined as the average number of new cases of an infection caused by one typical infected individual, in a population consisting of susceptibles only (Diekmann et al., 2010). In this paper we studied the stability of the steady states of the system in a disease free equilibrium and this basic reproductive number can be seen as a measure of the capacity of cats to spread toxoplasmosis. If $R_0 < 1$, the disease-free equilibrium is stable and toxoplasmosis always dies out. If $R_0 > 1$, there exists a unique endemic equilibrium which is globally stable and the disease persists if it initially exists, supported by the theoretical and numerical simulations. Since cats are the definite host of *T. gondii*, we divided the whole farm into 2 groups; group 1 with cats, rats and

farm environment as dynamics of pigs and humans do not affect the epidemic of toxoplasmosis and group 2 with all hosts together. We used the methodology in (Diekmann et al., 1990; Diekmann & Heesterbeek, 2000; van den Driessche & Watmough, 2002) to compute the basic reproductive numbers.

5.3.4 Scenario analysis

We performed three scenario analysis to evaluate the transmission dynamics of *T. gondii* infection. In scenario 1, the initial cat population was randomly changed by 5, 10, 20 and 25 and compared with the base model (base model had 15 cats) to evaluate the impact of variation in cat population on the spread of *T. gondii* infection in other hosts. The scenario 2 was to evaluate the effect of vaccination of cats on the environmental contamination as well as on the spread of infection in other hosts, rats, pigs and humans. Based on the probability of seroconversion estimated from a field trail of feline T-263 vaccine (Mateus-Pinilla et al., 1999), the vaccine efficacy (φ) was used as 48% in our model (**Table 5.1**). We assumed that the vaccinated cats would be immune for life and not shed oocysts ever. They would directly go into recovered subpopulation from susceptible (Eq. (A.12) – Eq. (A. 15)). In the last scenario, we evaluated the effect of the seasonal variation on the transmission of infection in all host populations as well as environmental contamination of the farm in our model. The field study on feral cats showed that the most births occur from March to May (spring, 47%), from June to August (summer, 34%) and with only 11% during September–November (fall/autumn) and 8% occurring during December–February (winter) (Warner, 1985). A one year survey was conducted to determine the influence of seasonal variation on the contamination of *T. gondii* oocysts in soil and determined that the prevalence of *T. gondii* oocysts in soil gradually declined from spring to winter (34.85% in spring,

28.57% in summer, 16.67% in fall/autumn and 11.11% in winter (Du et al., 2012)). This seasonal prevalence data was used to estimate the rate of oocysts shedding by cats in scenario 3. All data analysis and computations were performed in R statistical software, Version 3.6.2 (R Core Team, 2019). The system of ordinary differential equations were solved using "desolve" package and plots were generated by "matplot" function. The R code for this model will be available on request to the corresponding author.

5.4 Results

Our model evaluating the transmission dynamics of *T. gondii* infection in different hosts achieved equilibrium within a period of 1 year. The respective infection flow of *T. gondii* and distribution of subpopulations according to the infection status in cats, rats, pigs and humans are shown in **Figure 5.2**. The number of infected cat reached a maximum of 9 out of 15 (60%) in around 2 weeks (**Figure 5.2A**). The rat population achieved equilibrium in 6 weeks with number of infected rats reaching 128 out of 135 (95%) (**Figure 5.2B**). Similarly the pig population reached a steady state in 23 weeks with total 27 out of 30 (90%) pigs getting infected (**Figure 5.2C**). The infected human population showed a peak after 16 weeks with a total of 17 out of 50 (34%) (**Figure 5.2D**). With the calculated predation rate and transmission rate of infection from environment, the infection from *T. gondii* was expected to persist ($R_0 > 1$) in both the groups of hosts.

The results of scenario 1 evaluating the impact of variation in host population on the spread of infection are shown in **Table 5.2**. There was 5% decrease in the number of infected pigs when number of cats changed to 10 and 18.6% when the number of cats changed to five. Similarly, 3% and 12% decrease was observed in the number of infected humans with number of cats 10 and

five, respectively. The environmental contamination also declined by 13.6% with 10 cats and 38% with five cats as compared with the base model. Increasing the number of cats did not drastically increased the number of infected hosts or the environmental contamination.

The impact of vaccination of cats on environmental contamination as well as on the infection flow in other hosts are shown in **Table 5.3**. The 48% efficacy of vaccines in cats showed around 28.8% decrease in the proportion of farm getting contaminated by *T. gondii* oocysts (**Figure 5.3** and **Table 5.3**). Vaccination of cats showed 15% decrease in the number of infected of pigs and 8.6% decrease in the number of humans as compared with the base model data (**Table 5.3**). With highest oocysts contamination of the farm in the spring season (**Figure 5.4**), all hosts were observed to have higher number of infected individuals in summer season (**Table 5.4**).

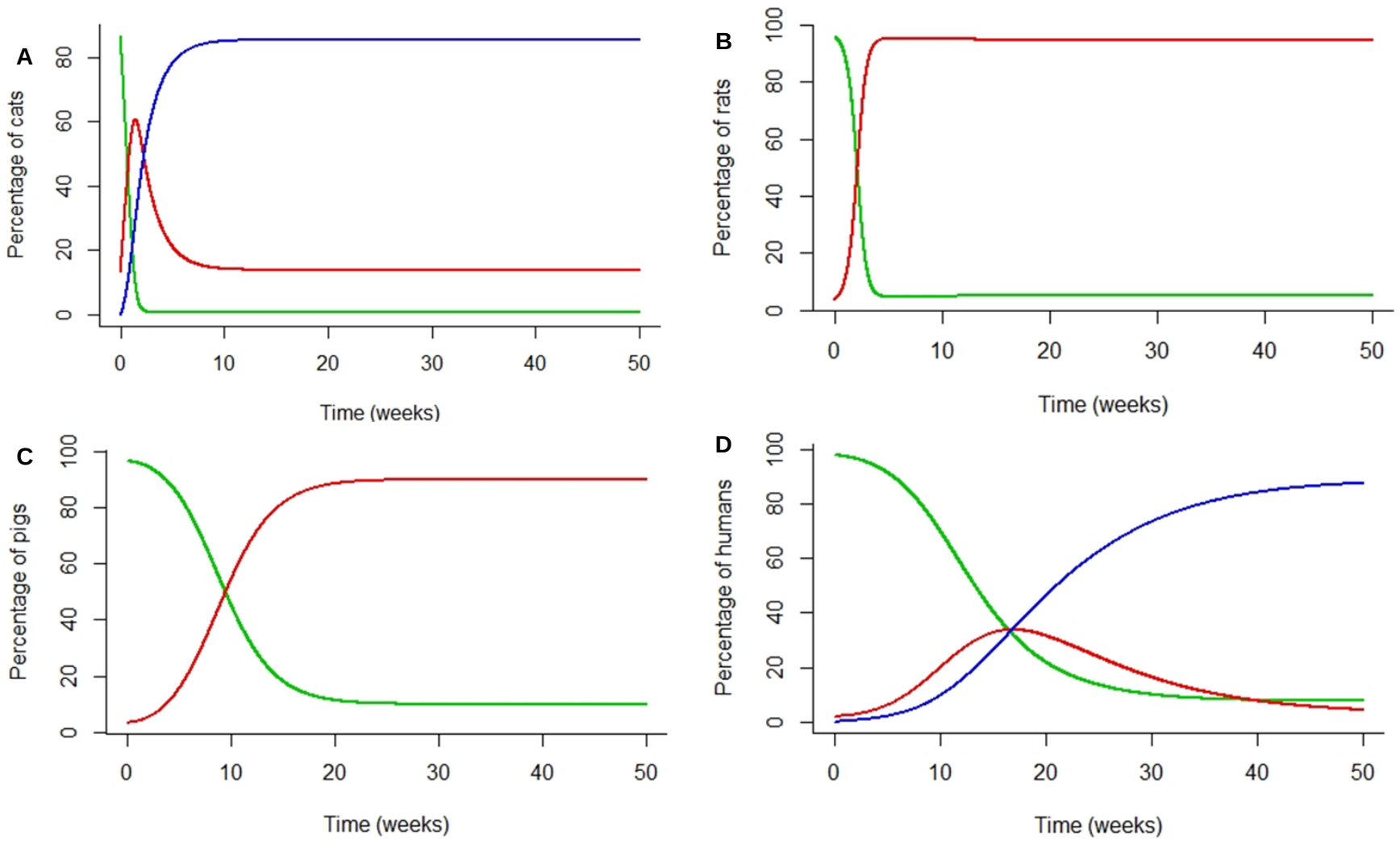

Figure 5.2 Dynamics of the different subpopulations of hosts (A, cats; B, rats; C, pigs; D, humans) due to the transmission of *T. gondii* infection over a period of 52 weeks (base model). Green line represents susceptible, red line represents infected and blue line represents recovered.

Table 5.2 Impact of variation of the initial cat population on the transmission of *T. gondii* infection in different hosts and on the environmental contamination (scenario 1)

Host Type	**Host subpopulation**	**N_c^* = 5**	**N_c = 10**	**N_c = 15 (base model)**	**N_c = 20**	**N_c = 25**
		Mean (95% CI#)	*Mean (95% CI)*	*Mean (95% CI)*	*Mean (95% CI)*	*Mean (95% CI)*
Cat	*Susceptible*	1.09 (0.04, 2.94)	2.21 (0.07, 7.82)	3.34 (0.12, 12.7)	5.46 (0.13, 17.6)	6.41 (0.16, 22.5)
	Infected	0.83 (0.69, 2.07)	1.66 (1.38, 3.74)	2.48 (2.07, 5.52)	3.32 (2.77, 7.3)	4.15 (3.46, 9.03)
	Recovered	3.07 (2.74, 4.27)	7.13 (5.35, 8.55)	10.2 (7.93, 12.8)	12.3 (11, 17.1)	15.4 (13.9, 21.4)
Rat	*Susceptible*	17.5 (9.61, 76.4)	13.6 (7.12, 57.8)	12.1 (6.21, 44.5)	11.2 (5.76, 32.1)	10.6 (5.5, 24.7)
	Infected	117.5 (58.7, 125.4)	121.4 (77.2, 128)	123 (90.5, 128.8)	123.8 (103, 129.2)	124.4 (110.3, 129.5)
Pig	*Susceptible*	12 (5.07, 28.4)	9.02 (3.5, 28.3)	7.91 (2.98, 28.1)	7.3 (2.72, 28)	6.93 (2.57, 27.8)
	Infected	18 (1.59, 24.9)	21 (1.74, 26.5)	22.1 (1.88, 27)	22.7 (2.04, 27.3)	23.1 (2.17, 27.4)
Human	*Susceptible*	24 (7.22, 48.3)	18.5 (4.58, 48.2)	16.3 (3.93, 48.1)	15.1 (3.58, 48)	14.4 (3.36, 48)
	Infected	6.96 (1.34, 11.6)	7.7 (1.44, 15)	7.92 (1.53, 16.7)	8.03 (1.63, 17.7)	8.09 (1.72, 18.3)
	Recovered	19.1 (0.35, 38.5)	23.8 (0.36, 42.5)	25.8 (0.37, 43.6)	27 (0.38, 44.1)	27.6 (0.38, 44.4)
Proportion of contaminated environment		0.41 (0.38, 0.50)	0.57 (0.55, 0.66)	0.66 (0.65, 0.75)	0.72 (0.71, 0.81)	0.76 (0.75, 0.85)

