PROTEOMIC HOST RESPONSE TO *TOXOPLASMA GONDII* STRAINS OF
DISTINCT VIRUENCE PHENOTYPES

by

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ABSTRACT

*Toxoplasma gondii* is an obligate intracellular protozoan parasite that can infect all warm-blood animals. In humans, it is estimated that one-third of world’s population is infected with *T. gondii*. Most *T. gondii* strains can be classified into three genotypes; type I, II and III. Type I strains (e.g. RH) are virulent strains with features of rapid growth. Type II (e.g. Me49) and type III strains (e.g. CTG) are avirulent, with slower growth and more readily form cysts in the brain in the chronic infection. In infected host cells, the parasite modulates the host immune system for their successful replication and dissemination. To investigate the host response to three different virulence phenotypes, we proposed to use a two dimensional-differential gel electrophoresis (2D-DIGE) based proteomic approach. Though preliminary studies, it was concluded that Me49 was not appropriate for this study due to their significantly slower growth rate within 24 hours p.i. Subsequent studies were then done comparing the type I RH strain vs. the type III CTG strain. Using the RH strain, kinetic analysis of the host cell response to infection was done, analyzing samples at 2, 12 and 24 hours p.i. Few protein changes were observed at 2 and 12 h p.i., and thus only 24 hours p.i. was analyzed via 2D-DIGE analysis. For 2D-DIGE analysis, quadruplicates of protein extracts were separated in 2D-DIGE based on pH and molecular weight. For RH strain, we found a total of 439 protein spots were found to be significantly affected by infection, with 247 spots with increased expression and 187 spots with decreased expression and fold-changes ranging from 0.16 to 13.1. For CTG strains total of 175 protein spots were found with significant expression changes. Among them, 48 spots were increased and 127 were decreased with expression changes ranging between 0.66 and 2.41. Interestingly, many decreased spots were seen in trains of spots indicating these may be due to a post-translational modification such as phosphorylation. For the further studies, the identification of protein spots is necessary to elucidate the interactions between host cell and *T. gondii* strains of distinct virulence phenotypes.
CHAPTER ONE

INTRODUCTION AND BACKGROUND ON *TOXOPLASMA GONDII*

*Toxoplasma gondii* is an obligate intracellular protozoan parasite that can infect all warm-blood animals including mammals and birds. The parasite is classified in the Phylum Apicomplexa, which is characterized by the presence of secretory organelles found at the anterior end of the organism called the apical complex. In addition to the apical complex, comprised of conoid and apical polar ring, parasites also contain secretory organelles called micronemes and rhoptries, and contain an organelle called the apicoplast which is a non-photosynthetic plastid with only a single plastid present per parasite cell (Figure 1). These unique secretory organelles play important roles during invasion and also in modification of host immune system\(^1,2\). The tachyzoite form of the parasite was isolated first from tissue in rodents in North Africa in 1908 by Charles Nicolle, and was originally named *Piroplasma quadrigeminum* because of the similarities in replication pattern with a parasite called *Piroplasma*\(^3\). In 1909, the parasite was classified in a new genus and named *Toxoplasma gondii* based on intensive microscopic observations of *in vivo* and *vitro* samples\(^4\). The genus name describes shape of the parasite. In Greek, “toxo” refers to the bow-like shape, of the parasite\(^5,6\). Since the first isolation of *T. gondii*, the parasite had been isolated from a wide range of animals, yet the complete life cycle was not elucidated until 1970 because of the drastic morphological changes at different life stages in the definitive and intermediate hosts\(^4\).
*T. gondii* has two lifecycles; a sexual life cycle in small intestine of the feline species (definitive host), and an asexual life cycle in other warm-blood animals (intermediate hosts) (Figure 2). There are three infectious forms of *T. gondii*; tachyzoites, the rapidly replicating form found in vacuoles; sporozoites, found in the oocyst; and bradyzoites, the slowly replicating form found in tissue cysts. The parasite’s infectious forms all have the bow- or crescent- like shape with an approximate size of 2 x 7 µm7. *T. gondii* infects host cells by a process called active invasion which involves secretion of parasite proteins from the apical complex and formation of the parasite vacuole membrane which serves as a membrane layer between the parasite and the host cell cytoplasm1,2. Infection of both definitive and intermediate hosts occurs when: 1) an oocyst is inhaled or ingested with contaminated water or food such as vegetables, or 2) a tissue cyst is ingested with undercooked meat from infected animals or 3) tachyzoites in the bloodstream migrate transplacentally and infect the fetus (Figure 3).

