

Molecular Epidemiology, Genotyping, and MAT Testing of *Toxoplasma gondii*



Jack Kang

Dr. Chunlei Su – Associate Professor

MICR 401 - Honors Thesis

Department of Microbiology

The University of Tennessee - Knoxville

Abstract:

Two experiments were carried out in this study: 1). Genotyping DNA samples of *Toxoplasma gondii* collected from the National Institute of Health (NIH) and 2). Performing Multiple Agglutination Tests (MAT) of feral cat samples collected from Knoxville, TN. Chicken DNA samples were genotyped using a series of PCR techniques followed by endonuclease restriction and an electrophoresis gel. Different molecular markers were amplified using specific primers to bind to certain sites of target DNA. This process will help allow the identification of strain types of the DNA samples in the given geographical area. After reviewing gel results, the data highlighted inconsistencies due to low DNA sample concentrations. Additionally, MAT tests were performed in order to better understand the initial scope of infection for *T. gondii*. Approximately 37% percent of the sample serums had tested positive for *T. gondii*.

Introduction:

Toxoplasma gondii is an obligate and intracellular parasite that causes the disease toxoplasmosis. It is a coccidian parasite in which an estimated ~30 percent of the world's population has been exposed to and chronically infected (Pernas, 2014). In 1908, Charles Nicolle and Louis Manceaux found the parasite as they were studying a protozoan organism in the tissues of a rodent called the gundi (Weiss, 2011). Today, *T. gondii* is one of the most well studied parasites because of its significance in the medical and veterinary field. For example, there are a multitude of research articles highlighting the molecular epidemiology, pathology, physiology, and cell biology of the parasite.

The sexual reproduction of *T. gondii* takes place in the intestines of felines, which is the definitive host. Asexual reproduction takes place in the intermediate host, including mammals and birds. A life cycle of *T. gondii* begins when a cat shed oocysts through their feces. Intermediate hosts become infected after ingesting soil, water, or plant material contaminated with oocysts (CDC, 2015). Parasites develop into chronic phase, which is in tissue cyst, localized in neural and muscle tissue cells (CDC, 2015). Cats can be infected after consuming intermediate hosts with tissue cysts such as mice. Humans can become infected mainly by the consumption of uncooked meat with tissue cysts and consumption of water or food contaminated with cat feces (oocysts). Developing fetus can also get infected from pregnant woman who has primary infection.

There are three infectious stages of *T. gondii*. The first stage is the tachyzoites, where cells are rapidly multiplying. Tachyzoites multiply asexually within the host cell by repeated endodyogeny (a specialized form of reproduction in which two progeny form within the parent parasite (Dubey, 2010). They are responsible for establishing the acute phase of infection during dissemination via the bloodstream to the various tissues (Pernas, 2014). The rate of invasion and growth can vary depending on the specific strain of *T. gondii* and host cells. After the tachyzoites makes its way into a host cell, there is a variable period of lag phase before the parasite divides and this phase is partly parasite dependent. The second stage is the bradyzoites, which is the encysted stage of the parasite in tissues and establishes the chronic phase of infection. These tissue cysts differ structurally from tachyzoites in that they have a nucleus situated towards the posterior end rather than a more centrally located nucleus. Tissue cysts serve as an integral part of the life cycle and can be formed in 3 days after inoculation. The last stage is the oocysts. Cats shed oocysts after ingesting any of the three infectious stages of *T. gondii*.

Geographically, *T. gondii* infection in humans and animals is widespread around the world and are more prevalent in some parts than others. Environmental conditionals may determine the degree of natural spread of *T. gondii* infection. Infection is more prevalent in warm and humid climates and low-lying areas than in cold, dry, and mountainous climates (Dubey, 2010). Additionally, cultural habitats and hygiene of people may play a role. For example, France has been suggested to have a high seroprevalence of *T. gondii* (Pappas, 2009). This can be due to the cooking and eating habits of the French, where eating raw or undercooked meat indicates a high standard of living (Dubey, 2010). On the contrary, countries such in Asia and Africa cook their meats well due to the presence of many other prominent parasites that people are afraid they may consume.

In this study, specific PCR-RFLP markers were chosen for genotyping because they have been widely used in identifying *T. gondii* isolates from a variety of hosts in different geographical regions. All ten markers together can help distinguish the three strains with high resolution. MAT Tests, on the other hand, helps to gain a better insight on how infected a DNA sample is by observing the presence of pellet (antigen).