* Nc: total number of cats on the farm; # CI: Confidence interval

Table 5.3 Comparison of the effect on spread of *T. gondii* infection in different hosts after vaccination of cats (scenario 2) with the base model

Host Type	**Host population**	**Base Model**			**Vaccination of cats**		
		Mean	*SD*[*]	*95% CI*[#]	*Mean*	*SD*	*95% CI*
Cat	*Susceptible*	3.34	1.35	(0.12, 12.7)	2.47	1.35	(0.21, 10.6)
	Infected	2.48	1.27	(2.07, 5.52)	1.08	0.56	(1.88, 3.65)
	Recovered	10.2	2.15	(7.93, 12.8)	12.5	1.66	(9.79, 12.9)
Rat	*Susceptible*	12.1	22.1	(6.21, 44.5)	16.4	24.1	(9.22, 83)
	Infected	123	22.1	(90.5, 128.8)	118.6	24.1	(52, 125.8)
Pig	*Susceptible*	7.91	8.49	(2.98, 28.1)	11.2	8.98	(4.16, 28.5)
	Infected	22.1	8.49	(1.88, 27)	18.8	8.98	(1.53, 25.8)
Human	*Susceptible*	16.3	16	(3.93, 48.1)	22.6	15.8	(5.89, 48.4)
	Infected	7.92	5.16	(1.53, 16.7)	7.24	3.74	(1.29, 12.8)
	Recovered	25.8	16.3	(0.37, 43.6)	20.1	14.5	(0.35, 40.1)
Proportion of contaminated environment		0.66	0.05	(0.65, 0.75)	0.47	0.03	(0.44, 0.50)

* SD: Standard deviation; # CI: Confidence interval

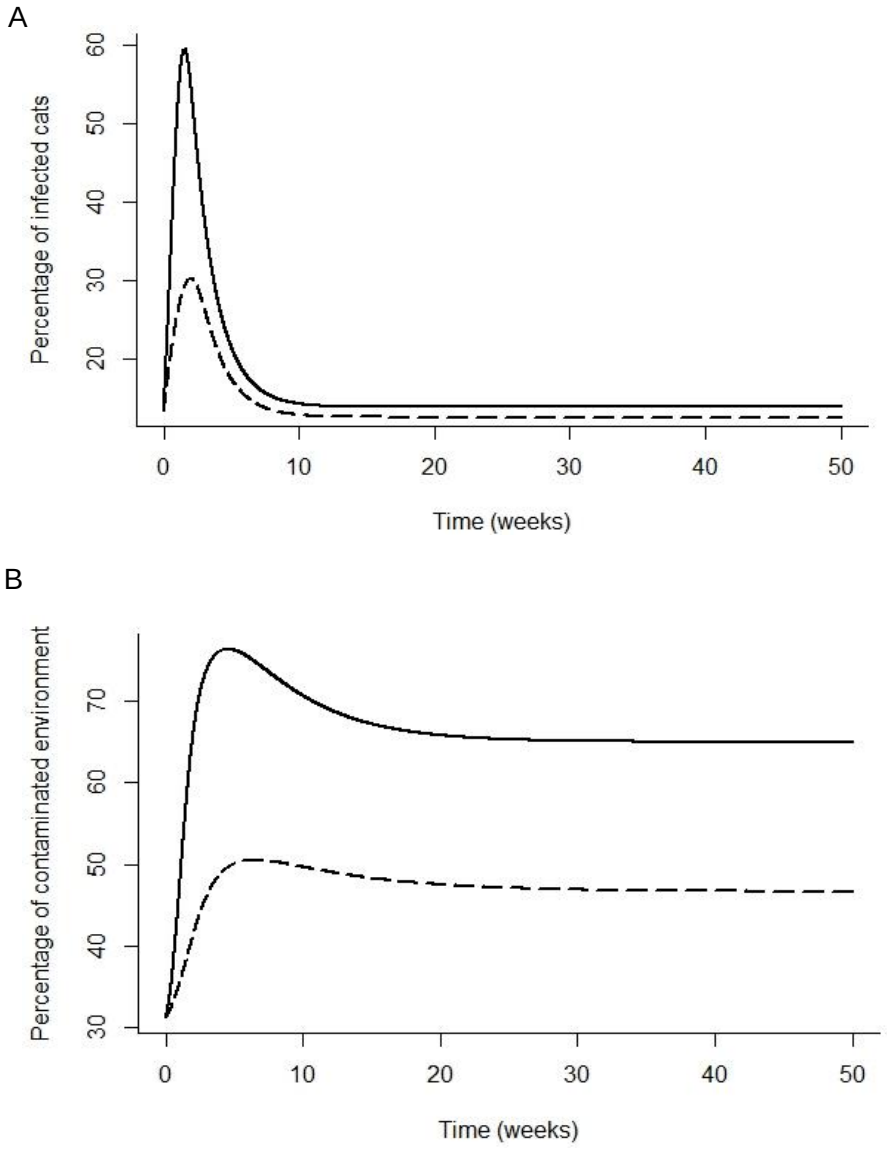

Figure 5.3 Dynamics of infected cats (A) and environmental contamination (B) with *T. gondii* after vaccination of cats. Straight line shows the results of base model and dashed line represents the results of scenario 2.

Table 5.4 Impact of seasonal variation on the infection flow of *T. gondii* in different hosts (scenario 3)

Season	Host population size	Mean	SD*	95% CI#
Spring	*Infected cats*	3.16	2.67	(1.1, 9.46)
	Infected rats	115	35.5	(12.1, 130.2)
	Infected pigs	12.4	8.67	(1.16, 25.2)
	Infected humans	8.53	7.17	(1.1, 18.7)
	Proportion of contaminated environment	0.89	0.1	(0.71, 0.97)
Summer	*Infected cats*	0.83	0.07	(0.78, 1.01)
	Infected rats	127.3	0.41	(127, 128.1)
	Infected pigs	27.3	0.40	(26.2, 27.5)
	Infected humans	15.8	3.44	(10.1, 20)
	Proportion of contaminated environment	0.79	0.03	(0.76, 0.85)
Fall / Autumn	*Infected cats*	0.28	0.14	(0.19, 0.63)
	Infected rats	124.6	1.6	(122, 125)
	Infected pigs	27	0.38	(26.3, 27.5)
	Infected humans	5.63	1.86	(3.22, 9)
	Proportion of contaminated environment	0.59	0.09	(0.46, 0.75)
Winter	*Infected cats*	0.25	0.02	(0.21, 0.26)
	Infected rats	118.8	1.48	(116.8, 121.4)
	Infected pigs	25.4	0.46	(24.7, 26.2)
	Infected humans	2.06	0.45	(1.52, 2.91)
	Proportion of contaminated environment	0.38	0.03	(0.33, 0.44)

* SD: Standard deviation; # CI: Confidence interval

5.5 Discussion

The objective of our study was to understand the transmission dynamics of *T. gondii* during its natural life cycle involving multiple hosts and to assess different strategic measures to mitigate the transmission. To our knowledge, this is the first compartmental model to study the spread of *T. gondii* infection that incorporated all major hosts on a pig farm, such as cats, rats, pigs and humans. Some mathematical models have been published that dealt with the transmission of *T. gondii*.

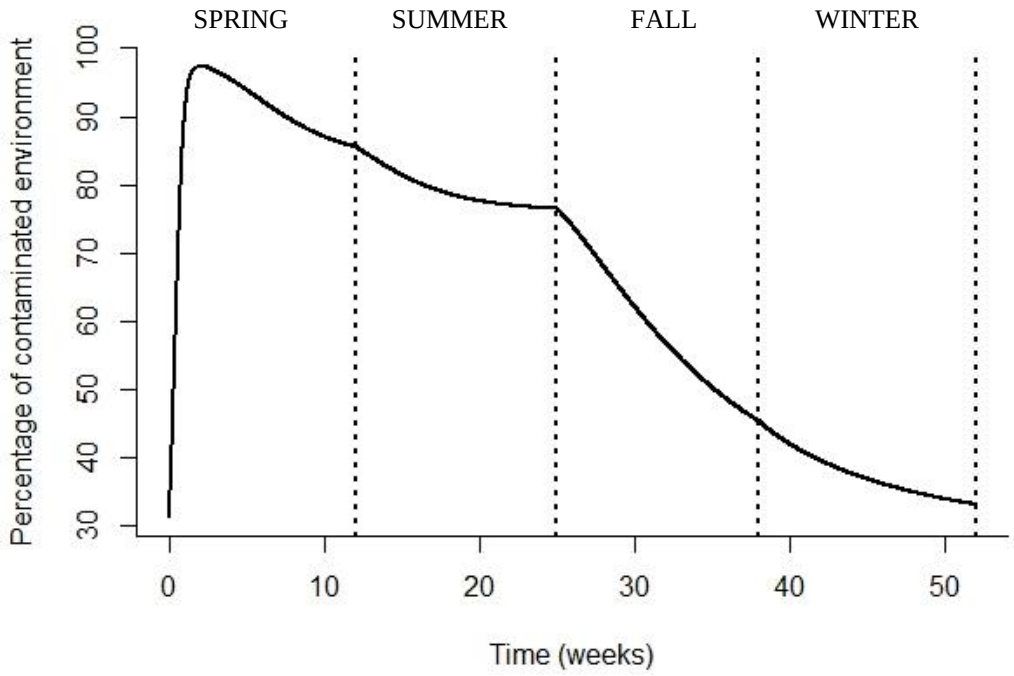

Figure 5.4 Seasonal variation of the percentage of farm environment getting contaminated with *T. gondii* oocysts over a year (52 weeks).

Aranda et al., 2008 were the first to study the evolution dynamics of *T. gondii* only in human population using a basic SIR (susceptible-infected-removed) model. González-Parra et al., 2009 and Arenas et al., 2010 also used SIR models to take into account of the role of cats and the effect of the vaccination of cats. They did not consider the effect of the presence of other hosts such as rats or pigs on the transmission of *T. gondii*. Since the predation of infected rats by cats (ingestion of tissue cysts) has been observed to have higher probability to cause infection in cats as compared to having contact with contaminated environment (ingestion of oocysts) (Dubey, 2006), we incorporated all major intermediate hosts.

