ABSTRACT

Title of Document: PREVALENCE, ISOLATION, AND GENETIC CHARACTERIZATION OF *TOXOPLASMA GONDII* IN CHICKEN

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Toxoplasma gondii (T. gondii) is one of the most successful parasites in the world because of its capability of infecting all warm-blooded animals. It has been reported that up to one third of the world population is infected with this parasite. Chickens are recognized as good indicators of the environmental *T. gondii* oocysts contamination because they obtain food from the ground. Thus, the prevalence of *T. gondii* in chicken provides more insight related to public health concern from *T. gondii*. Previous studies have shown a high isolation rate from free-range chickens raised in the United States. The objectives of this study were to evaluate the microbial safety and infection of *T. gondii* in free-range chickens available at the grocery stores and farms for the consumers to purchase and genotype *T. gondii* isolates. Chicken hearts were obtained from the local markets and also from the farms raising free-range chickens. Heart juice was obtained from cavities of each heart. Modified agglutination test (MAT) for detection of IgG antibodies was conducted with those heart juice samples with titer of 1:5, 1:25, and 1: 100. Each seropositive heart was pepsin digested and bioassayed into a group of two mice. Six weeks post inoculation (p.i.) mice were bled and euthanized to examine the

infection of *T. gondii*. In addition, multiplex multilocus nested PCR-RFLP was performed to genetically characterize *T. gondii* isolates with eleven PCR-RFLP markers including SAG1, SAG2, altSAT2, SAG3, BTUB, GRA6, c22-8, c29-a, L358, PK1, and Apico. One hundred fifty from a total of 997 samples (15.0%) were found seropositive for *T. gondii*. No viable *T. gondii* was isolated from chicken hearts that were sampled. A total of four genotypes were identified, including one new genotype and three previously identified genotypes. The results suggest that *T. gondii* oocysts could present in the environment and infect the food animals. *T. gondii* prevalence in chicken hearts could reflect the environmental contamination of *T. gondii* and prevalence information can be used to manage *T. gondii* infection risk.

PREVALENCE, ISOLATION, AND GENETIC CHARACTERIZATION OF *TOXOPLASMA GONDII* IN CHICKEN

By

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Chapter 1: Introduction

Toxoplasma gondii is a parasite that is capable of infecting all warm-blooded animals. *T. gondii* causes enormous economic losses every year. The Centers for Disease Control and Prevention (CDC) estimated that *T. gondii* was the top five pathogens causing domestically acquired foodborne illness resulting in hospitalization and the top two pathogens resulting in death (CDC, 2011). The United States Department of Agriculture (USDA), Economic Research Service (ERS) estimated that the cost of foodborne illness of *T. gondii* is 3.3 billion per year and it is ranked as the top two costly pathogen (USDA ERS, 2014).

Chickens are considered one of the most important food vehicles of *T. gondii* transmission in the epidemiology because of their special feeding behavior. Chickens feed from the ground, which makes them easier to get infected through oocysts from the environment. Chickens are also good indicators of the environmental *T. gondii* oocysts contamination. There is a significant consumption of chickens in the United States. The national chicken council estimates that the per capita yearly consumption of chicken is 41 kg and the consumption of pork and beef per capita yearly is 48 kg, in the United States (National Chicken Council, 2015).

The objectives of this study were to evaluate the microbial safety and infection of *T. gondii* in free-range chickens available at the grocery stores and farms for the consumers to purchase and to genotype *T. gondii* isolates.

Chapter 2: Literature Review

2.1 What is *T. gondii*?

T. gondii is a coccidian parasite. Feline family animals are the definitive host of *T. gondii*, and all warm-blooded animals are its intermediate hosts. Its special parasitic behavior makes it one of the most successful parasites in the world. It belongs to phylum: Apicomplexa (Levine, 1970); class: Sporozoasida (Leukart, 1879); subclass: Coccidiasina (Leukart, 1879); order: Eimeriorina (Leger, 1911); Family: Toxoplasmatidae (Biocca, 1956); Genus: *Toxoplasma* (Nicolle and Manceaux, 1909). There is only 1 species of *Toxoplasma, T. gondii. T. gondii* has four major stages in its lifetime: the tachyzoites (in groups or clones), the bradyzoites (in tissue cysts), the sporozoites (in oocysts) and the enteroepithelial stages (Dubey et al., 1998a).

2.1.1 Tachyzoites

Tachyzoites is the rapid reproduce asexual stage. It is crescent-shaped and approximately 2×6 μm. It has a pointed anterior (conoidal) end and a rounded posterior end (Dubey et al., 1998a). Tachyzoites invades the host cell by actively penetrating the cell plasmalemma or by phagocytosis (Bonhomme et al., 1992, Dubremetz et al., 1993). After that, tachyzoites becomes ovoid and is surrounded by a parasitoporous vacuole (PV), which is derived from both parasite and host cell. Soon after penetration, a tubulovesicular membranous network (TMN) develops within the PV (Dubey et al., 1998a). Tachyzoites multiply asexually inside the host cell by endodyogeny until the cell can no longer sustain the growth of tachyzoites and the host cell ruptures and releases all

of the tachyzoites. Thereafter, the released tachyzoites may penetrate the other cells and continue to replicate as tachyzoites or converts to slowly multiply bradyzoites stages within the infected cells. The rate of invasion and growth is mainly related to the strain of *T. gondii* and the host cells (Appleford and Smith, 1997). In cell culture, mouse virulent strains grow faster than "avirulent" strains. Tachyzoites has no structural difference between different strains of *T. gondii* (Howe and Sibley, 1995).

2.1.2 Bradyzoites

Bradyzoites is the slow replicate stage of *T. gondii*. It is found in the tissue cysts and formed as early as three days post inoculation (p.i.) in mice (Dubey and Frenkel, 1976). Tissue cysts are the structure where bradyzoites divide by endodyogeny inside. The size of tissue cysts is vary; it may as small as 5 μm in diameter and only contains two bradyzoites or as large as 100 μm long and contains thousands of bradyzoites (Dubey et al., 1998a). The cyst wall is elastic and thin (<0.5 μm thick) and consists of both host cell and parasite materials (Ferguson, 1987). Tissue cysts may form in visceral organs, but they are mostly found in neural and muscular tissues, such as brain, eyes, cardiac muscle, and skeletal (Dubey et al., 1988b). By altering the temperature and pH stress, the conversion of tachyzoites to bradyzoites may happen in vitro (Soete et al., 1994). For some particular strain, spontaneous cystogenesis was observed in vitro (Paredes-Santos et al., 2012). Bradyzoites is crescent shaped and approximately 1-3×5-8.5 μm in size and its structure is only slightly different from tachyzoites (Mehlhorn, 1980). Tissue cysts can periodically rupture and reinvade host cells (Reiter-Owona et al., 2000).

2.1.3 Oocysts

Felids excrete unsporulated oocysts after ingesting any of the infectious *T. gondii* stages, tachyzoites, bradyzoites, and sporozoites. Nearly all species of felids are capable of shedding *T. gondii* oocysts. *T. gondii* belongs to coccidian parasite because they have the same oocyst structure. Coccidia were the first protozoa discovered and they are one of the most important parasites of animals. The mature oocysts contains 4 sporocysts with 2 sporozoites in each sporocysts (total 8 sporozoites). Oocysts are approximately 10-12 μm in size and sub spherical to ellipsoidal in shape (Dubey et al., 1998a). The prepatent periods are 3-10 days after ingesting tissue cysts (Dubey et al., 1970), ≥13 days after ingesting tachyzoites (Dubey, 1998b), and ≥18 days after ingesting sporolated oocysts (Dubey, 1996). The pretatent periods are strain-independent (Dubey 2005b). In the experimental condition, all cats shed oocysts after ingesting tissue cysts, while fewer than 30% of cats excrete oocysts after ingesting tachyzoites or oocysts (Dubey, 1996). After the excretion of unsporulated oocysts, the oocysts need to sporulate before they are infective for animals. The sporulation periods vary due to the humidity and temperature of the environment. Humid environment and room temperature promotes sporulation. It takes 2-7 days for oocysts to sporulate under optimum condition.

Oocysts are very resistant in the environment; sporolated oocysts could live years in the soil. They could live outdoors in soil buried at the depth of 3-9 cm in Kansas for 18 month (Frenkel, 1975). *T. gondii* oocysts can also survive in sea water at room temperature up to 18 month (Lindsay et al., 2009).

T. gondii oocysts are shed by cats with their feces. Some cat feces remain unburied and some are brought to the surface by flies, cockroaches, earthworms, and by climatic conditions such as rain and snow. Oocysts can be carried into our homes on shoes contaminated with oocysts from street pavements. In the farms, oocysts can exists in the soil and water. Animals get infected with *T. gondii* by eating or drinking oocysts contaminated food and water. It is difficult to raise coccidian-free livestock that are herbivores.

2.1.4 Enteroepithelial stages

Enteroepithelial stages include both asexual and sexual stages of *T. gondii*. After cats ingest the tissue cysts, proteolytic enzymes in the stomach and small intestine digests the cyst wall and release bradyzoites. After that, bradyzoites intrudes the small intestine epithelial cells and start the asexual and sexual generations of *T. gondii*. There are five distinct morphological types A to E before gametogony begins (Dubey et al., 1988a) and late stages multiply by a special type of schizogony.