In the definitive host, the most common infection pathway is tissue cyst ingestion. Once the cyst is in the feline species, the cyst ruptures in the small intestine and the bradyzoite stage penetrates intestinal cells to replicate asexually, and eventually become merozoites. The parasites lyse their host cell and re-infect other host cells, and then differentiate into microgametes (male) and macrogametes (female) in intestinal host cells8. The male and female forms undergo sexual reproduction and produce a gametocyte, which develops into an oocyst that is shed in feces. It takes 3 – 7 days to shed oocysts in feces from tissue cyst ingestion, and it is reported that infected cats can shed more than 100 million oocysts in their feces9. In a few days after exposure to the
external environment, an oocyst becomes mature and becomes an infectious oocyst through a division process called sporogony, which produces sporozoites within the oocyst (Figure 2).

In the intermediate host, inhaling or ingestion of mature oocysts and ingestion of tissue cysts from raw or undercooked meat are the common infection pathways (Figure 3). When a tissue cyst or oocyst is ingested by an intermediate host, the parasites (bradyzoites or sporozoites) differentiate into tachyzoites in intestinal cells and replicate rapidly. This stage is called the acute infection. Then, tachyzoites circulate in the bloodstream and localize in organs such as brain, heart, lung, eye, and muscles. During this time, upon first exposure to the parasite, if the host is pregnant, the parasites can be transmitted transplacentally to the fetus. In localized organs, tachyzoites differentiate to bradyzoites, the slow replicating form and produce tissue cysts. This stage is called the chronic infection. It takes only 7 – 10 days after ingestion of an oocyst or tissue cyst for cysts to start forming in the tissues. Tissue cysts can be found in a variety of organs including liver, spleen, and pancreas, but the majority of cysts are found in skeletal muscle and the brain. The chronic infection lasts for the lifetime of the host with the parasite persisting primarily in cysts in the muscle and the brain. The chronic infection is typically asymptomatic, due to an effective immune response.

In 2008, T. gondii strains GT1, Me49, and VEG contig sequencing, annotation, and ontology association were completed. Those three strains represent three different genotypes with the genomic data available on ToxoDB (http://toxodb.org/toxo/). The genome project found that the T. gondii genome is approximately 63–65 Mbp and
consists of 14 chromosomes. The genome encodes about 7800 genes, and 18% of genes are expected to be stage specific. The genome is extremely large compared to another Apicomplexa genus, *Plasmodium falciparum*, the malaria parasite, which has 28 Mbp genome. This is because *T. gondii* genes are intron-rich and GC-rich compared to other Apicomplexa species. Also, housekeeping genes are more intron-rich than surface antigen genes. On average, genes are composed of 3.6 open reading frames (ORFs), and the average length of ORFs is about 100 residues.

Most *T. gondii* strains can be classified into three genotypes; type I, II and III. It is estimated that genome diversity between genotypes are 1~3%, and within genotypes, only differ by ~ 0.01%. Type I strains (e.g. RH, GT1) have features of rapid growth and high virulence. It is reported that only 10 organisms lead to death in a mouse in less than 10 days (LD<sub>100</sub> < 10). Type II (e.g. Me49, PLK) and type III strains (e.g. CTG, VEG) are less virulent readily forming cysts in brain and muscle tissue, with 100-1000 and >10,000 parasites leading to death in a mouse by 50% of chance (LD<sub>50</sub>), respectively. Regionally, Type II is highly predominant in Europe and type III is found more in South Europe. In North America and Africa, type II and III are seen commonly, while strains in South and Central America exhibit greater genotypic diversity. Finally in Asia, type III is most common. In human infection, type I is mainly related to acute outbreak and ocular toxoplasmosis. Type II is commonly found in toxoplasmosis and tends to cause chronic infection, while type III is also found in chronic infection.

*T. gondii* is a ubiquitous parasite with an estimated 1/3 of the world’s population infected. Although in healthy people the parasite infection does not manifest severe
symptoms, in immunosuppressed individuals the parasite can reactivate from the cyst stage in the brain and cause severe neurological disease. It is considered an important opportunistic infection in AIDS patients and other immunosuppressed individuals such as transplantation patients or those undergoing chemotherapy. In some regions, more than 60% of the population is chronically infected with the parasite as determined by presence of *T. gondii* antibodies in their serum (Figure 4)\textsuperscript{20}. Much of the early research was focused on the zoonotic aspect of infection, since the parasite has been isolated from wide variety of animals. Even now, *T. gondii* infection in animals is of concern because a common cause of infection in humans is consumption of undercooked meat containing tissue cysts. Also, in the ocean around California, 16% of the cause of death in sea otters was due to encephalitis caused by *T. gondii* infection\textsuperscript{21}. Thus, the parasite has influence not only as an opportunistic pathogen in humans but also as a zoonotic infection.