Methods:

Genotyping

A total of 18 NIH DNA samples (TX 54 and 60) in 1:5 dilutions were tested in the genotyping process. Additionally, 8 positive controls and 3 negative controls were included. The methodology used in this study was adopted from “Moving towards Integrated Genotyping and Diagnosis of Toxoplasmosis.”

To begin, a Multiplex Polymerase Chain Reaction (PCR) was performed to amplify and obtain more genomic DNA using a mix of primers (SAG1, 3'-SAG2, alternative SAG 2, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico). The following reagents were assembled for the multiplex PCR mix: distilled water (16.5 μ L), 10x Mg- PCR buffer (2.5 μ L), dNTPs (2.5mM; 2.0 μ L), $MgCl_2$ (25mM; 2.0 μ L), mixed external forward primer (25mM; 0.15 μ L), mixed external reverse primer (25mM; 0.15 μ L), and FastStart Taq (5U/ μ L); 0.20 μ L). The amount of each reagent is the standard value for one sample. Hence, the values were multiplied by 29 (total samples including +/- controls), bringing the total volume of the M-PCR mix to 681.5 μ L. Next, 23.5 μ L of the PCR mix and 1.5 μ L of the DNA samples were added into each microtube well on a PCR plate for all 29 samples based on Template 1. The samples were then heated in a thermocycler at 95°C for 4 minutes, then 30 cycles of 94°C for 30 seconds, 55°C for 1 min, 72°C for 2min, and lastly soaked at 15°C. The finished product from this M-PCR will be used in the following step of N-PCR.

After multiplex PCR, the DNA samples underwent a Nested PCR to further amplify DNA using specific primers in order to bind certain sites of the DNA. These primers will be the each individual primers for the markers used in the multiplex instead of all primers combined. The reagents used in this N-PCR mix were similar to the M-PCR, including distilled water (18.5 μ L), 10x Mg- PCR buffer (2.5 μ L), dNTPs (2.5mM; 2.0 μ L), nested forward primer (25mM; 0.15 μ L), nested reverse primer (25mM; 0.15 μ L), and Paq5000 polymerase (5U/ μ L); 0.20 μ L). For marker SAG1, the forward and reverse primers are SAG1-S2 and SAG1-Rext, respectively. For marker 5'-SAG2, the forward and reverse primers are 5-SAG2F and 5-SAG2R, respectively.

Only two markers were conducted due to error in DNA samples – see results. Again, the value of each reagent was multiplied by 29 (total samples including +/- controls), bringing the total volume of the N-PCR mix to 681.5 µL. The same amount of 23.5 µL PCR mix and 1.5 µL of extracted DNA sample were added into each microtube well on a PCR plate for all 29 samples. The samples were heated in a thermocycler at 95°C for 4 minutes, then 35 cycles of 94°C for 30 seconds, 60°C for 1 min, 72°C for 2min, and lastly soaked at 15°C. The finished product from this M-PCR will be used in the following step of restriction.

Following the two sets of PCR, the DNA samples underwent digestion via Restriction Fragment Length Polymorphism (RFLP). Restriction enzymes called endonucleases were used in order to cut specific parts of the DNA for analysis. The digestion mix included distilled water (14.6 µL), 10x NEB buffer (2.0 µL), and restriction enzyme(s) (0.1 µL). The restriction enzymes used for marker SAG1 were Sau96I and HaeII (double digest). The restriction enzyme used for 5'-SAG2 was MboI. 17 µL of digestion mix and 3 µL of N-PCR products were added to a microtube PCR plate. All samples were then incubated for 1 hour in a 37°C hot water bath.

Lastly, a 2.5% agarose gel was prepared for gel electrophoresis with the RFLP samples to confirm the gel results with the known positive control gel data. Gel electrophoresis separated the DNA fragments based on their size and works on the basis of electrical charge. The results are shown in Figures 1 and 2.

Template 1. Multiplex & Nested PCR Template (TX54 & TX60)

	1	2	3	4
A	Neg Control	TX54 CK627 OP	GT1	TX60 CK620
B	TX54 CK618OP	TX54 CK628 OP	PTG	TX60 CK621
C	TX54 CK619OP	TX54 CK629 OP	CTG	Neg Control
D	TX54 CK620OP	TX54 CK630 OP	TgCgCa1	TX60 CK622
E	TX54 CK621 OP	Neg Control	MAS	TX60 CK623
F	TX54 CK622 OP	TX54 CK631 OP	TgBr5	Empty
G	TX54 CK623 OP	TX60 CK618	TgbBr64	Empty
H	TX54 CK625 OP	TX60 CK619	TgToucan	Empty

Legend

Yellow = Positive Control

Green = Negative Control

Modified Agglutination Test (MAT Test)