After the development of types A to E, the sexual stages start two days after the ingestion of cysts by cats. Sexual stages happen only in enteroepithelial cells of felids. Gamonts are found throughout the small intestine above the nucleus of the epithelial cell near the tips of the villi, and most prevalently found in the ileum (Dubey et al., 1998a). Female gamonts (macrogamonts) are subspherical in shape while male gamonts (microgamonts) are ovoid to ellipsoidal. Macrogamonts has a structure call wall-forming bodies (WFB). There two types of WFB, type I and type II. Each microgamonts contains 10-30 microgametes. Microgametes use flagella to move to, penetrate, and fertilize macrogametes to form zygotes (Dubey et al., 1998a). After that, oocysts wall is formed

and then epithelial cells discharge the oocysts into the intestine lumen. Type I WEBs form 4 layers while type II WEBs form 5 layers of cyst wall.

2.2 Life Cycle of *T. gondii*

T. gondii has a very complicated life cycle with members of Felidae as the definitive host, and all warm-blooded animals as intermediate hosts. The infection of *T. gondii* may cause clinical disease, which is called toxoplasmosis, while the immune competent hosts are asymptomatic after the infection. Naturally, *T. gondii* is adapted to be transmitted biologically by carnivorism and cats shed oocysts mostly due to the ingesting of bradyzoites, while cats could also shed oocysts by the ingesting of the other two infectious stages. Although cats can only shed oocysts once in their lifetime and the shedding periods are 1-2 weeks, large numbers of oocysts are shed and stay in the environment. Intermediate hosts become infected because of the ingesting of sporulated oocysts together with soil, water, or plant materials. According to the CDC, there are four transmission routes for humans. Eating undercooked meat of animals harboring tissue cysts; consuming food or water contaminated with cat feces by contaminated environment samples; blood transfusion or organ transplantation; and transplacentally from mother to fetus (CDC). After infection with any infective stage, oocysts or bradyzoites transform into tachyzoites. Tachyzoites multiply and eventually encyst mainly in brain and muscle tissues (Dubey, 1998b). Transplacental transmission during pregnancy can cause serious problems, such as stillbirth, abortion, malformation fetus, and congenital toxoplasmosis.

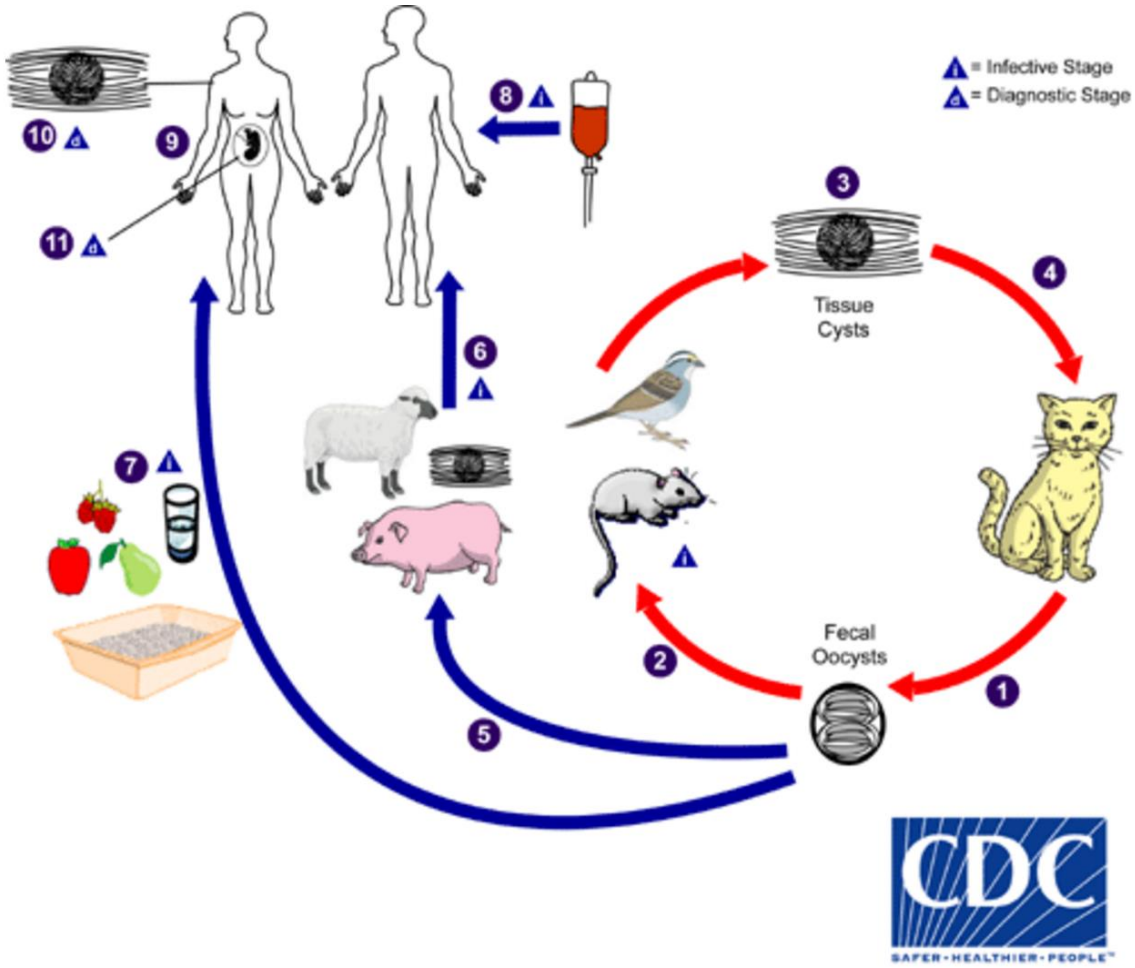

Figure 2.1 Main transmission routes that cause the infection with *T. gondii* by human beings. (Source: http://www.cdc.gov/parasites/toxoplasmosis/biology.html)

After ingestion of sporulated oocysts from the environment, sporozoites excysts in the gut, penetrate the intestinal epithelium, and then are carried to the lamina propria. Thereafter, sporozoites are distributed to the other part of body and divided into two tachyzoites. By six days postinoculation, bradyzoites and tissue cysts formed and the chickens are chronically infected with *T. gondii*. Once human beings ingest tissues with viable bradyzoites, they may get infected with the *T. gondii*.

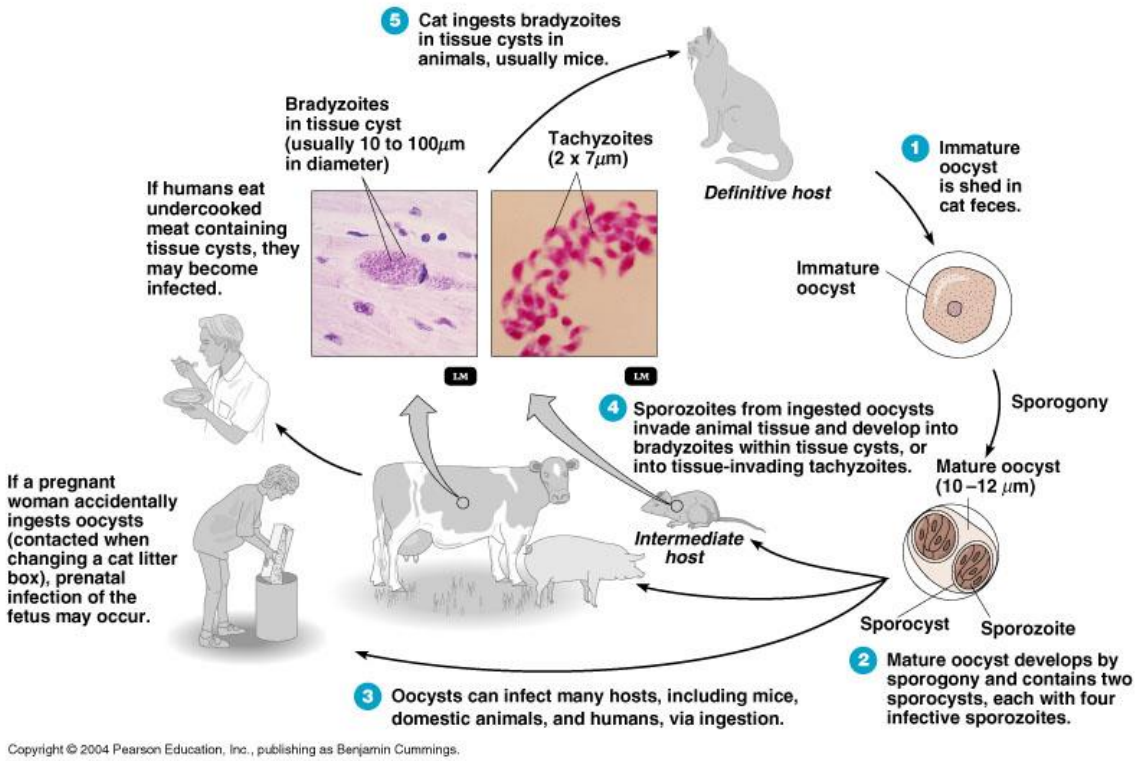

Figure 2.2 Life cycle of *T. gondii* with different stages, definitive host, and intermediate host. (Source: http://classes.midlandstech.edu/carterp/Courses/bio225/chap23/ss6.htm)

2.3 Modified agglutination test (MAT)

MAT was first developed by Fulton and Turk in 1959 and named direct agglutination test (Fulton and Turk, 1959). The test was then improved by Desmonts and Remington in 1980 and named by Dubey and Desmonts in 1987. No special conjugates or equipment are needed. MAT is very useful for the diagnosis of toxoplasmosis in animals and humans. Titers in the MAT parallel the result of the dye test both in human sera and in animal sera. The sensitivity and specificity of MAT has been verified by the comparison of serological data and isolations from naturally and experimentally infected pigs (Dubey et al., 1995; Dubey, 1997) and also free-range chickens (Dubey et al., 2016 and Casartelli-Alves et al., 2014).