Research on *T. gondii* became more significant when the AIDS epidemic occurred in the US in the beginning to mid-80’s. This is because encephalitis caused by the parasite infection was the second-most common cause of death in HIV patients. In these patients the parasite infection reactivated from the cyst stage in the brain, when the CD4 T cell counts fell below 400 cells/ml, leading to encephalitis and often resulting in death. However, when antiviral drugs such as protease inhibitors became commercially available in the mid-80’s, reactivated *T. gondii* infection become less of a problem as anti-retroviral therapy prevented the drop in the CD4 + T cell level in HIV+ patients\textsuperscript{22}. CD4+ T cells are also known as T helper (Th) cells and produce a variety of cytokines including interferon-γ which is the main mediator controlling resistance to the parasite. In
the last several decades, increasing evidence indicates a relationship between neuropsychiatric disorders and *T. gondii* infection. The association between Schizophrenia patients and the chronic infection is strongly supported by epidemiological data which has found higher rates of chronic parasite infection in individuals with schizophrenia, as compared to the non-Schizophrenic population\textsuperscript{23,24}. It has also been shown that other psychological problems such as bipolar, depression, anxiety, some behavioral and character changes, and Parkinson disease have correlation with chronic *T. gondii* infection\textsuperscript{25,26}.

To diagnose *T. gondii* infection, there are many methods are available, such as PCR, indirect fluorescence antibody tests (IFATs), immunosorbent agglutination assay, and immunofluorescent assay (IFA). Among those, enzyme-linked immunosorbent assay (ELISA) is the most commonly used method of diagnosis. Detection of both *T. gondii* specific IgM and IgG titers are used because of the complicated antibody response in infected individual. Upon the first infection, IgA and IgM are produced within a week and reach a plateau around a month after infection (Figure 5)\textsuperscript{7}. About one-quarter of infected people become IgM negative after 7 months of infection, yet they keep producing detectable level of IgM for a year or so\textsuperscript{27}. Thus, an IgM positive result on ELISA is usually indicative of an acute infection or of a recent infection that is becoming a chronic infection. On the other hand, an IgG positive titer as determined by ELISA, generally indicates a chronic infection because IgG is produced as long as the parasites are in a body without treatment\textsuperscript{7}. 
At the cellular level, *T. gondii* and host cells are battling for their own survival. The parasite secretes virulence factors such as ROP5, ROP16, ROP18, and protein phosphatase 2hnc (PP2hnc), and establishes the parasitophorous vacuole (PV) in the invading host cell\(^2\). Once the parasite is in the host, it secretes kinases and other effectors including ROP38 to modulate the host cell environment for their survival and replication via regulating transcription and signaling pathways, such as MAPK and STAT in the host cell (Figure 6)\(^2\). The host cell fights for its own survival and also for elimination of the parasite, producing cytokines which stimulate the host immune response and antimicrobial effector molecules such as nitric oxide and in mice, the IRG proteins which inhibit parasite growth.

To further our understanding of the interaction between parasite and host at the cellular and molecular level, proteomics offers a very powerful approach because it allows determination of expression of all proteins in the cell at a given time point. While there have been a wide variety of proteomic studies on the parasite, *T. gondii*, host proteomic studies have been neglected. Only three *T. gondii* infected host cell proteomic studies have been published. One is an *in vitro* study with human foreskin fibroblasts (HFFs) using pH4-7, 2D-DIGE, published in 2008\(^3\), the second is *in vivo* study with murine macrophage using pH4-7, conventional 2D gels, published in 2011\(^3\), and the third is an *in vivo* study of murine brain tissue infected with a cyst forming type II strain\(^3\). This host proteomic study was conducted based on 2D-DIGE with pH3-11 IEF, with improved CyDyes, and comparing the host cell proteomic response to strains of distinct virulence phenotypes, using RH (type I), Me49 (type II), and CTG (type III)
strains, in HFF cells. This study should help elucidate the host cell proteomic responses to *T. gondii* infection and lead to identification of candidate host cell proteins and pathways affected by virulent vs. avirulent strains. Identification of these candidate host proteins associated with virulent vs. avirulent infection will be of use for further studies directed toward understanding the host response to *Toxoplasma* infection and may indicate new pathways to target for therapeutic drug discovery to *Toxoplasma* infection.
Figure 1. *T. gondii* tachyzoite cell showing internal structures. Note the anterior end comprised of conoid and apical polar ring and the secretory organelles, the rhoptries and micronemes. From Baum, J. et al., *Nature reviews. Microbiology*, (2006)⁴⁰.