For the first round of test to identify seropositive samples, a total of 97 DNA serums were diluted 1:25 with 1x PBS in 1.5mL tubes (72 µL of PBS and 3 µL of sample serum). 50 µL of diluted samples were transferred to the first and fifth rows of a U-bottom 96 microtiter plate (which included 20 sample serums, 1 negative control, and 1 positive control. The rest of the wells had 25 µL of PBS added using a multichannel pipette. Next, a serial dilution was made to 1:200 for each sample (Template 2), but 1:3200 for negative and positive controls (Template 3). The last 25 µL of diluted serum samples were discarded. An antigen mixture was prepared, including alkaline buffer (2500 µL), 2-mercaptoethanol (35 µL), Evans blue dye (2mg/mL in H₂O; 50 µL), *T. gondii* MAT antigen (150 µL). 25 µL of the antigen mixture was immediately transferred into each well on the plate. Lastly, the plate was covered with sealing tape and

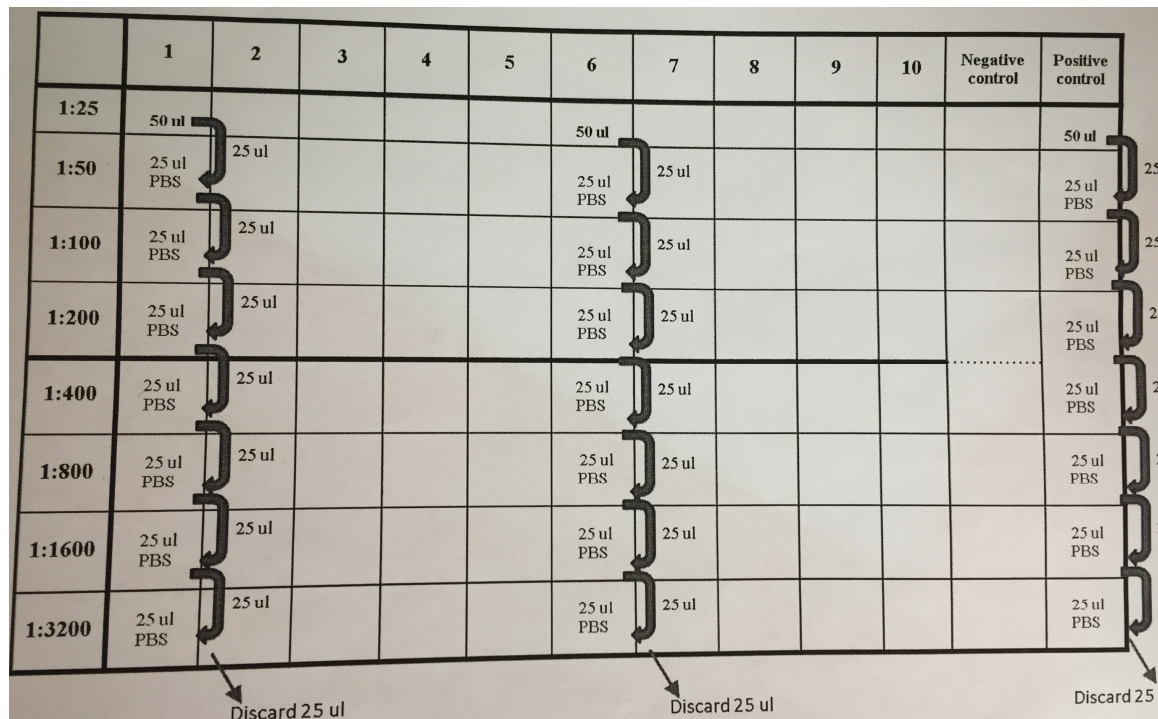
incubated at 37°C for 24 hours. After the results were collected, the plates were placed in the fridge at 4°C for another 24 hours. Results were checked again to verify the accuracy.

After testing was completed for all 97 samples, samples that tested positive were tested again in a 1:3200 serial dilution (Template 3). The same procedures were followed and results were collected at 24 hours after 37°C incubation and collected again after another 24 hours at 4°C.

Template 2. 1:200 serial dilution of serum samples in 96-well plate (20 total samples).