T. gondii modified agglutination test is an IgG specific method for the qualitative and quantitative diagnosis of *T. gondii* infection by testing serum. For MAT, sera are treated with 2-mercaptoethanol to remove non-specific IgM or IgM–like substances. This test detects only IgG antibodies; thus, it may give false negative results during early stages of acute infection. The cut-off value of MAT was adjusted to 1:5. In general, the rate of *T. gondii* increases with a magnitude of antibody titer but viable *T. gondii* has been isolated several times from chickens with a titer as low as 1:5 (Dubey et al., 2015, 2016). The heart juice obtained was already diluted in the packing plant, which made the low cut-off titer more reasonable.

Except MAT, there are many other serological tests that are used for the detection of IgG and IgM antibodies to *T. gondii*, which include latex agglutination (LA), indirect hemagglutination (IHA), Sabin-Feldman dye test (DT), indirect fluorescent antibody (IFA), enzyme-linked immunoabsorbent assay (ELISA), and complement fixation (CF). Among them, IFA, ELISA, and MAT have been modified to detect IgM antibodies. Different tests have different sensitivities and specificities for chicken sera samples. IFAT and MAT are two commonly used methods to detect anti-*T. gondii* antibodies in chicken. They have the highest sensitivity at the cut-off value of 16 (Casartelli-Alves et al., 2014). IHA is an insensitive test for chickens and DT could not be used to detect anti-*T. gondii* antibodies in chicken though it works well in the diagnosis of toxoplasmosis in humans (Dubey, 2010a). The results of ELISA are very consistent with MAT in chickens (Hamidineiat et al., 2014). Nowadays, several recombinant antigens are used for serological test of *T. gondii* to avoid the short come of ELISA. The most commonly used

recombinant antigens are GRA1 for chronic infection and GRA7 for acute infection (Alaganan et al., 2014 and Wang et al., 2013).

2.4 Bioassay

Cats and mice are two common animals used for bioassay of tissues in the isolation of *T. gondii*. Cats are more sensitive to *T. gondii* infection but they are more expensive. Cats can shed oocysts because of the ingestion of one single *T. gondii* cyst. Mice are cheaper and easier options and they are less likely to die of secondary infections.

Chickens, like the other intermediate host, in most case have chronic toxoplasmosis due to the infection. However, number of parasite in tissues of chronically infected animals is low, and bradyzoites are more likely to appear in the tissues than tachyzoites. Thus, it is necessary to digest the tissue to concentrate *T. gondii* in the inoculum. Tissue cyst wall is destroyed immediately by pepsin or trypsin, but released bradyzoites can survive up to 6 hour; tachyzoites are distoryed by pepsin but not trypsin (Jacobs et al., 1960a). As a result, during acid pepsin digestion process (60-90 min), homogenized host tissues are digested and many bacteria are killed without destroying bradyzoites of *T. gondii* in the inoculum. Trypsin is used for the digestion of brain tissues because brain tissues contain more fat tissues. But trypsin is more toxic to mice, samples need to be washed multiple time to remove traces of trypsin. All homogenizations should be performed under a hood to prevent splashing of material on the face of the operator.

Chicken hearts are the best organs for bioassay compared with brains and muscles (Dubey et al., 1988b) and (Dubey et al., 2015). Among 144 *T. gondii* positive chickens, 89.5% (129 of 144) of hearts, while only 49.2% (67 of 136) of brains, 44.1% (15 of 34)

of leg muscle and 18.6% (16 of 86) of pectoral muscle were found to be infected (Dubey et al., 1988b). Hence, we choose chicken hearts as out samples and do serology and bioassay with them. Swiss Webster (SW) mice were the animal models used in the experiment to isolation viable *T. gondii* from chicken heart samples. SW mice survived with the infection of none pathogenic mice. The blood samples of each SW mouse were collected and tested with MAT test. Brain smear of each SW mouse were made to confirm whether the mouse was infected by *T. gondii*. Interferon γ knockout (KO) mice were used to revive and subpassage *T. gondii*. *T. gondii* can replicate inside KO mice and kill the mice. Tachyzoites found in the lung of KO mice were easier and more efficient to cell culture in the cell line compared with cysts found in brain of SW mice.

2.5 Cell culture

T. gondii tachyzoites could be cultured in almost all warm-blooded cell lines. Fibroblasts are commonly used because they belong to spread out cells. In this experiment, CV-1 monkey kidney cell line was used to culture *T. gondii*. The growth of tachyzoites varies according to the cell line used and the strain of *T. gondii*. Mouse "virulent" strains cause more damage to the cells compared with "avirulent" strains because "avirulent" strains grow slowly and cause minimal cell damage. Unlike passage in mice, passage of tachyzoites in cell culture is not known to alter the virulence of the organism.

2.6 Multiplex multilocus nested PCR-RFLP

Many molecular diagnostic methods have been developed to genotype *T. gondii* and nested PCR-RFLP is proved to be a reproducible, sensitive, simple, fast, and cost-

effective method, and has been widely used to detection and characterization a variety of isolates from animals and humans. Nested PCR includes primary PCR and secondary PCR. In the primary, genetic loci are pre-amplified by multiplex PCR with external primers. In the secondary PCR, each locus is amplified with internal primers. Nested PCR intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites.

Until now, eleven markers of nested PCR-RFLP are developed to characterize *T. gondii*. In the early 2001, only 5 genetic markers were used for PCR-RFLP genotyping: B1, SAG1, SAG2, SAG3, and SAG4 (Grigg et al., 2001). More markers were developed later on and some markers were not used anymore. Genetic analysis of the SAG2 locus was performed to determine the prevalence of the different genotypes of *T. gondii*. In the ToxoDB, around two hundred PCR-RFLP genotypes of *T. gondii* were discovered. The eleven marker used in the study were SAG1, SAG2, SAG3, alt.SAG2, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico.

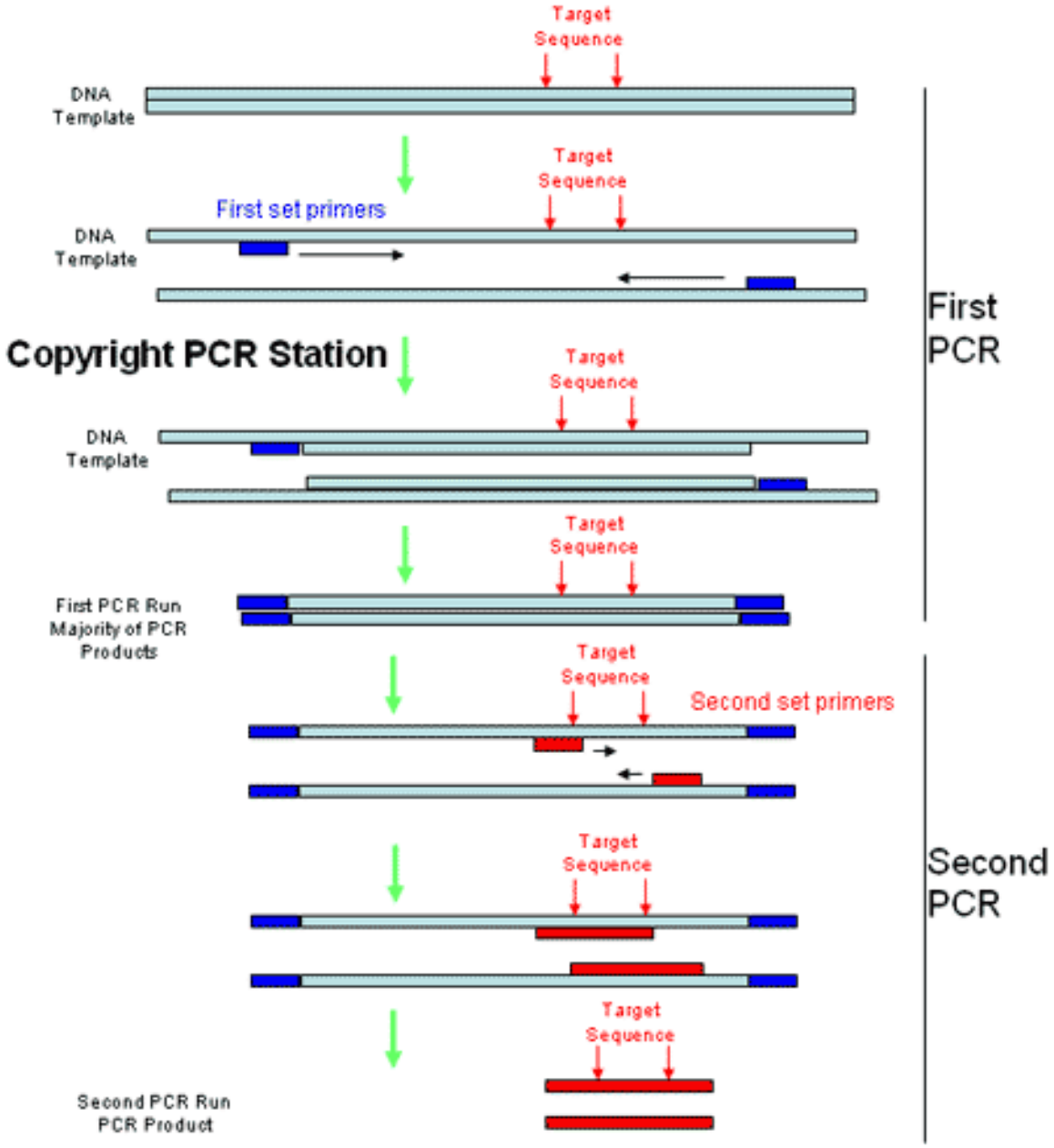

Figure 2.3 The schematic diagram of multiplex multilocus nested PCR-RFLP typing. (Source: http://scienceblogs.com/insolence/2011/09/06/oh-no-theres-dna-in-my-gardasil/)