Cats are everywhere and their role in increasing the risk of *T. gondii* infection for other hosts have been reported, therefore limiting the number of cats roaming on a farm can substantially decrease the infection (Weigel et al., 1995; Gotteland et al., 2014). Reducing the number of cats reduces the number of susceptible cats and therefore, it reduces future potential shedders of *T. gondii* oocysts into the environment. Thus, cat-population control appears to be a more effective strategy than vaccination of cats in the reduction of *T. gondii* infection in swine. Our results showed less number of infected pigs, rodents and humans in case of cats as low as 10 or five as compared to vaccinating the cats on the farm (**Table 5.2** and **Table 5.3**). The proportion of farm environment contaminated with *T. gondii* oocysts also declined more in case of limiting the number of cats on farm as compared to vaccinating them. No age and gender differentiation of cats was taken into account as both young and old cats, even suckling kittens, were found shedding *T. gondii* oocysts in nature (Dubey & Carpenter, 1993; Pena et al., 2008) and we assumed that the infection rate was same for all other hosts irrespective of age and gender.

Pigs were included in our model to observe the infection pattern in humans from having contact with oocysts contaminated environment as well as consuming pork meat contaminated with *T. gondii* tissue cysts. We assumed all meat consumed were under-cooked or raw to estimate the worst case scenario of infection in humans. A study on determining the early onset of *T. gondii* tissue cysts formation in experimentally infected pigs, observed tissue cysts formation as early as 7 days after infection (Rani et al., 2019). We started our simulation with 1 infected and 29 susceptible pigs. Our results showed the infection onset in pigs from 10 days (2/30 infected pigs) to reaching maximum (27/30 infected pigs) in 23 weeks (**Figure 5.2**). Farm environment was considered as the reservoir of *T. gondii* oocysts from where all hosts got infected. Given the multiple hosts and their complex dynamics, we attempted to build a deterministic model, which would be computationally reasonable for model calculations and easy to understand model outcomes and results. This model could serve as a precursor for more advanced and probabilistic models in the future to advance the knowledge about *T. gondii* transmission. It is important to mention the significance of the model from a health point of view since it could help design the optimal strategies to tackle *T. gondii* infection in the cat populations which are the main factor in the spread of the disease.

Decreasing oocyst-survival time, decreasing oocyst shedding by cats through vaccination and reducing the number of cats all can reduce the risk of infection with *T. gondii*. It is nearly impossible to alter environmental conditions to decrease oocyst survival. Our study suggests that a decrease in the initial number of farm cats had a greater impact on eliminating *T. gondii* infection in other hosts such rats, finishing pigs and humans than vaccination of cats. The seasonal variation showed that the summer season carried the highest number of infected hosts whereas the spring

season showed the highest proportion of farm environment being contaminated with *T. gondii* oocysts. The R_0 value determined the outcome of the disease being globally stable in all hosts. This model analyzed some of the dynamical behaviors of the *T. gondii* infection in the definite host (cat) as well as the intermediate host (e.g. rat, pig and human) populations.

Chapter 6. Summary and future studies

6.1 Summary

Toxoplasma gondii has been long recognized as a significant foodborne pathogen in terms of public health impact. This project has successfully evaluated the *T. gondii* infection formation and flow in different hosts, assessed the methods to mitigate the public health burden of toxoplasmosis posed by food animals as meat has been identified as an importance source of *T. gondii* infection in humans.

Chapter 2 focused the formation and distribution of *T. gondii* tissue cysts in muscle tissues of food animals. Food animals such as pigs, sheep and goats are the most popular meat consumed in the U.S. that are commonly infected with *T. gondii*. Previous studies have shown that pigs can be easily infected with *T. gondii*, and the parasite can persist in pork for more than two years. Based on the bioassay results, it has been concluded that the tissue cysts can be formed as early as seven days after the infection in experimentally infected pigs. The distribution of viable *T. gondii* varied according to the size of the sample analyzed with larger sample sizes resulting in higher isolation rates. The tissue cysts are unevenly distributed in the muscle samples of naturally infected lambs and goats. Small serving sizes (5 g and 10 g) of meat can contain viable parasite and have the potential to cause *T. gondii* infection if consumed raw or under-cooked.

In Chapter 3, the diagnostic accuracy of direct PCR and bioassay methods were evaluated, since several serological and molecular techniques like PCR have been developed to detect *T.*

gondii in animal samples but bioassay method is still the 'gold standard'. Meta-analysis provides evidence available in the literature to support an outcome. It has been observed that the odds of PCR results being false positive is 1.96 times higher as compared to the results obtained by the gold standard method (bioassay). The direct PCR has different sensitivities and specificities in detecting *T. gondii* in different sample sizes with larger samples having higher sensitivity and lower specificity, irrespective of animal type.

Chapter 4 estimated and validated the relationship of time and temperature for the inactivation of *T. gondii* in fresh cut meats. People often lack knowledge about how to prevent *T. gondii* infection, especially the risks associated with eating or handling raw or undercooked meat. According to our statistical analysis. The complete inactivation of *T. gondii* was computed at or above 64°C (147.2°F) and below -18°C (0°F). However, *T. gondii* tissue cysts may survive (3.32%) in cooked meat at the internal cooking temperature of 62.8℃ i.e. the USDA minimal internal cooking temperature for fresh pork and lamb. The tissue cysts can remain viable up to 30 days at 4°C (39°F). This information can be helpful in improving the risk models to further mitigate the public health burden of toxoplasmosis. Further reductions in infection could be achieved through spreading awareness at the consumer level regarding cooking and safe handling of raw or undercooked meat since primary prevention is the best way to lower the risk of toxoplasmosis.

Chapter 5 explored the dynamics of the transmission of *T. gondii* infection in different hosts such as cats, rats, pigs and humans on a farm using a deterministic dynamic compartmental model. Cats are the definite hosts and all other warm blooded species are intermediate hosts of *T. gondii*, which can become infected by ingestion of oocysts from environment or by ingestion of

tissues cysts by predation or meat consumption. Drastic decline in the environmental contamination as well as in the infection was observed in all hosts as the initial number of cats were decreased. Vaccination of cats also showed decrease in the number of infected hosts but not as good as limiting the number of cats on farm. This model helped understanding the life cycle of *T. gondii* with more focus on evaluation of mitigation measures.

6.2 Future studies

The current study represents our current best knowledge of *T. gondii* infection through ingestion of meat products. Several data gaps were identified and were elaborated in each chapter. Some possible areas of research pertaining to meat-borne *T. gondii* infection are proposed as follows.

1) More studies on understanding the distribution and concentration of *T. gondii* in muscle tissue of a naturally infected meat animals is needed. The formation of *T. gondii* tissue cysts in different animals infected with different stages of the parasite could better help understand the ecology of *T. gondii* infection in food animals.

2) An extensive meta-analysis of diagnostic test accuracy understanding and comparing the characteristics and underlying heterogeneities of different detection tests available for *T. gondii* as compared to the gold standard (bioassay) method is warranted. Separate pooled meta-analysis can be carried out for serological assays and PCR-based methods.

3) More experimental research is needed to investigate the effect of the USDA minimal internal cooking temperature on the survival of *T. gondii* in fresh pork and lamb that

has been reduced from 160°F (71.1°C) to 145°F (62.8°C) with a minimal three-minute rest time since 2011 (USDA FSIS, 2012). The current data have demonstrated that *T. gondii* tissue cysts can be inactivated immediately at internal temperature of 64°C. More experiments should be undertaken in order to verify this result, since it is critical in terms of safe handling of fresh meat.

4) The effects of irradiation, moisture enhancement and curing of meat have proved to be deleterious to *T. gondii* in meat products. Advanced survival module of *T. gondii* could aid in evaluating safe meat processing technology and estimating the inactivation of *T. gondii* tissue cysts in different meat and meat products.

5) More advanced stochastic transmission model of the farms that keep more than one type of animal needs to be developed. Many risk factors could influence the *T. gondii* infection rate in animals, such as the number of farm cats and mice, the virulent of strain, the spread of oocysts, the infection with oocysts and cysts, and the secondary infection. A transmission dynamic model helps us know by controlling which factors, the *T. gondii* infection rate could be most effectively decreased.

6) Furthermore, these transmission models need to be integrated to a quantitative microbial risk assessment (QMRA) model to further assess human infection risk through consumption of meat. This would allow risk assessors to investigate the influence of farm management, and to determine the preventive strategies to mitigate the risk during production.

References

Academics of Nutrition and Dietetics. (2011). *Consumer Knowledge of Home Food Safety Practices Survey*. http://www.eatright.org/~/media/homefoodsafety/multimedia/downloads/consumer knowledge of home food safety practices survey.ashx

Afonso, E., Lemoine, M., Poulle, M. L., Ravat, M. C., Romand, S., Thulliez, P., Villena, I., Aubert, D., Rabilloud, M., Riche, B., & Gilot-Fromont, E. (2008). Spatial distribution of soil contamination by Toxoplasma gondii in relation to cat defecation behaviour in an urban area. *International Journal for Parasitology*, *38*(8–9), 1017–1023. https://doi.org/10.1016/j.ijpara.2008.01.004

Aguirre, A. A., Longcore, T., Barbieri, M., Dabritz, H., Hill, D., Klein, P. N., Lepczyk, C., Lilly, E. L., McLeod, R., Milcarsky, J., Murphy, C. E., Su, C., VanWormer, E., Yolken, R., & Sizemore, G. C. (2019). The One Health approach to toxoplasmosis: Epidemiology, control, and prevention strategies. In *EcoHealth* (Vol. 16, Issue 2, pp. 378–390). Springer New York LLC. https://doi.org/10.1007/s10393-019-01405-7

Almeida, M., Alencar Júnio, L., Carmo, G., Araújo, W., Garcia, M., & Reis AK, V. (2005). Intra-family outbreak of toxoplasmosis, Santa Vitória do Palmar-RS [in Portuguese]. *Boletim Eletrônico Epidemiológico Da Secretária de Vigilância Em Saúde*, *18*, 1–7.

Andreoletti, O., Budka, H., Buncic, S., Colin, P., Collins, J. D., De Koeijer, A., Griffin, J., Havelaar, A., Hope, J., Klein, G., Kruse, H., Magnino, S., Martinez López, A., McLauchlin, J., Nguyen-Thé, C., Noeckler, K., Noerrung, B., Prieto Maradona, M., Roberts, T., & Vanopdenbosch, E. (2007). Scientific opinion of the panel on biological hazards. In *The EFSA Journal* (Vol. 583).