Row 1	1	2	3	4	5	6	7	8	9	10	Negative control	Positive control
Row 5	11	12	13	14	15	16	17	18	19	20		
1:25	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul
1:50	25 ul PBS	25 ul				25 ul PBS	25 ul					25 ul PBS
1:100	25 ul PBS	25 ul				25 ul PBS	25 ul					25 ul PBS
1:200	25 ul PBS	25 ul				25 ul PBS	25 ul					25 ul PBS
	Discard 25 ul					Discard 25 ul						25 ul PBS
1:25	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul		25 ul PBS
1:50	25 ul PBS	25 ul				25 ul PBS	25 ul					25 ul PBS
1:100	25 ul PBS	25 ul				25 ul PBS	25 ul					25 ul PBS
1:200	25 ul PBS	25 ul				25 ul PBS	25 ul					25 ul PBS
	Discard 25 ul					Discard 25 ul						25 ul PBS
	Discard 25 ul					Discard 25 ul						Discard 25 ul

Template 3. 1:3200 serial dilution of serum samples in 96-well plate (10 total samples).



Results:

Results collected from genotyping are shown in Figures 1a and 1b. The gel images of positive controls all looked normal and correct. However, all tested DNA samples indicated separated bands, suggesting some degree of error.

Results collected from MAT tests are organized in Tables 1 through 9, depicting which tested DNA samples were infected. Positive samples have no pellets present on the bottom of each well since antibodies (present in infected samples) are bound to the antigen. On the other hand, pellets were observed for negative samples as there are no antibodies in a negative sample, allowing the antigen to form a pellet on the bottom. MAT Testing of all 97 samples suggested that 37 samples tested positive. After a second MAT Test for all 37 positive samples, 36 were positive. Ultimately, results depict that 36 samples out of 97 were infected with *T. gondii*.

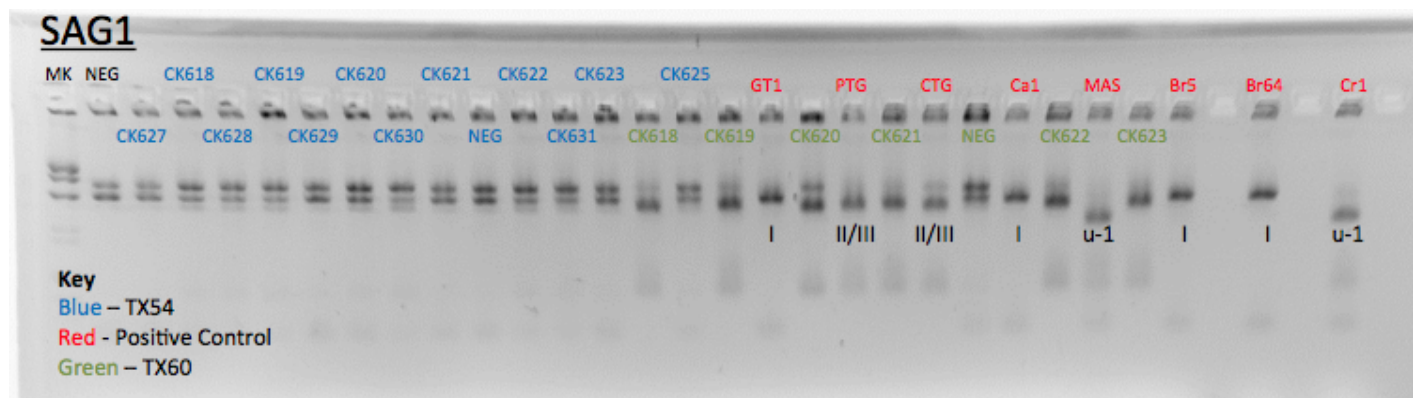


Figure 1a. Gel results for SAG1 marker, HaeIII + Sau96 double digest.