2.7 *T. gondii* in Chickens

Free-range chickens are important in epidemiology studies because they are transmission vehicles to cats and humans. Chickens are good indicator of the

environment *T. gondii* contamination and strains prevalent because of their habit of feeding from ground (Dubey, 2009; Pena et al., 2013). Thus, they are considered to be important sentinel animals as well as excellent animals for *T. gondii* epidemiology studies. In the study (Dubey et al., 2015), chickens were used as sentinel for the farm in New England. The seroprevalence of chickens could also be closely related to the vulnerability of ground water (Vieira et al., 2015). A waterborne outbreak of toxoplasmosis in Brazil in 2001 found that strains isolated from chickens foraging near the outbreak site matched the strains of individuals (Vaudaux et al., 2010).

A national meat survey in 2005, 2094 chicken breast samples from 28 major geographic areas of the United States was sampled (Dubey, 2005a). No viable *T. gondii* was isolated. However, this study did not negate the possibility that infected chickens may be a source of *T. gondii* for humans because chicken samples were injected with enhanced solution or frozen before (Dubey, 2010a). However, many small farms sell their chicken product directly to the local farmers markets, which increase the risk of *T. gondii*. In this study, fresh chicken heart samples (never frozen or enhanced) were used. Chicken hearts are good samples to isolate viable *T. gondii* from chicken. In one study in 2003, the hearts, brains and leg muscles of 26 chickens were bioassayed into mice. *T. gondii* was isolated from hearts of all 26, brains of three, and leg muscle of 11 (Dubey et al., 2015).

Table 2.2 is a summary of serological test and bioassay of *T. gondii* in chickens from different countries after 2009. Likewise, the results of serological test and bioassay of *T. gondii* in chickens from the United States and other countries before 2009 have been summarized by J. P. Dubey (Dubey, 2009). The range of seroprevalence in the table is

from 0% to 90%. The isolation rates of *T. gondii* are low in these studies with seropositive chickens: 2 in 77 (Lopes et al., 2016) and 1 in 43 (Tilahun et al., 2013). The infection rate in free-range chickens is higher compared to the one in caged chickens (Yang et al., 2012, Cong et al., 2012 and Yan et al., 2009).

Very few studies have been conducted so far to detect and isolate viable *T. gondii* in chickens. The prevalence of viable *T. gondii* ranged from 0 to 100% in the previous studies. The studies about the isolation of viable *T. gondii* from chicken are listed in Table 1. In the study in 2003 (Dubey et al., 2003), 22 strains were isolated from chicken in Ohio and Massachusetts. Seroprevalences were 16.9% (20/118) and 100% (11/11) and the isolation rates of the seropositive chickens were 55% (11/20) and 100% (11/11). Thirteen isolates of *T. gondii* from Ohio and Massachusetts were not virulent for mice. Out of thirteen isolates genotyped, 5 isolates were type II and 14 were type III; mixed types and type I isolates were not found (Dubey et al., 2003). At that time only one marker was used to identify the strain type. This study, isolations were revived and genotyped with 11 markers.

Chickens infected with *T. gondii* are usually asymptomatic and have little clinical signs. However, in 2007, an outbreak of clinical toxoplasmosis was reported on a farm in Illinois (Dubey 2007). Three chickens developed neurological signs and the *T. gondii* infection was confirmed by immunohistochemical staining.

Table 2.1 Isolation of *T. gondii* by bioassay in mice from chickens from different sources of chicken in the United States

States	Source	No. chickens	No. chickens bioassay positive	Tissue bioassayed	Strain designation/ Molecular data	Reference
Montana	FR	11	3 (27.2)	H + B	No/Yes	Dubey (1981)
Illinois	FR	11	11 (100)	H + B +M	Yes/Yes	Dubey et al. (2007)
Tennessee	FR	7	3 (42.8)	B	No/No	Gibson and Eyles (1957)
	FR	60	0 (0)	B		
Texas	FR	11	7 (71.4)	H + B	No/No	Foster et al. (1969)
Iowa	FR	12	11 (91.6)	H + B +M	No/No	McCulloch (1968)
New England	FR	11	8 (72.7)	Bioassay in cats	Yes/Yes	Lehmann et al., (2003)
Ohio	FR	118	11 (9.3)	H + B	Yes/Yes	Dubey et al. (2003)
Massachusetts	FR	11	11 (100)	Bioassay in cats	Yes/Yes	Dubey et al. (2003)
New England	FR	71	31 (43.7)	H+ B+ M	No/No	Dubey et al. (2015)
Maryland	SH	108	4 (3.7)	Ovary, B, M	No/No	Jacobs et al. (1966)
Nationwide	RS	2094	0 (0)	M	No/No	Dubey et al. (2005)

H: hearts; B: Brain; M: Muscle; FR: free-range; SH: slaughterhouse; RS: retail store.

Indoor or free ranged raised, soil oocysts contamination situation, ages of chickens, and farm management (rodent control and the presence of cats) could all influence the infection rate of chickens. The *T. gondii* infection rate of chickens could also be closely related to the vulnerability of ground water (Vieira et al., 2015). After chickens are slaughtered in the slaughterhouses, chilling, frozen, and inject with enhanced solution could kill or decrease viable *T. gondii* in chicken.

Chickens are raised in different ways. Caged and cage free chickens are all indoor chickens without the access to the outdoor. Caged chickens live inside the battery cages with restricted movement. Cage free chickens are free to walk, nest, and engage in other natural behaviors. Free-ranged chickens refer to the chicken raised in natural conditions with freedom of movement and could obtain food from the ground (Tilahun et al., 2013). Some studies find that free-range chicken have higher infection rate compared with indoor chickens (Yan et al., 2009), (Yang et al., 2012). The longer a chicken lives, the higher chance it has to be exposed to the environment *T. gondii* oocysts. Most of the chickens consumed in the U.S. are broilers (*Gallus domesticus*), which reach slaughter weight at five to seven weeks of age. Some other farm management, such as rodent control and presence of cats, could also influence the infection rate of *T. gondii* in the farm animals (Garcia-Bocanegra et al., 2010) because cats are definitive host of *T. gondii* and they can shade thousands of oocysts that could contaminate the environment.

Most chicken in the U.S. is chilled to freezing or completely frozen temperature at the packing plant (Chan et al., 2001), which should kill viable *T. gondii* in tissues (Kotula et al., 1991). Some other chicken products are injected with enhanced solution and could reduce the number of viable *T. gondii* in the products. The meat survey conducted in

2005 sampled 2094 chicken samples from the U.S. grocery stores and no viable *T. gondii* was isolated (Dubey et al., 2005a). One possible reason was that most of the chicken was injected with enhanced solution and killed most of the parasite.

Table 2.2 Serological prevalence and bioassay of *T. gondii* in chickens from different countries other than the United States

Country	Source	Test	No. Tested	No. positive (%)	Cut-off	No. Bioassay	Positive	Tissue	Reference
Argentina	FR	IFAT	32	17 (53)	1:50	7	6	B+H	Moré et al., 2012
Australia	FR	IFAT	20	18 (90.0)	1:16	ND			Chumpolbanchorn et al., 2013
Brazil	FR	IFAT	483	152 (31.5)	1:16	71	33	B+H	Feitosa et al., 2016
Brazil	FR	IFAT	60	15 (25.0)	1:16	60	5	B+H	Sousa et al., 2016
Brazil	FR	IFAT	430	380 (88.4)	1:16	ND			Magalhães et al., 2016
Brazil	FR	IFAT	212	86 (40.56)	1:16	ND			Fernandes et al., 2016
Brazil	FR	MAT	108	77 (71.03)	1:16	77	2	B+H	Lopes et al., 2016
Brazil	FR	ND				153	63	Pooled tissue sample	Casartelli-Alves et al., 2015
Brazil	FR	MAT	135	81 (60.0)	1:16	135	54	B+H+M	Casartelli-Alves et al., 2014
Brazil	FR	MAT	510	198 (38.8)	1:25	64	48	B+H+M	Neltram et al., 2012
Brazil	FR	ND				90	22	/	Soares et al., 2011
Brazil	FR	MAT	50	42 (84)	1:5	40	24	B+H	Dubey et al., 2010b
China	FR	MAT	700	132(18.86)	1:25	ND			Feng et al., 2016
China	FR	WB	110	16 (14.5)		ND			Sun et al., 2015
China	FR	ND				24	1	B	Wang et al., 2013
China	FR	MAT	206	23 (11.2)	1:25	ND			Yang et al., 2012
China	FR	MAT	108	11 (10.19)	1:5	ND			Cong et al., 2012
China	FR	ELIS	1176	356 (30.36)		ND			Zhao et al., 2012