Aranda, D. F., Villanueva, R. J., Arenas, A. J., & González-Parra, G. C. (2008). Mathematical modeling of toxoplasmosis disease in varying size populations. *Computers and Mathematics with Applications*, *56*(3), 690–696. https://doi.org/10.1016/j.camwa.2008.01.008

Arenas, A. J., González-Parra, G., & Villanueva Micó, R. J. (2010). Modeling toxoplasmosis spread in cat populations under vaccination. *Theoretical Population Biology*, *77*(4), 227–237. https://doi.org/10.1016/j.tpb.2010.03.005

Arkush, K. D., Miller, M. A., Leutenegger, C. M., Gardner, I. A., Packham, A. E., Heckeroth, A. R., Tenter, A. M., Barr, B. C., & Conrad, P. A. (2003). Molecular and bioassay-based detection of Toxoplasma gondii oocyst uptake by mussels (Mytilus galloprovincialis). *International Journal for Parasitology*, *33*(10), 1087–1097. https://doi.org/10.1016/S0020-7519(03)00181-4

Balasundaram, M. B., Andavar, R., Palaniswamy, M., & Venkatapathy, N. (2010). Outbreak of acquired ocular toxoplasmosis involving 248 patients. *Archives of Ophthalmology*, *128*(1), 28–32. https://doi.org/10.1001/archophthalmol.2009.354

Balfour, A. H., Fleck, D. G., Hughes, H. P. A., & Sharp, D. (1982). Comparative study of three tests (dye test, indirect haemagglutination test, latex agglutination test) for the detection of antibodies to Toxoplasma gondii in human sera. *Journal of Clinical Pathology*, *35*(2), 228–232. https://doi.org/10.1136/jcp.35.2.228

Balzan, S., Fasolato, L., Cardazzo, B., Berti, G., & Novelli, E. (2014). Cold chain and consumers' practices: Exploratory results of focus group interviews. *Italian Journal of Food Safety*, *3*(4), 4516. https://doi.org/10.4081/IJFS.2014.4516

Baril, L., Ancelle, T., Goulet, V., Thulliez, P., Tirard-Fleury, V., & Carme, B. (1999). Risk

factors for toxoplasma infection in pregnancy: A case-control study in France. *Scandinavian Journal of Infectious Diseases*, *31*(3), 305–309. https://doi.org/10.1080/00365549950163626

Batz, M. B., Hoffmann, S., & Morris, J. G. (2012). Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. *Journal of Food Protection*, *75*(7), 1278–1291. https://doi.org/10.4315/0362-028X.JFP-11-418

Berthier, K., Langlais, M., Auger, P., & Pontier, D. (2000). Dynamics of a feline virus with two transmission modes within exponentially growing host populations. *Proceedings of the Royal Society B: Biological Sciences*, *267*(1457), 2049–2056. https://doi.org/10.1098/rspb.2000.1248

Beverley, J. K. A. (1958). A rational approach to the treatment of toxoplasmic uveitis. *Transactions Ophthal. Soc.*, *78*, 109–121.

Bewick, V., Cheek, L., & Ball, J. (2005). Statistics review 14: Logistic regression. In *Critical Care* (Vol. 9, Issue 1, pp. 112–118). BioMed Central. https://doi.org/10.1186/cc3045

Bezerra, R. A., Carvalho, F. S., Guimarães, L. A., Rocha, D. S., Silva, F. L., Wenceslau, A. A., & Albuquerque, G. R. (2012). Comparison of methods for detection of Toxoplasma gondii in tissues of naturally exposed pigs. *Parasitology Research*, *110*(2), 509–514. https://doi.org/10.1007/s00436-011-2514-1

Boch, J., Rommel, M., Wchschr, K. J.-B. M. T., & 1964, undefined. (1964). Beitrage zur Toxoplasmose des Schweines. I. Ergebnisse künstlicher Toxoplasma-Infektionen be¡ Schweinen. *Berl. Münch. Tierärztl. Wchschr*, *77*, 161–167.

Bossi, P., & Bricaire, F. (2004). Severe acute disseminated toxoplasmosis. In *Lancet* (Vol. 364,

Issue 9434, p. 579). Elsevier Limited. https://doi.org/10.1016/S0140-6736(04)16841-4

Bowie, W. R., King, A. S., Werker, D. H., Isaac-Renton, J. L., Bell, A., Eng, S. B., & Marion, S. A. (1997). Outbreak of toxoplasmosis associated with municipal drinking water. *Lancet*, *350*(9072), 173–177. https://doi.org/10.1016/S0140-6736(96)11105-3

Boyer, K. M., Holfels, E., Roizen, N., Swisher, C., Mack, D., Remington, J., Withers, S., Meier, P., McLeod, R., & the Toxoplasmosis Study Group. (2005). Risk factors for Toxoplasma gondii infection in mothers of infants with congenital toxoplasmosis: Implications for prenatal management and screening. *American Journal of Obstetrics and Gynecology*, *192*(2), 564–571. https://doi.org/10.1016/J.AJOG.2004.07.031

Bruhn, C. M., & Schutz, H. G. (1999). Consumer food safety knowledge and practices. *Journal of Food Safety*, *19*(1), 73–87. https://doi.org/10.1111/j.1745-4565.1999.tb00235.x

CDC. (2018). *CDC - Toxoplasmosis*. https://www.cdc.gov/parasites/toxoplasmosis/

CDC NCHS. (2017). *CDC - NCHS - National Center for Health Statistics*. https://www.cdc.gov/nchs/index.htm

Chernick, M. R. (2011). *Bootstrap Methods A Guide for Practitioners and Researchers*. John Wiley & Sons. https://books.google.com/books?hl=en&lr=&id=UxDKh5Spwp8C&oi=fnd&pg=PT7&dq=Bootstrap+Methods+A+Guide+for+Practitioners+and+Researchers&ots=xDslzlUIW1&sig=-JYOZvZoP0nLn6XNTG7bLxd6880#v=onepage&q=Bootstrap Methods A Guide for Practitioners and

Cook, A. J., Gilbert, R. E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P. A., Foulon, W., Semprini, A. E., Dunn, D. T., & Dunn, D. T. (2000). Sources of toxoplasma infection in pregnant women: European multicentre case-control study. European Research Network on

congenital toxoplasmosis. *British Medical Journal*, *321*(7254), 142–147. https://doi.org/10.1136/bmj.321.7254.142

Coughlin, S. S., Trock, B., Criqui, M. H., Pickle, L. W., Browner, D., & Tefft, M. C. (1992). The logistic modeling of sensitivity, specificity, and predictive value of a diagnostic test. *Journal of Clinical Epidemiology*, *45*(1), 1–7. https://doi.org/10.1016/0895-4356(92)90180-U

Cowles, M. K., & Carlin, B. P. (1996). Markov Chain Monte Carlo convergence diagnostics: A comparative review. *Journal of the American Statistical Association*, *91*(434), 883–904. https://doi.org/10.1080/01621459.1996.10476956

da Silva, A. V., & Langoni, H. (2001). The detection of Toxoplasma gondii by comparing cytology, histopathology, bioassay in mice, and the polymerase chain reaction (PCR). *Veterinary Parasitology*, *97*(3), 193–200. https://doi.org/10.1016/s0304-4017(01)00404-6

Dabritz, H. A., & Conrad, P. A. (2010). Cats and toxoplasma: Implications for public health. In *Zoonoses and Public Health* (Vol. 57, Issue 1, pp. 34–52). https://doi.org/10.1111/j.1863-2378.2009.01273.x

de Meuter, F., Fameree, L., & Cotteleer, C. (1978). Influence de la virulence des souches toxoplasmiques pour la souris sur leur capacite de former des kystes chez le porc. *Ann. Soc. Belge Méd. Trop.*, *58*, 95–102. https://pascal-francis.inist.fr/vibad/index.php?action=getRecordDetail&idt=PASCAL7950195513

de Moura, L., Garcia Bahia-Oliveira, L. M., Wada, M. Y., Jones, J. L., Tuboi, S. H., Carmo, E. H., Ramalho, W. M., Camargo, N. J., Trevisan, R., Graça, R. M. T., Da Silva, A. J., Moura, I., Dubey, J. P., & Garrett, D. O. (2006). Waterborne toxoplasmosis, Brazil, from field to gene. *Emerging Infectious Diseases*, *12*(2), 326–329.

https://doi.org/10.3201/eid1202.041115

Demar, M., Ajzenberg, D., Maubon, D., Djossou, F., Panchoe, D., Punwasi, W., Valery, N., Peneau, C., Daigre, J.-L., Aznar, C., Cottrelle, B., Terzan, L., Darde, M.-L., & Carme, B. (2007). Fatal outbreak of human toxoplasmosis along the Maroni River: Epidemiological, clinical, and parasitological aspects. *Clinical Infectious Diseases*, *45*(7), e88–e95. https://doi.org/10.1086/521246

Demar, M., Hommel, D., Djossou, F., Peneau, C., Boukhari, R., Louvel, D., Bourbigot, A. M., Nasser, V., Ajzenberg, D., Darde, M. L., & Carme, B. (2012). Acute toxoplasmoses in immunocompetent patients hospitalized in an intensive care unit in French Guiana. *Clinical Microbiology and Infection*, *18*(7), E221–E231. https://doi.org/10.1111/j.1469-0691.2011.03648.x

Desmonts, G., & Couvreur, J. (1974). Toxoplasmosis in pregnancy and its transmission to the fetus. *Bulletin of the New York Academy of Medicine*, *50*(2), 146–159. http://www.ncbi.nlm.nih.gov/pubmed/4521728

Diekmann, O., & Heesterbeek, J. A. P. (2000). *Mathematical Epidemiology of Infectious Diseases: model building, analysis and interpretation* (Vol. 5). John Wiley & Sons.