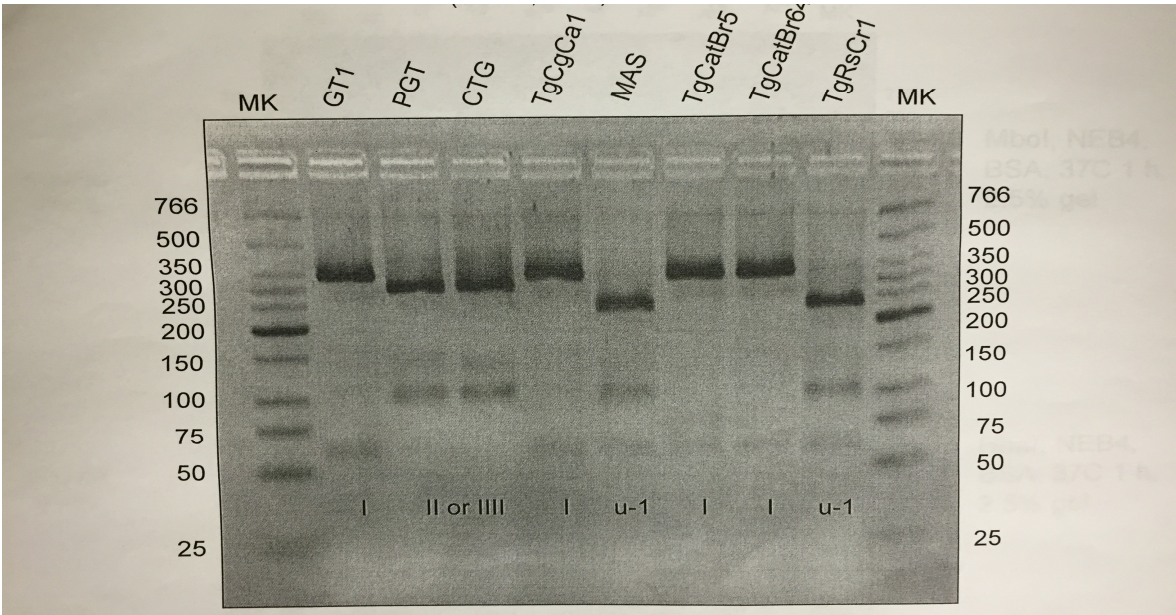


Figure 1b. Gel results for SAG1 marker (known positive controls).

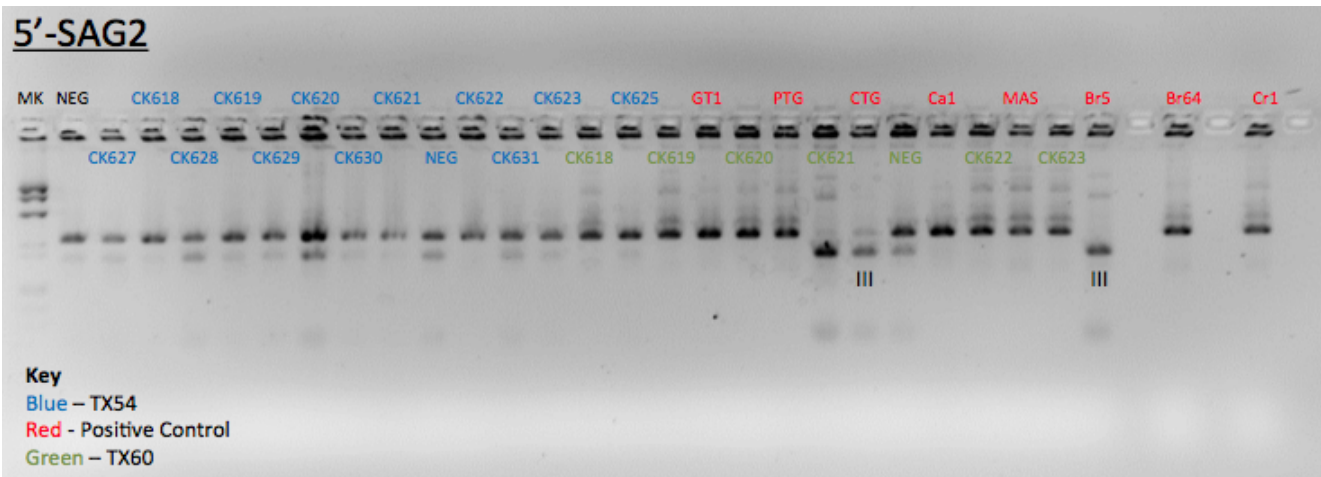


Figure 2a. Gel results for 5'-SAG2 marker, MboI digestion.