		A							
China	FR	MAT	361	41 (11.4)	1:5	ND			Yan et al., 2009
China	C	MAT	296	14 (4.7)	1:25	ND			Yang et al., 2012
China	C	MAT	305	19 (6.23)	1:5	ND			Cong et al., 2012
China	C	MAT	450	25 (5.6)	1:25	ND			Xu et al., 2012
China	C	MAT	244	10 (4.1)	1:5	ND			Yan et al., 2009
China	S	MAT	160	30 (18.8)	1:25	ND			Xu et al., 2012
Ethiopia	FR	DAT	183	41 (22.4)	1:40	41	29	B+H	Gebremedhin et al., 2014
Ethiopia	FR	MAT	601	183 (30.5)	1:60	ND			Gebremedhin et al., 2015
Ethiopia	FR	MAT	48	125 (38.4)	1:5	43	1	H	Tilahun et al., 2013
Egypt	S	ELISA	439	302 (68.6)		N/A		Pooled tissue sample	Barakat et al., 2012
Iran	FR	MAT	106	55 (51.89)	1:10	ND			Hamidinejat et al., 2014
Ireland	S	LAT	364	65 (18)	1:5	ND			Halová et al., 2013
Japan	S	LAT	235	0 (0)	1:64	ND			Matsuo et al., 2014
Mexico	FR	MAT	51	13 (25.5)	1:25	ND			Alvarado-Esquivel et al., 2012
	S		468	23 (4.9)	1:25				
Nigeria	FR	MAT	225	91 (40.4)	1:20	ND			Ayinmode et al., 2014

FR: free-range; S: slaughterhouse; C: caged; MAT: modified agglutination test; IFAT: indirect fluorescent antibody test; LAT: latex agglutination test; DAT: direct agglutination test; WB: western blot; ND: not done

2.8 *T. gondii* in other meat and meat animals

2.8.1 Pork and Pig

Pork is an important meat vehicle cause human *T. gondii* infections. It is estimated that 134 deaths and $1,219 million loss are due to the infection with *T. gondii* by the consumption of meat products (Batz et al., 2012). In the national meat survey conducted in 1983, 23% of market pigs and 42% of sows was seropositive with *T. gondii*. However, the *T. gondii* infection rates in the pigs and sows are not directly related with the prevalence of *T. gondii* in the pork products because many pork products sold in the retail stores are injected with enhanced solution, which could inactivate *T. gondii* cysts. In the national meat survey conducted in 2005, viable *T. gondii* was isolated from seven pork samples in a total of 2094 pork samples bought from retail stores from 28 major geographic areas in the United States (Dubey et al., 2005a). No viable *T. gondii* were isolated from beef and chicken in that meat survey because swine are highly susceptibility to *T. gondii* infection compared with cattles and poultries (Hill et al., 2013).

Organic pork products have higher risk compared with fresh pork from market pigs raised in confinement (Guo et al., 2015). The modern confinement management systems helped to considerably reduce the *T. gondii* infection rate in swine in the recent years. National Animal Health Monitoring System (NAHMS) surveys have shown a decline in Toxoplasma seroprevalence in sows from 20% in 1990 to 15% in 1995, to 6% in 2000 (Patton et al., 1996, 2002). However, seroprevalence in grower/finisher swine has remained stable over that period. The infection rates of *T. gondii* are also related with the age of the swine.

The consumption of pork products is around 18.6 kg per capital per year, which is stable over the last two decades. There is no inspection program for testing and detecting of *T. gondii* in pigs at slaughter in the US infected pigs enter the food chain (Hill et al., 2013). Consumers should cook pork products thoroughly before consumption.

2.8.2 Lamb, sheep and goats

Though lamb is not the major food that consumed in the US, it poses a major risk for human beings. Goat meat is one of the major meat for some ethnic groups. Many sheep and goats are raised outdoors and have the free access to the contaminated environment and the risk of getting infected with *T. gondii* with eating organic lamb meat is higher than organic pork and free-range chickens (Guo et al., 2015).

Prevalence of sheep and goats differed by animal age, geographic location, population density, grazing area, and farm management practice (Guo et al., 2015). Goats typically have lower infection rate compared with sheep because sheep tend to consume the all parts of the grasses including the roots and have higher change to be infected by *T. gondii* oocysts in the soil. Every year, abortions of sheep and goats result in great loss. Vaccine were invented and produced to reduce the lost due to abortions.

Viable *T. gondii* isolation was reported from both sheep and goats. *T. gondii* was isolated from two of 50 lamb chops from retail stores in California and from eight of 86 sheep diaphragms from slaughterhouse in Baltimore, Maryland (Jacobs et al., 1960b). Thereafter, more *T. gondii* was isolated from lamb and sheep. The number of goat slaughtered in the US is growing each year as the immigrant population is growing. In a recent study, 234 goat hearts were obtained from retail stored and tested for *T. gondii*

antibody using MAT (Dubey et al., 2011). The seroprevalence of those samples are 53.4%, 125 in 234 goats are positive with *T. gondii* antibody.

2.8.3 Beef and cattle

The ingestion of beef is not considered important in the epidemiology of *T. gondii* because of the resistance of cattle to the parasite (Hill and Dubey, 2013). In the national meat survey in 2005, no viable *T. gondii* was isolated from 2094 beef obtained from retail stores nationwide (Dubey et al., 2005a). Most of the serological tests do not work well with cattle sera and the seroprevalence results were not consistent with the appearance of tissue cysts. Cattle are resistant to the infection of *T. gondii* and thus are not considered as important hosts for *T. gondii* (Dubey, 2010a).

2.8.4 Goat milk and cheese

The risk of infection due to the consumption of raw goat milk or cheese is debatable. Viable *T. gondii* tachyzoites was isolated from goat milk (Dubey 2010a), but tachyzoites is less resistant to pepsin and trypsin digestion thus makes it less infectious (Dubey et al., 1998a). Viable *T. gondii* was also found from raw goat cheese made in the experimental condition (Dubey et al., 2014). Drinking unpasteurized goat milk can cause fatal toxoplasmosis in humans (Chiari and Neves, 1984; Riemann et al., 1975; and Sacks et al., 1982) and are extremely dangerous for pregnant women (Patton et al., 1990 and Jones et al., 2009).

2.8.5 Processed meat products

Salted, fermented, smoked, or other processing are not adequate to inactive all *T. gondii* in meat products, thus the consumption of processed meat products is considered

as a risk factor of *T. gondii* infection. In the study of fresh pork sausage, thirteen of the 149 sausage meat samples were positive by bioassay (Dias et al., 2005). No viable T. gondii was found from ham made from naturally infected pigs after curing for 14 months (Bayarri et al., 2010).

2.8.6 Seafood

T. gondii oocysts may washed into sea water with the rainfall. Oysters, mussels, or clams may be contaminated with *T. gondii*. The studies of survival of *T. gondii* oocysts in eastern oysters indicate that *T. gondii* can survive for several months in oysters (Lindsay et al., 2009) and oysters can readily remove *T. gondii* oocysts from seawater (Lindsay et al., 2004). Infected filter feeders may serve as a source of *T. gondii* for marine mammals and possibly humans.

Chapter 3: Research Objective

3.1 Objective 1

Assess the prevalence of *T. gondii* in chickens and evaluate the microbial safety and infection of *T. gondii* in free-range chickens available at the grocery stores and farms for the consumers to purchase. Chicken hearts were purchased from six local markets, one farm in Maryland and from Ohio Amish town. Seropositivity of the chicken heart is not only reflecting *T. gondii* prevalence in chickens, but also used as an indicator of the environmental oocysts contamination during chicken production.

3.2 Objective 2

Genotype *T. gondii* from isolates. A total number of thirteen isolates from previous study in chickens were revived. Each of the isolated strain was genotyped by nested PCR with genetic markers SAG1, SAG2, alt-SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico.

Chapter 4: Materials and Methods

4.1 Sampling

Chicken heart samples from six local markets and one farm in Maryland and from Ohio Amish town were collected. One or two markets or farms was visited each week, to ensure that multiple samples were collected. 0.454~0.907 kg (one to two pounds) of chicken hearts were purchased at each sampling and kept at 4°C during transportation to the laboratory for processing within 48 hour. A total of 19 to 95 chicken hearts were bought each time. Each chicken heart was put into an individual plastic bag before the other processes. A total number of 997 chicken hearts were processed.

4.2 Modified agglutination test (MAT)

A large volume of serum diluting buffer and antigen diluting buffer was prepared ahead of the test. Serum diluting buffer (1): Around 900ml deionized water was used to dissolve 42.5 g NaCl, 1.54 g NaH_2PO_4, and 5.4 g Na_2HPO_4. The pH was adjusted to 7.2. Deionized water was added to bring the volume to 1 liter. This 5× stock solution was stored in a refrigerator. This stock solution was diluted 1:5 to give 0.01 M phosphate buffered saline (PBS) (1 part stock and 4 parts deionized water). PBS was filtered just before use through a 0.22μM membrane. Antigen diluting buffer (2): A total of 900 ml deionized water was used to dissolve 7.01 g sodium chloride, 3.09 g boric acid, and 2.0 g sodium azide. Additional 24 ml 1 N NaOH was added and adjusted the pH to 8.95. Deionized water was added to bring the volume to 1 liter. This stock solution was stored at room temperature. For the working antigen diluting buffer, 0.4 g bovine serum

albumin (BSA) was dissolved in 100 ml borate buffer and their concentration was adjusted the tachyzoites concentration to 2×10^4 /□l. This solution was stored in a refrigerator.