Diekmann, O., Heesterbeek, J. A. P., & Metz, J. A. J. (1990). On the definition and the computation of the basic reproduction ratio R0 in models for infectious diseases in heterogeneous populations. *Journal of Mathematical Biology*, *28*(4), 365–382. https://doi.org/10.1007/BF00178324

Diekmann, O., Heesterbeek, J. A. P., & Roberts, M. G. (2010). The construction of next-generation matrices for compartmental epidemic models. *Journal of the Royal Society Interface*, *7*(47), 873–885. https://doi.org/10.1098/rsif.2009.0386

do Carmo, E., Marins Póvoa, M., Salgado Monteiro, N., Rodrigues Marinho, R., Maria Nascimento, J., Nazaré Freitas, S., Nazaré Carneiro Bichara, C., & Municipality, A. (2010). Human toxoplasmosis outbreak in the Monte Dourado District. *Rev Pan-Amaz Saude*, *1*(1), 61–66. https://doi.org/10.5123/S2176-62232010000100009

Doganci, L., Tanyuksel, M., Araz, E. R., Besirbellioglu, B. A., Erdem, U., Ozoguz, C. A., Yucel, N., & Ciftcioglu, A. (2006). A probable outbreak of toxoplasmosis among boarding school students in Turkey. *Clinical Microbiology and Infection*, *12*(7), 672–674. https://doi.org/10.1111/j.1469-0691.2006.01449.x

Du, F., Feng, H. L., Nie, H., Tu, P., Zhang, Q. L., Hu, M., Zhou, Y. Q., & Zhao, J. L. (2012). Survey on the contamination of Toxoplasma gondii oocysts in the soil of public parks of Wuhan, China. *Veterinary Parasitology*, *184*(2–4), 141–146. https://doi.org/10.1016/j.vetpar.2011.08.025

Du, F., Zhang, Q., Yu, Q., Hu, M., Zhou, Y., & Zhao, J. (2012). Soil contamination of Toxoplasma gondii oocysts in pig farms in central China. *Veterinary Parasitology*, *187*(1–2), 53–56. https://doi.org/10.1016/j.vetpar.2011.12.036

Dubey, J. P. (1980). Persistence of encysted Toxoplasma gondii in caprine livers and public health significance of toxoplasmosis in goats. *Journal of the American Veterinary Medical Association*, *177*(12), 1203–1207. https://doi.org/https://www.cabdirect.org/cabdirect/abstract/19812263782

Dubey, J. P. (1988). Long-term persistence of Toxoplasma gondii in tissues of pigs inoculated with T gondii oocysts and effect of freezing on viability of tissue cysts in pork. *American Journal of Veterinary Research*, *49*(6), 910–913. http://www.ncbi.nlm.nih.gov/pubmed/3400928

Dubey, J. P. (1992). Isolation of Toxoplasma gondii from a Naturally Infected Beef Cow. *The Journal of Parasitology*, *78*(1), 151. https://doi.org/10.2307/3283705

Dubey, J. P. (1995). Duration of Immunity to Shedding of Toxoplasma gondii Oocysts by Cats. *The Journal of Parasitology*, *81*(3), 410–415. https://doi.org/10.2307/3283823

Dubey, J. P. (1998). Refinement of pepsin digestion method for isolation of Toxoplasma gondii from infected tissues. *Veterinary Parasitology*, *74*(1), 75–77. https://doi.org/10.1016/S0304-4017(97)00135-0

Dubey, J. P. (2006). Comparative infectivity of oocysts and bradyzoites of Toxoplasma gondii for intermediate (mice) and definitive (cats) hosts. *Veterinary Parasitology*, *140*(1–2), 69–75. https://doi.org/10.1016/j.vetpar.2006.03.018

Dubey, J. P. (2009). History of the discovery of the life cycle of Toxoplasma gondii. *International Journal for Parasitology*, *39*(8), 877–882. https://doi.org/10.1016/J.IJPARA.2009.01.005

Dubey, J. P. (2010). *Toxoplasmosis of animals and humans* (2nd ed.). CRC Press. https://doi.org/10.1201/9781420092370

Dubey, J. P., Andrews, C. D., Lind, P., Kwok, O. C., Thulliez, P., & Lunney, J. K. (1996). Antibody responses measured by various serologic tests in pigs orally inoculated with low numbers of Toxoplasma gondii oocysts. *American Journal of Veterinary Research*, *57*(12), 1733–1737. http://www.ncbi.nlm.nih.gov/pubmed/8950427

Dubey, J. P., & Beattie, C. P. (1988). *Toxoplasmosis of animals and man.* CRC Press, Inc. https://www.cabdirect.org/cabdirect/abstract/19892292620

Dubey, J. P., & Carpenter, J. L. (1993). Neonatal toxoplasmosis in littermate cats. *Journal of the American Veterinary Medical Association*, *203*(11), 1546–1549.

http://www.ncbi.nlm.nih.gov/pubmed/8288476

Dubey, J. P., & Desmonts, G. (1987). Serological responses of equids fed Toxoplasma gondii oocysts. *Equine Veterinary Journal*, *19*(4), 337–339. https://doi.org/10.1111/j.2042-3306.1987.tb01426.x

Dubey, J. P., Hill, D. E., Fournet, V., Hawkins-Cooper, D., Cerqueira-Cézar, C. K., Murata, F. H. A., Verma, S. K., Kwok, O. C. H., Rani, S., Fredericks, J., Adams, B., Jones, J. L., Wiegand, R. E., Ying, Y., Guo, M., Su, C., & Pradhan, A. K. (2020). Low prevalence of viable Toxoplasma gondii in fresh, unfrozen, American pasture-raised pork and lamb from retail meat stores in the United States. *Food Control*, *109*, 106961. https://doi.org/10.1016/j.foodcont.2019.106961

Dubey, J. P., Hill, D. E., Jones, J. L., Hightower, A. W., Kirkland, E., Roberts, J. M., Marcet, P. L., Lehmann, T., Vianna, M. C. B., Miska, K., Sreekumar, C., Kwok, O. C. H., Shen, S. K., & Gamble, H. R. (2005). Prevalence of viable Toxoplasma gondii in beef, chicken, and pork from retail meat stores in the United States: risk assessment to consumers. *Journal of Parasitology*, *91*(5), 1082–1093. https://doi.org/10.1645/GE-683.1

Dubey, J. P., Hill, D. E., Rozeboom, D. W., Rajendran, C., Choudhary, S., Ferreira, L. R., Kwok, O. C. H., & Su, C. (2012). High prevalence and genotypes of Toxoplasma gondii isolated from organic pigs in northern USA. *Veterinary Parasitology*, *188*(1–2), 14–18. https://doi.org/10.1016/j.vetpar.2012.03.008

Dubey, J. P., & Jones, J. L. (2008). Toxoplasma gondii infection in humans and animals in the United States. In *International Journal for Parasitology* (Vol. 38, Issue 11, pp. 1257–1278). Pergamon. https://doi.org/10.1016/j.ijpara.2008.03.007

Dubey, J. P., & Kirkbride, C. A. (1989). Economic and public health considerations of

congenital toxoplasmosis in lambs. *Journal of the American Veterinary Medical Association*, *195*(12), 1715–1716. http://www.ncbi.nlm.nih.gov/pubmed/2599957

Dubey, J. P., Kotula, A. W., Sharar, A., Andrews, C. D., & Lindsay, D. S. (1990). Effect of high temperature on infectivity of Toxoplasma gondii tissue cysts in pork. *The Journal of Parasitology*, *76*(2), 201–204. https://doi.org/10.2307/3283016

Dubey, J. P., Lindsay, D. S., & Speer, C. A. (1998). Structures of Toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. In *Clinical Microbiology Reviews* (Vol. 11, Issue 2, pp. 267–299). American Society for Microbiology Journals. https://doi.org/10.1128/cmr.11.2.267

Dubey, J. P., Lunney, J. K., Shen, S. K., Kwok, O. C. H., Ashford, D. A., & Thulliez, P. (1996). Infectivity of low numbers of Toxoplasma gondii oocysts to pigs. *The Journal of Parasitology*, *82*(3), 438. https://doi.org/10.2307/3284082

Dubey, J. P., Murrell, K. D., Fayer, R., & Schad, G. A. (1986). Distribution of Toxoplasma gondii tissue cysts in commercial cuts of pork. *Journal of the American Veterinary Medical Association*, *188*(9), 1035–1037. http://www.ncbi.nlm.nih.gov/pubmed/3710886

Dubey, J. P., Rajendran, C., Ferreira, L. R., Martins, J., Kwok, O. C. H., Hill, D. E., Villena, I., Zhou, H., Su, C., & Jones, J. L. (2011). High prevalence and genotypes of Toxoplasma gondii isolated from goats, from a retail meat store, destined for human consumption in the USA. *International Journal for Parasitology*, *41*(8), 827–833. https://doi.org/10.1016/j.ijpara.2011.03.006

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

International Journal for Parasitology, *38*(8–9), 999–1006. https://doi.org/10.1016/j.ijpara.2007.11.012

Dubey, J. P., Thulliez, P., & Powell, E. C. (1995). Toxoplasma gondii in Iowa Sows: Comparison of Antibody Titers to Isolation of T. gondii by Bioassays in Mice and Cats. *The Journal of Parasitology*, *81*(1), 48. https://doi.org/10.2307/3284004

Dumètre, A., & Dardé, M.-L. (2003). How to detect *Toxoplasma gondii* oocysts in environmental samples? *FEMS Microbiology Reviews*, *27*(5), 651–661. https://doi.org/10.1016/S0168-6445(03)00071-8

Dunn, D., Wallon, M., Peyron, F., Petersen, E., Peckham, C., & Gilbert, R. (1999). Mother-to-child transmission of toxoplasmosis: Risk estimates for clinical counselling. *Lancet*, *353*(9167), 1829–1833. https://doi.org/10.1016/S0140-6736(98)08220-8

Eduardo, M., Katsuya, E., Ramos, S., & Pavanello, E. (2007). Investigation of the outbreak of toxoplasmosis associated with the consumption of raw meat dish ("steak tartar"), in the municipalities of São Paulo [in Portuguese]. *Boletim Epidemiológico Paulista*, *4*, 1–6.

Egger, M., Smith, G. D., Schneider, M., & Minder, C. (1997). Bias in meta-analysis detected by a simple , graphical test measures of funnel plot asymmetry. *Bmj*, *315*(7109), 629–634. https://doi.org/10.1136/bmj.315.7109.629

Ekman, C. C. J., Chiossi, M. F. do V., Meireles, L. R., Andrade Júnior, H. F. de, Figueiredo, W. M., Marciano, M. A. M., & Luna, E. J. de A. (2012). Case-control study of an outbreak of acute toxoplasmosis in an industrial plant in the state of São Paulo, Brazil. *Revista Do Instituto de Medicina Tropical de Sao Paulo*, *54*(5), 239–244. https://doi.org/10.1590/s0036-46652012000500001

Erlich, H. A. (2015). *PCR technology: Principles and applications for DNA amplification*.

Palgrave Macmillan. https://doi.org/10.1007/978-1-349-20235-5

Esteban-Redondo, I., Maley, S. W., Thomson, K., Nicoll, S., Wright, S., Buxton, D., & Innes, E. A. (1999). Detection of Toxoplasma gondii in tissues of sheep and cattle following oral infection. *Veterinary Parasitology*, *86*(3), 155–171. https://doi.org/10.1016/S0304-4017(99)00138-7

FAO. (2014). *"Top Ten" list of foof-borne parasites released*. http://www.fao.org/news/story/en/item/237323/icode/

FDA. (2017). *Audits/FDA temperature databases*. FoodRisk.Org. https://www.foodrisk.org/resources/display/20

Ferguson, D. J. P. (2009). Toxoplasma gondii: 1908-2008, homage to Nicolle, Manceaux and Splendore. *Memorias Do Instituto Oswaldo Cruz*, *104*(2), 133–148. https://doi.org/10.1590/S0074-02762009000200003

Fisher, N. I., & Hall, P. (1991). Bootstrap algorithms for small samples. *Journal of Statistical Planning and Inference*, *27*(2), 157–169. https://doi.org/10.1016/0378-3758(91)90013-5

Foerster, C., Figueroa, G., & Evers, E. (2015). Risk assessment of *Listeria monocytogenes* in poultry and beef. *British Food Journal*, *117*(2), 779–792. https://doi.org/10.1108/BFJ-03-2014-0131

Frenkel, J. K., Dubey, J. P., & Miller, N. L. (1970). Toxoplasma gondii in cats: Fecal stages identified as coccidian oocysts. *Science*, *168*(3929), 353–353. https://doi.org/10.1126/science.168.3929.353-a

Gajadhar, A. A., Scandrett, W. B., & Forbes, L. B. (2006). Overview of food-and water-borne zoonotic parasites at the farm level. In *Rev. sci. tech. Off. int. Epiz* (Vol. 25, Issue 2).