Figure 2. Life cycle of *T. gondii*. From Robert-Gangneux & Dardé, *Clinical microbiology reviews*, (2012)⁷.
Figure 3. Infection route of *T. gondii* in different hosts
From Robert-Gangneux & Dardé, *Clinical microbiology*

Figure 4. World distribution of *T. gondii* seroprevalence
Figure 5. Kinetics of four isotypes of antibody response to T. gondii infection on time course

Figure 6. Host cell-*T. gondii RH strain* interactions.
Orange arrows indicate sup-regulation, and orange dots indicate down-regulation.
CHAPTER TWO

PRELIMINARY DATA AND BACKGROUND STUDIES

Multiplicity of Infection (MOI) Experiments

Introduction

Multiplicity of infection is the ratio between host cells and infectious agent, in this case, *T. gondii*. It is commonly used in virology and for other intracellular pathogens when determining the optimal infectious dose to obtain a high degree of infection. These experiments were designed to establish the best infection method and MOI for three genotypically different strains of *Toxoplasma* for proteomic analysis. The criterions for infection were to: 1) maximize the infection rate (> 90 %), 2) obtain single or double infections per host cell and 3) avoid hyper infection (≥ 5 vacuoles/host cell). To determine the best MOI to use, multiple MOI’s were applied to three different strains which represent each genotype group; RH (type I), Me49 (type II), CTG (type III). These three strains have different virulence phenotypes, infection rates, and doubling times. The best MOI was determined by microscopic observation at 24 hours post-infection (p.i) and counting of percent infected cells, number of parasites/vacuole and number of vacuoles/host cell.
Materials and Methods

Cell Culture, Parasite Preparation and Infection. Human foreskin fibroblasts (HFFs) were used as host cells for *T. gondii*. HFFs were cultured in Dulbecco Modified Essential Medium (DMEM; CellGro\textsuperscript{®}) supplemented with heat-inactivated 10 % fetal calf serum (Atlanta Biologicals), heat-inactivated at 56 °C for 30 minutes, 2 mM L-glutamine with GlutaMAX (GibcoGMGibcoMAX0) with antimycotics (CellGro\textsuperscript{®}) containing penicillin, streptomycin, and amphorericin B, and 0.15 % (w/v) sodium bicarbonate (CellGro\textsuperscript{®}) and cultured in a 5 % CO\textsubscript{2} incubator at 37 °C. Tachyzoites stages of *T. gondii* of three different strains; RH (RH-YFP, ATCC 50940), Me49 (ATCC 50611), and CTG (ATCC 50842), were independently maintained by serial passage in monolayer of HFFs in growth medium.