Heart juice was extracted from atria and ventricles of each chicken heart. The juice was collected and stored at 4°C for modified agglutination test (MAT). Heart juice samples were diluted with serum diluting buffer (1) in small test tubes (1.2 ml in strips of 8 or 12) with a multichannel pipette, starting at 1:5. Microtiter plates mere also used for making serum dilutions. Antigen mixture was prepared as follows: For each plate, 2.5 ml antigen diluting buffer (2), 35 µl 2-mercaptoethanol, 50 µl evans blue dye solution (2 mg/ml water), and 0.15 ml antigen (formalin-fixed whole parasites) were mixed together. Agglutination was done in U bottom 96 well microtiter plates. After mixing, 25µl antigen mixture were pipetted to each well immediately. A same volume of serum dilutions were added into the wells and mixed gently with the antigen by repeated pipetting action.

A positive control was included in each plate. Because the fluid got from atria and ventricle of each heart was already washed at the slaughterhouse, low dilutions of 1:5, 1:25, and 1:100 were used.

The plates were covered with sealing tape. Plates were gently agitated to allow for complete mixing and incubated for 12 h at room temperature in a moisture atmosphere. A magnifying mirror was used to help read the results. A clear-cut button-shaped deposit of parasite suspension at the bottom of the well was interpreted as a negative reaction, and a complete carpet of agglutinated parasites was considered a positive result. The cut off value was 1:5.

Technical details are described in several previous studies (Dubey et al., 1987) and (Desmonts et al., 1980). The MAT has been modified to detect *T. gondii* IgM antibodies in serum.

4.3 Bioassay

The heart tissues were trimmed with disposable lancets on nonporous, hard plastic cutting boards. Muscle was cut into small (1-2 cm) pieces and stored in plastic cups. Each heart was approximately 10g. Whole heart tissues and 50 ml of saline were added into blender and blended for about 30 sec at middle speed. To save expense and time in cleaning the lid of the blender every time to avoid cross contamination, the lid of the blender was lined with a disposable plastic sheet (commercially available 16.5 x 14 cm sandwich bags are convenient to use). The tissue homogenate were poured into a wide mouth polypropylene centrifuge bottle (Nalgene). Each bottle was labeled with the animal number using a good adhesive tape and water-resistant marker. These labels were transferred throughout the procedure to help reduce mislabelling. Homogenates can be left at room temperature for 1-3 hours until all specimens have been processed.

A same volume of freshly prepared, prewarmed (37ºC) acid pepsin solution (pepsin, 1:10,000, 2.6g; NaCl, 5.0g; HC1, 7.0ml; and water to make 500 ml, pH 1.10-1.20) was added into the prewarmed (37ºC) homogenate. The mixture was incubated at 37ºC in a shaking water bath for 60 min. The source and purity of pepsin used was probably not critical, but porcine stomach pepsin (1:10,000 biological activity, Spectrum Chemical Mfg. Corp., New Brunswisk, NJ, USA) was used in the experiment.

The homogenate was filtered through 2 layers of gauze and centrifuged in the same bottle at 1200 × g for 10 minutes. The supernatant was poured off. The sediment was suspended in 10 ml of phosphate buffered saline (PBS, pH 7.2) using disposable plastic pipettes. The homogenate was transferred in a 50 ml centrifuge tube with a conical bottom and then neutralized with 12 to 15 ml of freshly prepared 1.2% sodium bicarbonate (pH 8.3) with phenol red as a pH indicator until the color changed to orange. After mixing, saline was added to a volume of 50 ml and centrifuge at 1180 × g for 10 minutes. The supernatant was poured off and same volume of saline (1-2 ml) that contains 1000 units penicillin and 100 µg of streptomycin per ml was added.

A maximal 1 ml of tissue homogenate was subcutaneously inoculated into each of a group of two mice (Swiss-Webster weighing 20-25 g) over the back using a 1.5 inch long 21-23 gauge needle. Each mouse was identified with a rodent ear tag. Approximately 120-240 min were expected to elapse from the time homogenizing tissue in saline to inoculation. All inoculated mice were examined for *T. gondii* infection. Six weeks p.i., SW mice were bled and serum were tested with MAT. After the serology test, mice were sacrificed and every mouse brain was microscopically examined for *T. gondii* cysts.

T. gondii stages (tachyzoites, bradyzoites) in tissues were killed by water and by heating to 60ºC, and so blenders, cutting boards, and other materials were cleaned with soap and hot water, then rinsed with cold water and finally sterilized and dried before use for the next specimen.

The second batch of 60 chicken hearts was bioassayed in one cat. The top part of hearts with fat was chopped off with disposable lancets on nonporous, hard plastic cutting

boards. All 60 hearts were added into one blender and blended for about 30 seconds at a middle speed to make meat paste, which was fed to one cat for 3 to 4 days until the cat ate up all of the samples.

Feces of the cat were collected everyday from the fourth day after infection. Around 10 gram of feces of the cat were emulsified in approximately 40 ml of sucrose solution (specific gravity 1.28), and centrifuged at 1,180 g for 10 minutes. A drop of the film from the very top of the fecal float was examined microscopically for oocysts between a coverslip and a slide. The cat was bled and euthanized on day 21 and the blood sample was used to test *T. gondii* antibody.

4.4 Revive cryopreserved *T. gondii* isolates

Cell cultures or mice tissues of *T. gondii* isolations from previous studies in 2003 were cryopreserved in the nitrogen tank. A total of 13 samples were successfully revived from a total of 17 isolations that were preserved in the nitrogen tank. 8 of them are from Ohio and 5 of them are from one New England pig farm.

Cryotubes were found and taken out from nitrogen tanks according to tank, canister, and stick numbers in the notebook. Cryotubes were immediately transferred to 37 °C water bath and fast defreeze samples for five minutes until the cryopreserved tissues were thoroughly melted. Each sample was inoculated subcutaneously into one mouse (interferon γ knockout mouse weighing 20-25 g) over the back using a 1.5 inch long 21-23 gauge needle.

After subcutaneously inoculation, if the mice died within 14 days, imprints of the lungs were examined for tachyzoites. After 14 days, organisms usually begin to

disappear from the visceral organs and *T. gondii* was most likely to be demonstrated in the brain. Smear of brain tissues were examined for bradyzoites. The survived mice bled and sacrificed after six weed p.i.. MAT was done with the blood and brains were microscopically examined for *T. gondii* cysts.

When the lungs or brains were examined as positive for *T. gondii*, the tissues were homogenized in a mortar, and were then suspended in saline. If bacterial contamination was suspected, tissues or fluids were mixed with antibiotics in 0.9% NaCl solution (saline) to give a concentration of 1000 units of penicillin and 100 μg of streptomycin per ml (antibiotic saline) without reducing the infectivity. Contaminated specimens suspended in antibiotics were allowed to stand at room temperature for 1 h.

4.5 Cell culture

Positive mouse tissues were seeded on to CV-1 cell culture flasks and tachyzoites were harvested from the medium. The flasks were kept for half month after the tachyzoites grow up. The entire medium was collected and centrifuged to get the compacted tachyzoites. The tachyzoites were used for DNA extraction.

4.6 DNA extraction

T. gondii DNA was extracted from cultured tachyzoites by using the commercial kit (DNeasy blood & tissue kit (250), Cat# 69506, Qiagen). First of all, in an eppendorf 180 μl ATL buffer and 20 μl Proteinase K were added into each tachyzoites sample to digest the protein. The eppendorf was mixed by vortex and incubated at 56 °C overnight in water bath.

After the incubation, samples were vortexed and 200 µl AL buffer was added and then vortex to mix properly. In addition to AL buffer, 200 µl Ethanol (96%-100%) were added and again mixed by vortex. The above mixture including any precipitate was pipetted into the column placed in 2 ml collection tube and centrifuged for 1 min at 8000 rpm. Discard the flow through and collection tube. The column was placed in new collection tube and 500 µl AW1 buffer was added. The collection tube was centrifuged for 1 min at 8000 rpm and discarded the flow through. The column was placed in a new collection tube and 500 µl AW2 beffer was added and centrifuge for 3 min at 14000 rpm. The flow through and collection tube was discarded. The column was placed in 1.5 ml eppendorf tube and 100-200 µl AE buffer was add. The column was incubated for 1 min at room temperature and then centrifuged for 1 min at 8000 rpm. The received solution was taken as DNA and store at -10 °C for multiplex multipocus nested PCR-RFLP.

4.7 Multiplex multilocus nested PCR-RFLP

Multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) typing using a set of the eleven genetic markers SAG1, SAG2, SAG3, alt.SAG2, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico were used for genotyping (Su et al., 2010). Three PCR controls, Strains RH (Type I), PTG (Type II), and CTG (Type III) were used as primary reference strains for genotyping. Additional positive control strains MAS, TgCgCa1 (Couger) and negative controls without DNG template were included to monitor contamination and efficiency of PCR amplification and genotyping. In summary, the sequence of the multiplex multilocus nested PCR-RFLP was as follows: multiplex PCR, nested PCR, restriction digestion, gel electrophoresis and phylogenetic analysis.