Gilbert, S. E., Whyte, R., Bayne, G., Paulin, S. M., Lake, R. J., & van der Logt, P. (2007).

Survey of domestic food handling practices in New Zealand. *International Journal of Food Microbiology*, *117*(3), 306–311. https://doi.org/10.1016/J.IJFOODMICRO.2007.05.004

Gillies, C., & Clout, M. (2003). The prey of domestic cats (Felis catus) in two suburbs of Auckland City, New Zealand. *Journal of Zoology*, *259*(3), 309–315. https://doi.org/10.1017/S095283690200328X

Gisbert Algaba, I., Geerts, M., Jennes, M., Coucke, W., Opsteegh, M., Cox, E., Dorny, P., Dierick, K., & De Craeye, S. (2017). A more sensitive, efficient and ISO 17025 validated Magnetic Capture real time PCR method for the detection of archetypal Toxoplasma gondii strains in meat. *International Journal for Parasitology*, *47*(13), 875–884. https://doi.org/10.1016/j.ijpara.2017.05.005

González-Parra, G. C., Arenas, A. J., Aranda, D. F., Villanueva, R. J., & Jódar, L. (2009). Dynamics of a model of toxoplasmosis disease in human and cat populations. *Computers and Mathematics with Applications*, *57*(10), 1692–1700. https://doi.org/10.1016/j.camwa.2008.09.012

Gotteland, C., Gilot-Fromont, E., Aubert, D., Poulle, M. L., Dupuis, E., Dardé, M. L., Forin-Wiart, M. A., Rabilloud, M., Riche, B., & Villena, I. (2014). Spatial distribution of Toxoplasma gondii oocysts in soil in a rural area: Influence of cats and land use. *Veterinary Parasitology*, *205*(3–4), 629–637. https://doi.org/10.1016/j.vetpar.2014.08.003

Grigg, M. E., Ganatra, J., Boothroyd, J. C., & Margolis, T. P. (2001). Unusual abundance of atypical strains associated with human ocular toxoplasmosis. *The Journal of Infectious Diseases*, *184*(5), 633–639. https://doi.org/10.1086/322800

Guo, M., Buchanan, R. L., Dubey, J. P., Hill, D. E., Lambertini, E., Ying, Y., Gamble, H. R., Jones, J. L., & Pradhan, A. K. (2015). Qualitative assessment for Toxoplasma gondii

exposure risk associated with meat products in the United States. *Journal of Food Protection*, *78*(12), 2207–2219. https://doi.org/10.4315/0362-028X.JFP-15-270

Guo, M., Lambertini, E., Buchanan, R. L., Dubey, J. P., Hill, D. E., Gamble, H. R., Jones, J. L., & Pradhan, A. K. (2017). Quantifying the risk of human Toxoplasma gondii infection due to consumption of fresh pork in the United States. *Food Control*, *73*, 1210–1222. https://doi.org/10.1016/J.FOODCONT.2016.10.038

Guo, M., Mishra, A., Buchanan, R. L., Dubey, J. P., Hill, D. E., Gamble, H. R., Jones, J. L., & Pradhan, A. K. (2016). A systematic meta-analysis of Toxoplasma gondii prevalence in food animals in the United States. *Foodborne Pathogens and Disease*, *13*(3), 109–118. https://doi.org/10.1089/fpd.2015.2070

Hamidinejat, H., Nabavi, L., Mayahi, M., Ghourbanpoor, M., Borojeni, P., Fard, N., & Shokrollahi, M. (2014). Comparison of three diagnostic methods for the detection of Toxoplasma gondii in free range chickens. In *Tropical Biomedicine* (Vol. 31, Issue 3).

Hide, G. (2016). Role of vertical transmission of Toxoplasma gondii in prevalence of infection. In *Expert Review of Anti-Infective Therapy* (Vol. 14, Issue 3, pp. 335–344). Taylor and Francis Ltd. https://doi.org/10.1586/14787210.2016.1146131

Hill, D., & Dubey, J. P. (2002). Toxoplasma gondii: transmission, diagnosis and prevention. *Clinical Microbiology and Infection*, *8*(10), 634–640. https://doi.org/10.1046/j.1469-0691.2002.00485.x

Hill, D.E., & Dubey, J. P. (2013). Toxoplasma gondii prevalence in farm animals in the United States. *International Journal for Parasitology*, *43*(2), 107–113. https://doi.org/10.1016/J.IJPARA.2012.09.012

Hill, Dolores E., Chirukandoth, S., Dubey, J. P., Lunney, J. K., & Gamble, H. R. (2006).

Comparison of detection methods for Toxoplasma gondii in naturally and experimentally infected swine. *Veterinary Parasitology*, *141*(1–2), 9–17. https://doi.org/10.1016/j.vetpar.2006.05.008

Hill, Dolores E, & Dubey, J. P. (2016). Toxoplasma gondii as a parasite in food: analysis and control. *Microbiol Spectrum*, *4*(4), 0011. https://doi.org/10.1128/microbiolspec.PFS-0011-2015

Hoffmann, S., Batz, M. B., & Morris, J. G. (2012). Annual cost of illness and Quality-Adjusted Life Year losses in the United States due to 14 foodborne pathogens. *Journal of Food Protection*, *75*(7), 1292–1302. https://doi.org/10.4315/0362-028X.JFP-11-417

Howe, D. K., & Sibley, D. L. (1995). *Toxoplasma gondii Comprises Three Clonal Lineages: Correlation of Parasite Genotype with Human Disease*. https://academic.oup.com/jid/article-abstract/172/6/1561/820647

Huedo-Medina, T. B., Sánchez-Meca, J., Marín-Martínez, F., & Botella, J. (2006). Assessing heterogeneity in meta-analysis: Q statistic or I 2 Index? *Psychological Methods*, *11*(2), 193–206. https://doi.org/10.1037/1082-989X.11.2.193

Hunter, C. A., & Sibley, L. D. (2012). Modulation of innate immunity by Toxoplasma gondii virulence effectors. In *Nature Reviews Microbiology* (Vol. 10, Issue 11, pp. 766–778). Nature Publishing Group. https://doi.org/10.1038/nrmicro2858

Irwig, L., Tosteson, A. N. A., Gatsonis, C., Lau, J., Colditz, G., Chalmers, T. C., & Mosteller, F. (1994). Guidelines for meta-analyses evaluating diagnostic tests. In *Annals of Internal Medicine* • (Vol. 120). https://annals.org

Janitschke, K., & Wormuth, H. (1970). Serological, Clinical Parasitological Investigations of Specific Pathogen-Free Piglets before and after Infection with Toxoplasma gondii [in

German]. *Zentralbl. BakterioI. I. Abt. Orig*, *215*, 123–131.

Jiang, W., Sullivan, A. M., Su, C., & Zhao, X. (2012). An agent-based model for the transmission dynamics of Toxoplasma gondii. *Journal of Theoretical Biology*, *293*, 15–26. https://doi.org/10.1016/j.jtbi.2011.10.006

Jones, J. L., & Dubey, J. P. (2012). Foodborne toxoplasmosis. *Clinical Infectious Diseases*, *55*(6), 845–851. https://doi.org/10.1093/cid/cis508

Jones, J. L., Kruszon-Moran, D., Elder, S., Rivera, H. N., Press, C., Montoya, J. G., & McQuillan, G. M. (2018). Toxoplasma gondii infection in the United States, 2011–2014. *The American Journal of Tropical Medicine and Hygiene*, *98*(2), 551–557. https://doi.org/10.4269/ajtmh.17-0677

Jones, J. L., Kruszon-Moran, D., & Wilson, M. (2003). Toxoplasma gondii infection in the United States, 1999-2000. *Emerging Infectious Diseases*, *9*(11), 1371–1374. https://doi.org/10.3201/eid0911.030098

Jones, J., Lopez, A., & Wilson, M, J Schulkin, Gibbs, R. (2001). Congenital toxoplasmosis: a review. *Obstetrical & Gynecological Survey*, *56*(5), 296–305. https://journals.lww.com/obgynsurvey/fulltext/2001/05000/congenital_toxoplasmosis__a_review.25.aspx

Jones, Jeffrey L., Dargelas, V., Roberts, J., Press, C., Remington, J. S., & Montoya, J. G. (2009). Risk factors for Toxoplasma gondii infection in the United States. *Clinical Infectious Diseases*, *49*(6), 878–884. https://doi.org/10.1086/605433

Jones, Jeffrey L., & Holland, G. N. (2010). Short report: Annual burden of ocular toxoplasmosis in the United States. *American Journal of Tropical Medicine and Hygiene*, *82*(3), 464–465. https://doi.org/10.4269/ajtmh.2010.09-0664

Jones, Jeffrey L, Kruszon-Moran, D., Sanders-Lewis, K., & Wilson, M. (2007). Toxoplasma gondii infection in the United States, 1999 2004, decline from the prior decade. *The American Journal of Tropical Medicine and Hygiene*, *77*(3), 405–410. http://www.ncbi.nlm.nih.gov/pubmed/17827351

Jones, Jeffrey L, Ogunmodede, F., Scheftel, J., Kirkland, E., Lopez, A., Schulkin, J., & Lynfield, R. (2003). Toxoplasmosis-related knowledge and practices among pregnant women in the United States. *Infectious Diseases in Obstetrics and Gynecology*, *11*(3), 139–145. https://doi.org/10.1080/10647440300025512

Kapperud, G., Jenum, P. A., Stray-Pedersen, B., Melby, K. K., Eskild, A., & Eng, J. (1996). Risk factors for Toxoplasma gondii infection in pregnancy: results of a prospective case-control study in Norway. *American Journal of Epidemiology*, *144*(4), 405–412. https://doi.org/10.1093/oxfordjournals.aje.a008942

Ketchem, R., & Rix, M. (2016). *Does farm size matter in swine production?* National Hog Farmer. https://www.nationalhogfarmer.com/reproduction/does-farm-size-matter-swine-production

Kim, K. W., Lee, J., Choi, S. H., Huh, J., & Park, S. H. (2015). Systematic review and meta-analysis of studies evaluating diagnostic test accuracy: A practical review for clinical researchers–part I. general guidance and tips. In *Korean Journal of Radiology* (Vol. 16, Issue 6, pp. 1175–1187). Korean Radiological Society. https://doi.org/10.3348/kjr.2015.16.6.1175