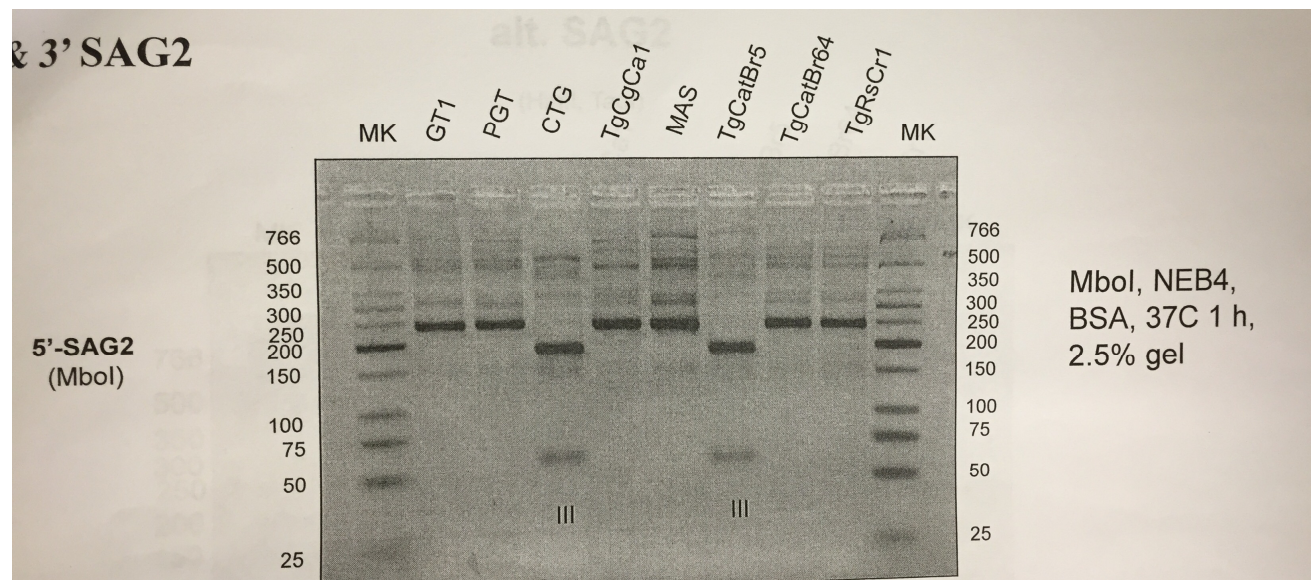


Figure 2b. Gel results for 5'-SAG2 marker (known positive controls).

Table 1. MAT Test results of first 20 samples.

BOX #6 (Set 1)												Jack Kang 3/9/15
Antigen 2/21/2014												
	1	2	3	4	5	6	7	8	9	10	11	12
	521-4	521-5	521-6	521-7	521-8	521-9	521-10	521-1	521-11	521-12	Negative Control FBS 2/21/14	Positive Control 1:500
A 1:25	-	-	-	+	-	-	+	-	-	+	-	+
B 1:50	-	-	-	+	-	-	+	-	-	+	-	+
C 1:100	-	-	-	+	-	-	+	-	-	+	-	+
D 1:200	-	-	-	+	-	-	+	-	-	+	-	+
	520-1	520-2	520-3	520-4	520-5	520-6	521-2	521-3	521-4	516-1		
E 1:25	+	-	-	-	-	+	+	-	-	-	-	+
F 1:50	+	-	-	-	-	+	+	-	-	-	-	+
G 1:100	+	-	-	-	-	+	+	-	-	-	-	+
H 1:200	+	-	-	-	-	+	+	-	-	-	-	+
Notes:												
* First observation made after 24 hours at 37°C (data above)												
* Second observation made after another 24 hours at 4°C (data in blue)												

Table 2. MAT Test results for second set of 20 samples.

BOX #6 (Set 2)												Jack Kang
Antigen 2/21/2014												3/23/15
	1	2	3	4	5	6	7	8	9	10	11	12
	516-2	516-3 #1	516-3 #2	517-1	518-2	518-1	519-1	519-2	519-3	513-7	Negative Control FBS 2/21/14	Positive Control 1:500
A	1:25	+	-	-	-	-	-	-	+	+	-	+
B	1:50	+	-	-	-	-	-	-	+	+	-	+
C	1:100	+	-	-	-	-	-	-	+	+	-	+
D	1:200	+	-	-	-	-	-	-	+	+	-	+
	514-1	514-2	514-3 #1	514-3 #2	514-4	514-6	514-7	515-1	515-2	507-4		
E	1:25	-	-	-	-	-	+	+	+	+	-	-
F	1:50	-	-	-	-	-	+	+	+	+	-	-
G	1:100	-	-	-	-	-	+	+	+	+	-	-
H	1:200	-	-	-	-	-	+	+	+	+	-	-
Notes:												
* First observation made after 24 hours at 37°C (data above)												
* Second observation made after another 24 hours at 4°C (data in blue) ; First column #4 (517-1) turned positive + after second observation												

Table 3. MAT Test results for third set of 20 samples.