Genetic loci were pre-amplified by multiplex PCR with external primers and each locus were amplified by nested PCR using internal primers. The multiplex PCR reaction solution contained: 17 μl nuclease-free water, 2.5 μl 10× standard Taq reaction buffer, 2 μl $MgCl_2$, 0.5 μl each of the dNTPs, 0.8 μl each of the external forward and reverse primers, 0.2 μl of FastStart DNA polymerase (Roche, Indianapolis, IN) and 2 μl of DNA samples. The treatment time and temperature of the reaction mixture were: 95°C for 4 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min and 72°C for 2 min. The nested PCR reaction solution contained 8.5 μl nuclease-free water, 12.5 μl Taq master mix buffer (QIAGEN), 1.0 μl each of internal forward and reverse primers, 2.0 ml of diluted multiplex PCR products. The nested PCR cycle were: 95°C for 4 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 1.5 min (for marker Apico, the annealing temperature was at 58°C instead of 60 °C).

For RFLP typing, 6 ml of nested PCR products were treated with restriction enzymes and the digested samples were resolved in agarose gel to reveal strain-specific DNA banding patterns (Su et al., 2006). For each marker, specific restriction enzymes were used to digest 6 ml of secondary PCR products. After digestion, the DNA fragments were separated by agarose gel electrophoresis. Each distinctive DNA fragments patterns that were result from the presence of sequence polymorphism were compared to the reference type I, II, and III strains to decide its genotype at that locus. The genotype of each isolate was determined by the genotype results of all eleven loci. The primers and restriction enzymes used in this study for Mn-PCR-RFLP typing of *T. gondii* isolates are listed in Table 4.1.

Table 4.1 Summary of RFLP-markers, primers and restriction enzymes used, size of the primers, and digestion conditions

Marker	PCR primers 5'-3'	Restriction Enzymes	Size (bp)	Digestion Tm/Time	References
SAG1	SAG1F:CAATGTGCACCTGTAGGAAGC SAG1R:GTGGTTCTCCGTCGGTGTGAG	Sau96 I, Hae II	390	37°C/1H	Grigg et al. (2001)
3'-SAG2	3'SAG2F:ATTCTCATGCCTCCGCTTC 3'SAG2R:AACGTTTCACGAAGGCACAC	Hha I	222	37°C/1H	Grigg et al. (2001)
5'-SAG2	5'SAG2F:GAAATGTTTCAGGTTGCTGC 5'SAG2R:GCAAGAGCGAACTTGAACAC	Mbo I	242	37°C/1H	
SAG3	P43S1:CAACTCTCACCATTCCACCC-3 P43AS1:GCGCGTTGTTAGACAAGACA	Nci I	311	37°C/1H	Grigg et al. (2001)
L358	L358-F2:AGGAGGCGTAGCGCAAGT L358-R2:CCCTCTGGCTGCAGTGCT	Hae III, Nla III	418	37°C/1H	Khan et al (2005) and Su et al. (2006)
GRA6	GRA6-F1:TTTCCGAGCAGGTGACCT GRA6-R1x:TCGCCGAAGAGTTGACATAG	Mse I	344	37°C/1H	Fazaeli et al. (2000) and Su et al. (2006)
PK1	PK1-F:CGCAAAGGGAGACAATCAGT PK1-R:TCATCGCTGAATCTCATTGC	Ava I, Rsa I	903	37°C/1H	Khan et al (2005) and Su et al. (2006)
c29-2	c29-2F:AGTTCTGCAGAGTGTCGC c29-2R:TGTCTAGGAAAGAGGCGC	HpyCH4 IV, Rsa I	446	37°C/1H	Khan et al. (2005) and Su et al. (2006)
Apico	Apico-F:TGCAAATTCTTGAATTCTCAGTT ApicoR:GGGATTCGAACCCTTGATA	Afl II, Dde I	640	37°C/1H	Su et al. (2006)
BTUB	Btb-F:GAGGTCATCTCGGACGAACA Btb-R:TTGTAGGAACACCCGGACGC	BsiE I, Taq I	411	60°C/30M, 65°C/30M	Khan et al (2005) and Su et al. (2006)
alt-SAG2	SAG2-Fa:ACCCATCTGCGAAGAAAACG SAG2-Ra:ATTTCGACCAGCGGGAGCAC	Hinf I, Taq I	546	37°C/30M, 65°C/30M	Lehmann et al. (2000) and Su et al. (2006)
c22-8	c22-8F:TCTCTCTACGTGGACGCC c22-8R:AGGTGCTTGGATATTCGC	BsmA I, Mbo II	521	37°C/30M, 55°C/30M	Khan et al. (2005) and Su et al. (2006)

Chapter 5: Results and Disscussion

5.1 Serological and bioassay results

A total of 997 chicken heart samples were purchased from six local markets in Maryland, one farm in Maryland and Amish town in Ohio. *T. gondii* antibodies were detected from 150 samples with MAT at titer 1:5 or higher. All 31 chicken hearts obtained from one farm in Maryland were seronegative of *T. gondii*. No parasite was isolated through bioassay. The second batch of 60 chicken hearts from Store A was bioassayed in one cat. The cat did not shed oocysts and *T. gondii* antibodies were not detected by MAT.

Chicken heart samples purchased from local grocery stores A-F. They were expected to be neither frozen nor injected with the enhanced solution. The heart samples from Ohio were collected from an Amish town H. The fresh chicken heart samples from Maryland were bought directly from a local Farm G. In that farm, the free-range chickens are raised. Lamb, beef, goat, pork, and fresh eggs products from their outdoor animals are also sold.

The titers of serum positive samples were low. Only eight of the samples have a titer of 1: 25 and none of them has a titer of 1:100. Details of sampling are listed in table 5.1. Only two batches of samples have titer higher than 1:5. The *T. gondii* seropositive rates of chicken hearts from the same market but different batches were not consistent. In the same market, it could be as low as 0% and as high as 28.3%.

Table 5.1 Details of every sampling, including sampling time, places, MAT results, and seropositive rates with the cut-off titer of 1: 5

Date	**1:5**	**1:25**	**1:100**	**Total**	**Places**	**Positive%**
Nov 14, 2014	15	4	0	53	Store A	28.3
Jan 29, 2015	0	0	0	60	Store A	0
Jun 11, 2015	1	0	0	19	Store B	5.3
Jun 19, 2015	0	0	0	31	Farm G	0
Jun 27, 2015	8	0	0	48	Store A	16.7
Jul 08, 2015	24	4	0	71	Store C	33.8
Jul 10, 2015	2	0	0	55	Store A	3.6
Jul 14, 2015	15	0	0	60	Store C	25.0
Jul 16, 2015	5	0	0	60	Store D	8.3
Aug 06, 2015	12	0	0	82	Store C	14.6
Aug 11, 2015	0	0	0	54	Amish town H	0
Sep 03, 2015	10	0	0	77	Store C	13.0
Sep 09, 2015	16	0	0	95	Store C	16.8
Sep 15, 2015	9	0	0	67	Store E	13.4
Sep 23, 2015	3	0	0	46	Store F	6.5
Sep 30, 2015	6	0	0	65	Store C	9.2
Oct 07, 2015	24	0	0	54	Store E	44.4
Total	150	8	0	997		15.0

5.2 Multiplex multilocus nested PCR-RFLP

Among the seventeen samples isolated from chickens in Ohio and New England, thirteen isolations were revived. The DNA was collected from the successfully revived samples and genotyped because only one marker was used to identify the strain of the parasite from Ohio and isolates from New England were not genotyped. In this study we used 11 markers: SAG1, SAG2, alt-SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico.

The source of eight isolates from Ohio was Scioto County Fair, from where free-range chickens were purchased. The specific locations of farms were not known. Three genotypes were found in the revived cryopreserved samples: four ToxoDB#2, one ToxoDB#3 and three ToxoDB#268, which is a new genotype.

The other five isolates were from a pig farm in New England. This farm was selected to conduct the study based on its high seroprevalence of pigs (Gamble et al., 1999). A total number of eleven free-range adult domestic chickens were examined for *T. gondii* infection (Lehmann et al., 2003). *T. gondii* were isolated by bioassay brain, heart, and muscles from the leg and breast into eleven cats. They all have the same genotype ToxoDB#1.

Table 5.2 Multiplex multilocus nested PCR-RFLP ToxoDB number and results with eleven genetic markers SAG1, SAG2, SAG3, alt.SAG2, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico

Isolates	**SAG1**	**SAG2**	**alt.SAG2**	**SAG3**	**BTUB**	**GRA6**	**C22-8**	**C29-2**	**L358**	**PK1**	**Apico**	**ToxoDB**
OH, Highland	II or III	III	III	III	III	III	III	III	III	III	III	#2
OH, Highland	II or III	III	III	III	III	III	III	III	III	III	III	#2
OH, Warren	II or III	III	III	III	III	III	III	III	III	III	III	#2
OH, Warren	II or III	III	III	III	III	III	III	III	III	III	III	#2
OH, Pike	II or III	III	III	III	I	III	III	III	III	III	III	New, #268
OH, Hamilton	II or III	III	III	III	I	III	III	III	III	III	III	New, #268
OH, Hamilton	II or III	III	III	III	I	III	III	III	III	III	III	New, #268
OH, Madison	II or III	II	II	II	II	II	II	II	II	II	I	#3
NE	II or III	II	II	II	II	II	II	II	II	II	II	#1
NE	II or III	II	II	II	II	II	II	II	II	II	II	#1
NE	II or III	II	II	II	II	II	II	II	II	II	II	#1
NE	II or III	II	II	II	II	II	II	II	II	II	II	#1
NE	II or III	II	II	II	II	II	II	II	II	II	II	#1

OH: Ohio, NE: New England.