Kotula, A. W., Dubey, J. P., Sharar, A. K., Andrews, C. D., Shen, S. K., & Lindsay, D. S. (1991). Effect of freezing on infectivity of Toxoplasma Gondii tissue cysts in pork. *Journal of Food Protection*, *54*(9), 687–690. https://doi.org/10.4315/0362-028X-54.9.687

Kravetz, J. D., & Federman, D. G. (2005). Toxoplasmosis in pregnancy. *The American Journal of Medicine*, *118*(3), 212–216. https://doi.org/10.1016/J.AMJMED.2004.08.023

Landis, J. R., & Koch, G. G. (1977). An application of hierarchical kappa-type statistics in the assessment of majority agreement among multiple observers. *Biometrics*, *33*(2), 363. https://doi.org/10.2307/2529786

Latorre, A. A., Pradhan, A. K., Van Kessel, J. A. S., Karns, J. S., Boor, K. J., Rice, D. H., Mangione, K. J., Gröhn, Y. T., & Schukken, Y. H. (2011). Quantitative risk assessment of listeriosis due to consumption of raw milk. *Journal of Food Protection*, *74*(8), 1268–1281. https://doi.org/10.4315/0362-028X.JFP-10-554

Lee, J., Kim, K. W., Choi, S. H., Huh, J., & Park, S. H. (2015). Systematic review and meta-analysis of studies evaluating diagnostic test accuracy: A practical review for clinical researchers–part II. Statistical methods of meta-analysis. In *Korean Journal of Radiology* (Vol. 16, Issue 6, pp. 1188–1196). Korean Radiological Society. https://doi.org/10.3348/kjr.2015.16.6.1188

Lee, S.-Y., & Song, X.-Y. (2004). Evaluation of the Bayesian and maximum likelihood approaches in analyzing structural equation models with small sample sizes. *Multivariate Behavioral Research*, *39*(4), 653–686. https://doi.org/10.1207/s15327906mbr3904_4

Lélu, M., Langlais, M., Poulle, M. L., & Gilot-Fromont, E. (2010). Transmission dynamics of Toxoplasma gondii along an urban-rural gradient. *Theoretical Population Biology*, *78*(2), 139–147. https://doi.org/10.1016/j.tpb.2010.05.005

Lélu, Maud, Villena, I., Dardé, M.-L., Aubert, D., Geers, R., Dupuis, E., Marnef, F., Poulle, M.-L., & Gotteland, C. (2012). Quantitative estimation of the viability of Toxoplasma gondii oocysts in soil. *Am Soc Microbiol*, *78*(15), 5127–5132. https://doi.org/10.1128/AEM.00246-

12

Li-Cohen, A. E., & Bruhn, C. M. (2002). Safety of consumer handling of fresh produce from the time of purchase to the plate: A comprehensive consumer survey. *Journal of Food Protection*, *65*(8), 1287–1296. https://doi.org/10.4315/0362-028X-65.8.1287

Lindsay, D. S., Collins, M. V, Mitchell, S. M., Wetch, C. N., Rosypal, A. C., Flick, G. J., Zajac, A. M., Lindquist, A., & Dubey, J. P. (2004). Survival of Toxoplasma gondii oocysts in eastern oysters (Crassostrea virginica). *The Journal of Parasitology*, *90*(5), 1054–1057. https://about.jstor.org/terms

Liu, Q., Wang, Z. D., Huang, S. Y., & Zhu, X. Q. (2015). Diagnosis of toxoplasmosis and typing of Toxoplasma gondii. In *Parasites and Vectors* (Vol. 8, Issue 1, pp. 1–14). BioMed Central Ltd. https://doi.org/10.1186/s13071-015-0902-6

Luptakova, L., Valencakova, A., Balent, P., Malcekova, B., & Petrovova, E. (2012). Evaluation of detection of Toxoplasma gondii DNA in animal blood samples by quantitative PCR. *Central European Journal of Biology*, *7*(3), 431–435. https://doi.org/10.2478/s11535-012-0025-4

Mateus-Pinilla, N. E., Dubey, J. P., Choromanski, L., & Weigel, R. M. (1999). A Field Trial of the Effectiveness of a Feline Toxoplasma gondii Vaccine in Reducing T. gondii Exposure for Swine. *The Journal of Parasitology*, *85*(5), 855–860. https://doi.org/10.2307/3285821

Mateus-Pinilla, N. E., Hannon, B., & Weigel, R. M. (2002). A computer simulation of the prevention of the transmission of Toxoplasma gondii on swine farms using a feline T. gondii vaccine. *Preventive Veterinary Medicine*, *55*(1), 17–36. https://doi.org/10.1016/S0167-5877(02)00057-0

McCabe, R. E. (2001). *Antitoxoplasma chemotherapy. In Toxoplasmosis: a comprehensive*

clinical. Cambridge University Press. https://books.google.com/books?hl=en&lr=&id=6UoYLNBUJ3kC&oi=fnd&pg=PA319&dq=antitoxoplasma+chemotherapy&ots=n1fbFKEJ0s&sig=6sEXSf93tK8DotdTgsEeYxug_sk

Montoya, J. G., & Liesenfeld, O. (2004). Toxoplasmosis. *Lancet*, *363*(9425), 1965–1976. https://doi.org/10.1016/S0140-6736(04)16412-X

Montoya, J. G., & Remington, J. S. (1996). Toxoplasmic chorioretinitis in the setting of acute acquired toxoplasmosis. *Clinical Infectious Diseases*, *23*(2), 277–282. https://doi.org/10.1093/clinids/23.2.277

Morais, R. dos A. P. B., Freire, A. B. C., Barbosa, D. R. L., Silva, L. de C. T. da, Pinheiro, A. F., Costa, S. S. da, Ramos, F. L. de P., Bichara, C. N. C., Lima, L. J. B., Silva, A. V. da, Souza, S. R. P. de, Piqueira, L. P., Gonçalves, N. V., Póvoa, M. M., & Carmo, E. L. do. (2016). Acute toxoplasmosis outbreak in the Municipality of Ponta de Pedras, Marajó archipelago, Pará State, Brazil: clinical, laboratory, and epidemiological features. *Revista Pan-Amazônica de Saúde*, *7*(esp), 143–152. https://doi.org/10.5123/s2176-62232016000500016

Nicole, C., & Manceaux, L. (1908). Sur un protozoaire nouveau du gondii. *C. R. Seances Acad. Sci.*, *147*, 763–766.

Nissapatorn, V., Lee, C., Fatt Quek, K., Leong, C. L., Mahmud, R., & Abdullah, K. A. (2004). Toxoplasmosis in HIV/AIDS patients: A current situation. *Japanese Journal of Infectious Diseases*, *57*, 160–165. http://www0.nih.go.jp/JJID/57/160.html

Nutter, F. B., Levine, J. F., & Stoskopf, M. K. (2004). Reproductive capacity of free-roaming domestic cats and kitten survival rate. *Journal of the American Veterinary Medical Association*, *225*(9), 1399–1402. https://doi.org/10.2460/javma.2004.225.1399

Opsteegh, M., Langelaar, M., Sprong, H., den Hartog, L., De Craeye, S., Bokken, G., Ajzenberg, D., Kijlstra, A., & der Giessen, J. van. (2010). Direct detection and genotyping of Toxoplasma gondii in meat samples using magnetic capture and PCR. *International Journal of Food Microbiology*, *139*(3), 193–201. https://doi.org/10.1016/j.ijfoodmicro.2010.02.027

Opsteegh, M., Prickaerts, S., Frankena, K., & Evers, E. G. (2011). A quantitative microbial risk assessment for meatborne Toxoplasma gondii infection in The Netherlands. *International Journal of Food Microbiology*, *150*(2–3), 103–114. https://doi.org/10.1016/J.IJFOODMICRO.2011.07.022

Ozgonul, C., & Besirli, C. G. (2017). Recent developments in the diagnosis and treatment of ocular toxoplasmosis. *Ophthalmic Research*, *57*(1), 1–12. https://doi.org/10.1159/000449169

Palanisamy, M., Madhavan, B., Balasundaram, M., Andavar, R., & Venkatapathy, N. (2006). Outbreak of ocular toxoplasmosis in Coimbatore, India. *Indian Journal of Ophthalmology*, *54*(2), 129–131. https://doi.org/10.4103/0301-4738.25839

Pena, H. F. J., Gennari, S. M., Dubey, J. P., & Su, C. (2008). Population structure and mouse-virulence of Toxoplasma gondii in Brazil. *International Journal for Parasitology*, *38*(5), 561–569. https://doi.org/10.1016/j.ijpara.2007.09.004

Peters, J. L., Sutton, A. J., Jones, D. R., Abrams, K. R., & Rushton, L. (2008). Contour-enhanced meta-analysis funnel plots help distinguish publication bias from other causes of asymmetry. *Journal of Clinical Epidemiology*, *61*(10), 991–996. https://doi.org/10.1016/j.jclinepi.2007.11.010

Peyron, F., Mc Leod, R., Ajzenberg, D., Contopoulos-Ioannidis, D., Kieffer, F., Mandelbrot, L., Sibley, L. D., Pelloux, H., Villena, I., Wallon, M., & Montoya, J. G. (2017). Congenital

toxoplasmosis in France and the United States: One parasite, two diverging approaches. *PLOS Neglected Tropical Diseases*, *11*(2), e0005222. https://doi.org/10.1371/journal.pntd.0005222

Pino, L. E., Salinas, J. E., & López, M. C. (2009). Description of an epidemic outbreak of acute toxoplasmosis in immunocompetent patients from Colombian Armed Forces during jungle operations. *Infectio*, *13*(2), 83–91. https://doi.org/10.1016/s0123-9392(09)70729-5

Plummer, M. (2003). *JAGS: A program for analysis of Bayesian graphical models using Gibbs sampling* (No. 125; 124). http://www.ci.tuwien.ac.at/Conferences/DSC-2003/

Price, C., Wilkins, P. P., Kruszon-Moran, D., Jones, J. L., & Rivera, H. N. (2014). Toxoplasma gondii Seroprevalence in the United States 2009–2010 and Comparison with the Past Two Decades. *The American Journal of Tropical Medicine and Hygiene*, *90*(6), 1135–1139. https://doi.org/10.4269/ajtmh.14-0013

R Core Team. (2018). *A language and environment for statistical computing. R Foundation for Statistical Computing*. https://www.r-project.org/

Rani, S., Cerqueira-Cézar, C. K., Murata, F. H. A., Kwok, O. C. H., Dubey, J. P., & Pradhan, A. (2020). Distribution of Toxoplasma gondii Tissue Cysts in Shoulder Muscles of Naturally Infected Goats and Lambs. *Journal of Food Protection*. https://doi.org/10.4315/jfp-20-024

Rani, S., Cerqueira-Cézar, C. K., Murata, F. H. A., Sadler, M., Kwok, O. C. H., Pradhan, A. K., Hill, D. E., Urban, J. F., & Dubey, J. P. (2019). Toxoplasma gondii tissue cyst formation and density of tissue cysts in shoulders of pigs 7 and 14 days after feeding infected mice tissues. *Veterinary Parasitology*, *269*, 13–15. https://doi.org/10.1016/j.vetpar.2019.04.004

Rasmussen, C. E., & Ghahramani, Z. (2003). Bayesian Monte Carlo. In *Advances in neural information processing systems*. http://www.gatsby.ucl.ac.uk

Remington, J., Meleod, R., & Desmots, G. (1995). *Toxoplasmosis In Regmington JS, and klicn I (eds.) Infectious Diseases of The Fetus and Newborn Infant. 4th (ed.) Philadelphia*.