BOX #6 (Set 3)												Jack Kang
Antigen 2/21/2014												3/25/15
	1	2	3	4	5	6	7	8	9	10	11	12
	512-1	512-2	512-3	511-4	513-1	513-2	513-3	513-4	513-5	513-6	Negative Control FBS 2/21/14	Positive Control 1:500
A	1:25	-	-	-	+	+	-	+	-	+	-	+
B	1:50	-	-	-	+	+	-	+	-	+	-	+
C	1:100	-	-	-	+	+	-	+	-	+	-	+
D	1:200	-	-	-	+	+	-	+	-	+	-	+
	503-1	507-1	507-3	507-5	508-1	508-3	508-4	506-3	506-2			
E	1:25	+	-	+	+	+	+	+	-	-	-	-
F	1:50	+	-	+	+	+	+	+	-	-	-	-
G	1:100	+	-	+	+	+	+	+	-	-	-	-
H	1:200	+	-	+	+	+	+	+	-	-	-	-
Notes:												
* First observation made after 24 hours at 37°C (data above)												
* Second observation made after another 24 hours at 4°C (data in blue) ; First column #9 (513-5) turned positive + after second observation												

Table 4. MAT Test results for fourth set of 20 samples.

BOX #6 (Set 4)												Jack Kang
Antigen 4/25/2014												3/25/15
	1	2	3	4	5	6	7	8	9	10	11	12
	504-4	504-3	504-2	504-1	500-6	503-7	503-6	503-5	503-4	503-3	Negative Control FBS 2/21/14	Positive Control 1:500
A	1:25	+	-	+	+	+	-	+	-	+	-	+
B	1:50	+	-	+	+	+	-	+	-	+	-	+
C	1:100	+	-	+	+	+	-	+	-	+	-	+
D	1:200	+	-	+	+	+	-	+	-	+	-	+
	505-1	505-2	505-3	505-4	505-5	505-6	505-7	503-8	503-10	503-11		
E	1:25	+	+	-	-	-	-	+	+	-	-	-
F	1:50	+	+	-	-	-	-	+	+	-	-	-
G	1:100	+	+	-	-	-	-	+	+	-	-	-
H	1:200	+	+	-	-	-	-	+	+	-	-	-
Notes:												
*First observation made after 24 hours at 37C												
*Second observation made after another 24 hours at 4C (data in blue); Column #4 (Sample 504-1) turned positive after second observation												

Table 5. MAT Test results for fifth/last set of 20 samples.

Table 8. MAT Test results for third set of positive samples.

BOX #6 (Positives - Set 3)												Jack Kang	
Antigen 4/25/2014												4/15/15	
		1	2	3	4	5	6	7	8	9	10	11	12
		508-1	508-3	508-4	504-4	504-2	504-1	500-6	503-7	503-5	503-3	Negative Control FBS 2/21/14	Positive Control 1:500
A	1:25	+	+	+	+	+	+	+	+	+	+	-	+
B	1:50	+	+	+	+	+	+	+	+	+	+	-	+
C	1:100	+	+	+	+	+	+	+	+	+	+	-	+
D	1:200	+	+	+	+	+	+	+	+	+	+	-	+
E	1:400	+	+	+	+	+	-	+	+	-	-	-	-
F	1:800	+	+	+	+	+	-	+	+	-	-	-	-
G	1:1600	+	+	+	+	+	-	+	+	-	-	-	-
H	1:3200	+	+	+	+	+	-	+	+	-	-	-	-
Notes:													
*First observation made after 24 hours at 37C													
*Second observation made after another 24 hours at 4C													

Table 9. MAT Test results for fourth set of positive samples.

BOX #6 (Positives - Set 4)										Jack Kang
Antigen 4/25/2014										4/16/15
		1	2	3	4	5	6	7	8	9
		505-1	505-2	503-8	503-10	502-2 #3	503-9	510-1	Negative Control FBS 2/21/14	Positive Control 1:500
A	1:25	+	+	+	+	+	+	+	-	+
B	1:50	+	+	+	+	+	+	+	-	+
C	1:100	+	+	+	+	+	+	+	-	+
D	1:200	+	+	+	+	+	+	+	-	+
E	1:400	+	+	+	+	+	-	+	-	-
F	1:800	+	+	+	+	+	-	+	-	-
G	1:1600	+	+	+	+	+	-	+	-	-
H	1:3200	+	+	+	+	+	-	+	-	-
Notes:										
*First observation made after 24 hours at 37C										
*Second observation made after another 24 hours at 4C										

Table 10. Box #6 MAT Test Summary (Sample Collected: 7/17/2010)

Sample ID	Titer
521-4	Negative
521-5	Negative
521-6	Negative
521-7	• 3200
521-8	Negative
521-9	Negative
521-10	• 3200