5.3 Discussion

In the study, cats and mice were used to isolate viable *T. gondii*. Cats and mice are two common animals used for bioassay of tissues in the isolation of *T. gondii*. Cats are more sensitive to *T. gondii* infection but they are more expensive. Cats can shed oocysts because of the ingestion of one single *T. gondii* cyst. Mice are a cheaper and easier option and they are less likely to die from secondary infections. During the pepsin digestion process of heart tissues, cysts ruptured and bradyzoites were released. It is unknown that how many bradyzoites were inactivated during pepsin digestion and how many bradyzoites could cause the infection of mice by *T. gondii*.

Chicken hearts are the best organs for bioassay compared with brains and muscles (Dubey et al., 1988b) and (Dubey et al., 2015). Among 144 *T. gondii* positive chickens, 89.5% (129 of 144) of hearts, while only 49.2% (67 of 136) of brains, 44.1% (15 of 34) of leg muscle and 18.6% (16 of 86) of pectoral muscle were found to be infected (Dubey et al., 1988b). Moreover, heart juice could be obtained from the inside part of chicken. It is hard to obtain juice from other tissues and then do serological tests. Hence, we choose chicken hearts as out samples and do serology and bioassay with them.

Though no viable *T. gondii* was isolated from sampled chicken hearts, the isolation rates were high from the previous studies. The results from chickens in the farms indicate that *T. gondii* prevalence of free-range chicken may not be as high as previously expected. The low isolation rate could be attributed to the processing after the chickens were slaughtered, such as washing, low temperature storage. *T. gondii* prevalence in chicken hearts can reflect the environmental contamination of *T. gondii*.

The MAT results suggest that the *T. gondii* oocysts are ubiquitously existed in the environment.

In the national meat survey of 2005, 2096 chicken breast samples were bioassayed and ELISA was used to detect infection of *T. gondii*. Negative outcome of bioassay and high positive rate of ELISA (1.3%) were the result of the freezing process of chicken meat that could inactivate *T. gondii*. To avoid the inactivation of parasite by freezing or enhanced solution, in this study, fresh chicken heart samples (neither frozen nor enhanced) were used. Chicken hearts are good samples to isolate viable *T. gondii* from chicken because they have higher isolation rate compared to other parts of chicken. In one study in 2003, the hearts, brains and leg muscles of 26 chickens were bioassayed into mice. *T. gondii* was isolated from all of the 26 hearts, three brains, and 11 leg muscle (Dubey et al., 2015). However, the result, which was quite contradictory, was that no viable *T. gondii* was isolated via bioassay while the seroprevalence was 15.05% by MAT. This seroprevalence was much higher compared to the results of national meat survey in 2005. The low isolation rate indicates that other reasons may also contribute to the inactivation of *T. gondii*. The chicken hearts sold in some stores were fresh. Chicken heart samples from Store A, Farm G, and Amish Town H were never frozen or injected with enhanced solution. However we are not sure that the chicken heart samples we bought were not frozen before. That might be the reason to explain the low isolation rate.

The cut-off titer may influence the results and conclusion. The cut-off titer was set to 1:5 because the samples we tested were diluted heart juice rather than true serum. If 1:25 cut-off titer was used in the study, the seroprevalence was 0.8%, which is very

similar to the result of the national meat survey in 2005. Moreover, heart juice instead of the real serum was used for serological test, which might cause the false positive results.

As mentioned earlier, 2 in 77 (Lopes et al., 2016) and 1 in 43 (Tilahun et al., 2013) isolation rates of *T. gondii* reported were not high in studies involving seropositive chickens. Seropositive chicken hearts were bioassayed in mice. It is interesting that contradicting conclusions are drawn according to the results of serology and bioassay: one conclusion from Lopes is drawn according to serology results. These results reveal high prevalence (71.3%) of *T. gondii* infection in free-range chickens throughout the place, which indicate an important degree of oocyst environmental contamination and the existence of considerable risk of *T. gondii* transmission to humans (Lopes et al., 2016); on the contrary, the low isolation rate of the other study indicates a very low environmental contamination with *T. gondii* oocysts around that area. Whichever result is more representative to the degree of environmental contamination and the risks of *T. gondii* transmission to humans needs to be explored.

No samples from the free-range chickens obtained from farms in Ohio and Maryland were found seropositive, which indicates that those farms are less likely to be contaminated by *T. gondii*. “Free-range” refers to animals that are allowed to roam for food, rather than confined in an enclosed system. Free-range chickens are important in epidemiology studies because they are transmission vehicles to cats and humans. The demand for pasture-raised, free-ranged, backyard raised, and organic meats is increasing annually. *T. gondii* infection rate is usually higher in free-range chickens. Chickens are a good indicator of the environment *T. gondii* contamination and strains prevalent because of their habit of feeding from ground (Dubey, 2009; Pena et al., 2013). Thus, they are

considered to be important sentinel animals as well as excellent animals for *T. gondii* epidemiology studies. The low seroprevalence rate is a good indicator of little *T. gondii* contamination in this area.

The age of chickens could also influence the seroprevalence. In the United States, the average slaughter age for chickens is 47 days. Those free-range chickens from one farm in Maryland might be too young and did not have enough time to assess to the environment. The location where the chickens roamed for food could also influence the *T. gondii* seroprevalence in those free-range chickens. Although those chickens had free access to the outdoors, they were confined in a small area instead of the whole farm. The contamination of *T. gondii* oocysts was not evenly distributed in the whole farm.

No viable *T. gondii* was isolated from chicken heart obtained from Amish markets. Meats from Amish markets are popular in mid-Atlantic region, where consumers (general public) can purchase variety of meat products that are produced by the Amish community, including some special products such as chicken heart and rabbit meat. Unlike commercial meat production system in the U.S., the husbandry operation in Amish community is similar to organic or free-range rearing system. Prevalence of *T. gondii* was found greater in organic-raised meat animals when compared to their indoor-raised counterparts (Hill and Dubey, 2013). Chickens from Amish community are raised in backyards. However, the chicken hearts in the Amish market were possibly from indoor chickens, which has no difference from the local market.

The PCR-RFLP result shows a population diversity of *T. gondii* genotypes. Only one genotype was discovered in one farm, which indicates the monotonicity of *T. gondii* strains in one area. No Type I *T. gondii* was found. From the 13 isolates, four genotypes

were found, one of which was a new genotype. The genotypes of *T. gondii* are related to its virulence to human beings. New strains of *T. gondii* may have different phenotypic properties, some possibly highly virulent to human hosts. The virulence study of the new strain ToxoDB#268 in mice model could be done in the future.

Very few studies have been done to genetically identify *T. gondii* isolates from chickens by PCR-RFLP. In the study that clinical signs of toxoplasmosis were developed at three chickens on a farm in Illinois, *T. gondii* was isolated from all 11 chickens bioassayed in mice. The isolations were designed TgCkUsII 1-11 (Dubey et al., 2007). The same genetic loci were used to identify the isolates and all isolates belonged to ToxoDB#1. Genetic characterization of the isolates from our study suggests higher genetic diversity of *T. gondii* from chickens in the United States.

Chapter 6: Conclusions and Suggestions for Future Research

In this study, a total of 997 chicken heart samples were purchased and examined for *T. gondii* infection by MAT and bioassay. *T. gondii* antibodies were detected from 150 samples with MAT at titer 1:5 or higher. However, no parasite was isolated through bioassay. A total number of thirteen isolates from free-range chickens in Ohio and New England were genotyped. Three genotypes were found in isolates from Ohio: four ToxoDB#2, one ToxoDB#3 and three ToxoDB#268, which is a new genotype. The other five isolate from New England have the same genotype ToxoDB#1.

Two local Amish markets were sampled in this study. Some of those chickens were raised in a same way as the modern chicken farms. *T. gondii* prevalence of pregnant women in an Amish community in Lancaster, Pennsylvania was reported significantly higher than the national average. It was found that 59 out of 114 (52%) pregnant Amish women was seropositive of *T. gondii* infection (Boyer et al., 2005). In one of the Amish family where one congenital infant was born, six out of eight people were seropositive and the infection was associated with *T. gondii* oocysts (Hill et al., 2011). The high prevalence may result from the Amish traditional self-sufficient agriculture system. Meat is one of the important sources for *T. gondii* infection. The study of seroprevalence and the isolation rate of *T. gondii* from free-range chickens in Amish communities is needed in the future.

Meats from Amish markets are popular in mid-Atlantic region, where consumers (general public) can purchase variety of meat products that are produced by the Amish community, including some special products such as chicken heart and rabbit meat. The

study of evaluating the microbial safety and infection of *T. gondii* in other special meat products could be conducted in the future.

Furthermore, a transmission dynamic model of the farms that keep more than one type of animal could be done. 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.

The isolation rates from *T. gondii* seropositive chicken are not consistent in the previous studies. Whether serology or bioassay is more representative to the degree of environmental contamination and the risks of *T. gondii* transmission to humans, more studies need to be conducted.

Although chickens are considered important in epidemiology studies as they are transmission vehicles to cats and humans, the genetic studies of *T. gondii* isolates from chickens in the Unites States are very few. Therefore, more isolates from chickens need to be genetically characterized in order to do the epidemiology analysis. Moreover, virulence study of the new strain ToxoDB#268 is not done. New strains of *T. gondii* may have different phenotypic properties, some possibly highly virulent to human hosts. The emerging genotypes may have an evolution of virulence.

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