Renoiner, E. I. M., Siqueira, A. A., Garcia, M. H., Alves, R. M., Cardoso, M. E., & Ferreira, A. B. P. L. (2007). Outbreak of acquired toxoplasmosis, Anápolis-GO [in Porutguese]. *Boletim Eletrônico Epidemiológico Da Secretária de Vigilância Em Saúde*, *8*, 1–6. https://scholar.google.com/scholar?hl=en&as_sdt=0%2C21&q=Outbreak+of+acquired+toxoplasmosis%2C+Anápolis-GO%2C&btnG=

Robert-Gangneux, F., & Dardé, M.-L. (2012). Epidemiology of and diagnostic strategies for toxoplasmosis. *American Society for Microbiology*, *25*(2), 264–296. https://doi.org/10.1128/CMR.05013-11

Robertson, L. J., van der Giessen, J. W. B., Batz, M. B., Kojima, M., & Cahill, S. (2013). Have foodborne parasites finally become a global concern? In *Trends in Parasitology* (Vol. 29, Issue 3, pp. 101–103). Elsevier. https://doi.org/10.1016/j.pt.2012.12.004

Samico-Fernandes, E. F. T., Samico-Fernandes, M. F. T., De Albuquerque, P. P. F., De Almeida, J. C., Santos, A. D. S., Da Rocha Mota, A., Neto, O. L. D. S., & Mota, R. A. (2017). Toxoplasma gondii in backyard pigs: Seroepidemiology and mouse bioassay. *Acta Parasitologica*, *62*(2), 466–470. https://doi.org/10.1515/ap-2017-0054

San Miguel, J. M., Gutiérrez-Expósito, D., Aguado-Martínez, A., Gonzá Lez-Zotes, E., Pereira-Bueno, J., Gómez-Bautista, M., Rubio, P., Ortega-Mora, L. M., Collantes-Fernández, E., & Álvarez-García, G. (2016). Effect of different ecosystems and management practices on Toxoplasma gondii and Neospora caninum infections in wild ruminants in Spain. *Journal of Wildlife Diseases*, *52*(2), 293–300. https://doi.org/10.7589/2015-07-176

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V, Widdowson, M.-A., Roy, S. L., Jones,

J. L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States--major pathogens. *Emerging Infectious Diseases*, *17*(1), 7–15. https://doi.org/10.3201/eid1701.p11101

Schares, G., Herrmann, D. C., Beckert, A., Schares, S., Hosseininejad, M., Pantchev, N., Globokar Vrhovec, M., & Conraths, F. J. (2008). Characterization of a repetitive DNA fragment in Hammondia hammondi and its utility for the specific differentiation of H. hammondi from Toxoplasma gondii by PCR. *Molecular and Cellular Probes*, *22*(4), 244–251. https://doi.org/10.1016/J.MCP.2008.04.003

Shenep, J. L., Barenkamp, S. J., Brammeier, S. A., & Gardner, T. D. (1984). An outbreak of toxoplasmosis on an Illinois farm. *The Pediatric Infectious Disease Journal*, *3*(6), 518–522. https://doi.org/10.1097/00006454-198411000-00007

Sibley, L. D., & Boothroyd, J. C. (1992). Virulent strains of Toxoplasma gondii comprise a single clonal lineage. *Nature*, *359*(6390), 82–85. https://doi.org/10.1038/359082a0

Silva, R., & Langoni, H. (2009). Molecular detection of Toxoplasma gondii in tissue samples of slaughtered Brazilian sheep. *Sustainable Animal Husbandry: Prevention Is Better than Cure, Volume 2. Proceedings of the 14th International Congress of the International Society for Animal Hygiene (ISAH), Vechta, Germany, 19th to 23rd July 2009*, 797–800.

Šimundić, A.-M. (2009). Measures of diagnostic accuracy: Basic definitions. *EJIFCC*, *19*(4), 203–211. http://www.ncbi.nlm.nih.gov/pubmed/27683318

Song, F., Khan, K. S., Dinnes, J., & Sutton, A. J. (2002). Asymmetric funnel plots and publication bias in meta-analyses of diagnostic accuracy. *International Journal of Epidemiology*, *31*(1), 88–95. https://doi.org/10.1093/ije/31.1.88

Sudan, V., Jaiswal, A. K., & Shanker, D. (2013). Recent trends in the diagnosis of

toxoplasmosis. *Clinical Reviews and Opinions*, *5*(2), 11–17. https://doi.org/10.5897/CRO11.022

Switaj, K., Master, A., Skrzypczak, M., & Zaborowski, P. (2005). Recent trends in molecular diagnostics for Toxoplasma gondii infections. In *Clinical Microbiology and Infection* (Vol. 11, Issue 3, pp. 170–176). Blackwell Publishing Ltd. https://doi.org/10.1111/j.1469-0691.2004.01073.x

Tenter, A. M., Heckeroth, A. R., & Weiss, L. M. (2000). Toxoplasma gondii: from animals to humans. *International Journal for Parasitology*, *30*(12–13), 1217–1258. https://doi.org/10.1016/S0020-7519(00)00124-7

Torgerson, P. R., & Mastroiacovo, P. (2013). The global burden of congenital toxoplasmosis: a systematic review. *Bulletin of the World Health Organization*, *91*(7), 501–508. https://doi.org/10.2471/BLT.12.111732

USDA APHIS. (2011). *Swine Industry Manual National Animal Health Emergency Management System FAD PReP*. https://doi.org/https://www.aphis.usda.gov/animal_health/surveillance_toolbox/docs/fadprep_swine_industry_manual.pdf Accessed: 2020-03-29

USDA FSIS. (2012). *Safe Minimum Internal Temperature Chart*. www.fsis.usda.gov.

van den Driessche, P., & Watmough, J. (2002). Reproduction numbers and sub-threshold endemic equilibria for compartmental models of disease transmission. *Mathematical Biosciences*, *180*(1–2), 29–48. https://doi.org/10.1016/S0025-5564(02)00108-6

Villena, I., Durand, B., Aubert, D., Blaga, R., Geers, R., Thomas, M., Perret, C., Alliot, A., Escotte-Binet, S., Thébault, A., Boireau, P., & Halos, L. (2012). New strategy for the survey of Toxoplasma gondii in meat for human consumption. *Veterinary Parasitology*, *183*(3–4),

203–208. https://doi.org/10.1016/j.vetpar.2011.08.001

Wallace, G. D. (1969). Sabin-Feldman dye test for toxoplasmosis. The use of sodium citrate in accessory factor, and a method for collecting and storing blood on paper discs. *The American Journal of Tropical Medicine and Hygiene*, *18*(3), 395–398. https://doi.org/10.4269/ajtmh.1969.18.395

Wang, Z.-D., Liu, H.-H., Ma, Z.-X., Ma, H.-Y., Li, Z.-Y., Yang, Z.-B., Zhu, X.-Q., Xu, B., Wei, F., & Liu, Q. (2017). Toxoplasma gondii infection in immunocompromised patients: a systematic review and meta-analysis. *Frontiers in Microbiology*, *8*, 389. https://doi.org/10.3389/fmicb.2017.00389

Warner, R. E. (1985). Demography and movements of free-ranging domestic cats in rural Illinois. *The Journal of Wildlife Management*, *49*(2), 340–346. https://doi.org/10.2307/3801527

Weigel, R. M., Dubey, J. P., Siegel, A. M., Kitron, U. D., Mannelli, A., Mitchell, M. A., Mateus-Pinilla, N. E., Thulliez, P., Shen, S. K., Kwok, O. C. H., & Todd, K. S. (1995). Risk factors for transmission of Toxoplasma gondii on swine farms in Illinois. *The Journal of Parasitology*, *81*(5), 736–743. https://doi.org/10.2307/3283964

Weiss, L. M., & Dubey, J. P. (2009). Toxoplasmosis: A history of clinical observations. *International Journal for Parasitology*, *39*(8), 895–901. https://doi.org/10.1016/J.IJPARA.2009.02.004

Wilson, Nana O., Hall, Rebecca L., Montgomery, Susan P., Jones, J. L. (2015). Trichinellosis surveillance — United States, 2008–2012. In *Morbidity and Mortality Weekly Report*. https://www.cdc.gov/mmWr/preview/mmwrhtml/ss6401a1.htm

Wingstrand, A., Lind, P., Haugegaard, J., Henriksen, S., Bille-Hansen, V., & Sørensen, V.

(1997). Clinical observations, pathology, bioassay in mice and serological response at slaughter in pigs experimentally infected with Toxoplasma gondii. *Veterinary Parasitology*, *72*(2), 129–140. https://www.sciencedirect.com/science/article/pii/S0304401797000344

Work, K., Eriksen, L., Fennestad, K. L., Møller, T., & Siim, J. C. (2009). Experimental toxoplasmosis in pregnant sows. *Acta Pathologica Microbiologica Scandinavica Section B Microbiology and Immunology*, *78B*(2), 129–139. https://doi.org/10.1111/j.1699-0463.1970.tb04279.x

Ying, Y., Verma, S. K., Kwok, O. C. H., Alibana, F., Mcleod, R., Su, C., Dubey, J. P., & Pradhan, A. K. (2017). Prevalence and genetic characterization of Toxoplasma gondii in free-range chickens from grocery stores and farms in Maryland, Ohio and Massachusetts, USA. *Parasitology Research*, *116*(5), 1591–1595. https://doi.org/10.1007/s00436-017-5420-3

Zhang, H., Thekisoe, O. M. M., Aboge, G. O., Kyan, H., Yamagishi, J., Inoue, N., Nishikawa, Y., Zakimi, S., & Xuan, X. (2009). Toxoplasma gondii: Sensitive and rapid detection of infection by loop-mediated isothermal amplification (LAMP) method. *Experimental Parasitology*, *122*(1), 47–50. https://doi.org/10.1016/j.exppara.2009.01.012