521-4	Negative
521-11	Negative
521-12	• 3200
520-1	• 3200
520-2	Negative
520-3	Negative
520-4	Negative
520-5	Negative
520-6	Negative
521-2	• 3200
521-3	Negative
521-4	Negative
516-1	Negative
516-2	• 3200
516-3 #1	Negative
516-3 #2	Negative
517-1	Negative
518-2	Negative
518-1	Negative
519-1	Negative
519-2	• 3200
519-3	• 3200
513-7	Negative
514-1	Negative
514-2	Negative
514-3 #1	Negative
514-3 #2	Negative
514-4	Negative
514-6	• 3200
514-7	• 3200
515-1	• 3200
515-2	• 3200
507-4	Negative
512-1	Negative
512-2	Negative
512-3	Negative
511-4	• 3200
513-1	• 3200
513-2	Negative
513-3	200
513-4	Negative
513-5	• 3200
513-6	• 3200
504-4	Negative
504-3	• 3200
504-2	200

504-1	• 3200
500-6	• 3200
503-7	Negative
503-6	200
503-5	Negative
503-4	200
503-3	200
503-1	Negative
507-1	Negative
507-2	• 3200
507-3	• 3200
507-5	• 3200
508-1	• 3200
508-3	• 3200
508-4	Negative
506-3	Negative
506-2	• 3200
505-1	• 3200
505-2	Negative
505-3	Negative
505-4	Negative
505-5	Negative
505-6	• 3200
505-7	• 3200
503-8	Negative
503-10	Negative
503-11	Negative
500-5	Negative
500-4	Negative
500-3	Negative
500-2	Negative
500-1	Negative
501-2	• 3200
501-1	Negative
502-2#3	200
502-1	Negative
503-9	Negative
509-2	Negative
504-5	• 3200
509-1	Negative
510-1	Negative
511-3	Negative
511-1	
506-1	

Discussion:

The unsuccessful genotyping results from the gel were most likely due to low DNA sample concentrations. The reason is because all the bands of positive controls on the gel were correct after comparing it to known positive controls. This means that this would not be due to technique error. After further analysis, it was concluded that the separated bands indicate minimal contamination due to low DNA concentrations. Minimal contamination would have been negligible of a sample of higher concentration. Therefore, future studies should utilize original DNA concentration instead of 1:5 dilutions.

The second MAT test helps to confirm all positive samples from initial MAT Test. Only 1 out of all 37 positive samples turned out negative, so approximately 97% (36/37) initial positive samples tested positive again during the second confirmation test. The one sample that turned negative can be an error in reading the initial MAT test results, as there can be fog forming underneath the plate when taken out of the 4°C fridge. Overall, this represents that 37% (36/97) of feral cat samples in Knoxville were infected with *T. gondii*. This data is reasonable because it is estimated that *T. gondii* infects about one-third of the world's human population (Perna, 2014).

For future studies, DNA samples from different years can be tested since infection rates differ as time changes. Furthermore, testing samples from varying environmental conditions/features can also be an important study. For example, infection rates of organisms may change when comparing a rural area to an urban landscape. To better understand and verify if an organism has an infection, the host's organ tissue can be harvested and inoculated in mice (in the hopes the some tachyzoites are inoculated). This would be a good verification since virulence was first tested in mice; and thus, symptoms are more understood in mice. Lastly, the sample size in this study was low (97 samples). Hence, future studies can also incorporate testing of more samples (approximately 2,000 samples) through a longer span of time to help improve consistency and accuracy of results.

References:

- "Biology of Toxoplasma Gondii." Centers for Disease Control and Prevention (CDC). Global Health – Division of Parasitic Diseases and Malaria, 17 Mar. 2015. Web. 22 Apr. 2015. <<http://www.cdc.gov/parasites/toxoplasmosis/biology.html>>.
- Dubey, J. P. *Toxoplasmosis of Animals and Humans*. 2nd Ed. Boca Raton, FL: CRC, 2010. Print.
- Pappas, G; Roussos, N; Falagas, ME (October 2009). "Toxoplasmosis snapshots: global status of Toxoplasma gondii seroprevalence and implications for pregnancy and congenital toxoplasmosis." *International Journal for Parasitology* 39 (12): 1385–94.
- Pernas, L.; Ramirez, R.; Holmes T.; Montoya, J.; Boothroyd; J. (March 2014). "Immune Profiling of Pregnant Toxoplasma- Infected US and Colombia Patients Reveals Surprising Impacts of Infection on Peripheral Blood Cytokines." *The Journal of Infectious Diseases* 2014; 210:923–31•.
- Weiss, LM; Kim, K (2011). *Toxoplasma Gondii: The Model Apicomplexan. Perspectives and*

Methods. London: Academic Press/Elsevier. pg 49–80.