To determine MOI, HFFs were grown on a 2-well slide for approximately a week to obtain a confluent monolayer, and then infected with *T. gondii* that was harvested from previously infected HFFs. To harvest the parasite, the infected HFFs were washed with fresh culture medium to remove free parasites in medium, scraped from the bottom of a flask, transferred into a syringe and passed through a 27-gauge needle to break individual HFF cells and parasite vacuoles. To harvest *T. gondii*, cells were passed through 3.0 \( \mu \text{m} \) polycarbonate filter, which traps host cell debris but allows the parasites to pass through. *T. gondii* were collected by centrifugation at 1200 rpm (~180 xg) for 15 minutes. The pellet was resuspended in 1 ml growth medium, and the number of parasites determined using a hemocytometer.
For the infection, HFFs on 2-well slides were freshly fed, and then infected with MOI of 1:2, 1:5, and/or 1:10, depending on the strain. For RH strain MOI’s of 1:2 and 1:5 were used, and for type II and III strains MOI of 1:2, 1:5 and 1:10 were used. For each MOI, the number of parasites needed was calculated from the estimated density of host cells on a slide (Table 1). Several different ways of infection were applied to determine the best method of infection: 1) continuous, 2) pulse-chase, 3) spin seeding + continuous, and 4) spin seeding + pulse-chase. These different methods of infection were tested using the RH strain. The “continuous” infection method set time zero (t = 0) upon introduction of *T. gondii* to the culture. With the pulse-chase method the parasites were washed 2 h after addition of the parasites to the cells to remove *T. gondii* that had not infected the host cells. For the “spin-seeding” method, slides were centrifuged at 1000 rpm (~180 xg) for 1 minute. In spin seeding, centrifugal force is used to bring parasites into contact with the host cells instead of gravitational force to settle parasite on host cells, as occurs for the continuous and pulse-chase methods. For the “spin-seeding + pulse-chase” method, parasites were washed 2 h after addition of the parasites to the cells to remove *T. gondii* that had not infected the host cells. For all four methods, *T. gondii* infection and growth were checked 24 hours post infection (p.i.)
Immunofluorescent Assay (IFA). Cells on the 2-well slides were washed with phosphate buffered saline (PBS) at 24 hours p.i., and fixed on the slide with 4% paraformaldehyde (Electron Microscopy Science) for 15 minutes. Cells were washed with three PBS 5 minute washes at room temperature. To permeabilize cells and to block non-specific binding, cells were exposed to 0.1% Triton X-100 diluted in PBS with 1% bovine serum albumin (BSA, Fisher Scientific) for 30 minutes. Cells were then incubated with a primary antibody which was mouse-anti-p30 monoclonal antibody (Meridian Life Science, Inc.). Antibodies were diluted to 1: 100 in PBS with 1% BSA and applied on cells. Cells were incubated at room temperature for an hour, and then washed three times with PBS for 5 minutes each time. Cells were then incubated in the secondary antibody, Alexa Fluor®488 F(ab')₂ fragment of rabbit-anti mouse IgG (Invitrogen), diluted to 1: 100 in PBS with 1% BSA solution. Then, cells were washed with PBS for three times, with 5 minutes each wash, with keeping slides in the dark during washes. After washing, the slide was dried in the dark and coverslips were carefully mounted on small volume of ProLong Gold reagent with DAPI (Invitrogen) while avoiding air bubbles. The slide was dried in the dark overnight, and the coverslip sealed to the slide with fingernail polish.

This immunolabeling was done only for Me49 and CTG because the RH strain was green fluorescently tagged by gene modification. For the RH strain experiment, the cells on slides were fixed with 4% paraformaldehyde and then coverslips were mounted with ProLong Gold Reagent with DAPI.

Slides were observed under a fluorescent microscope and pictures were taken at five fields in each well. The number of host cells and parasites/vacuole were counted to
determine percent infection, parasite growth in 24 hours respectively and the number of vacuoles/host cell counted to assess degree of infection with >5 vacuoles/host cell determined as a “hyper-infected” cell.

Results of MOI Experiments

The multiplicity of infection (MOI) experiments were designed to establish the best infection method and MOI for three genotypically different strains for sample collection. The optimal infection would maximize the infection rate (> 90 %) and minimize the multiple infections per host cell to avoid hyper infection (≥ 5 vacuoles/ host cell) at 24 hours after infection. Multiple MOI’s were applied to the three different strains that representing each genotype group, RH (type I), Me49 (type II), CTG (type III), which have different virulence phentoypes, infection rates, and doubling times. The best MOI was determined by microscopic observation at 24 hours post-infection (p.i). The RH strain was used to assess the best method of infection with the following four infection methods tested: 1) continuous, 2) pulse-chase, 3) spin seeding + continuous, and 4) spin seeding + pulse-chase, and based upon these results the best infection method was chosen for use with the other strains (Figure 7).

The slides were observed under a microscope with 400X magnification with three pictures were taken at the same field; phase contrast under transmitted light, DAPI used to host nuclei, and visualized *T. gondii* with *Alexa Fluor®* 488 or GFP labeled in case of RH strain. The fluorescence images were overlaid on the phase contrast to assess the effect of infection on integrity of the host cell monolayer. Also, an overlay picture of DAPI and *Toxoplasma* stains were done to aid in the counting of the number of host cell,
numbers of vacuoles, and numbers of parasites/vacuole. Since the parasites form vacuoles as they invade the host and replicate within the vacuole, the number of vacuoles indicates the initial percent infection. The number of parasites within the vacuole indicates the rate of growth with RH strain replicating every 6-7 hours, and with the avirulent strains expected to replicate every 7-8 hours. Thus for the RH strain, vacuoles are expected to have about 8 -16 parasites per vacuole at 24 hours p.i., while type II and III strains are expected to have 4-8 parasites/vacuoles at 24 hours p.i..

**RH Strain.** Microscopic observation of MOI of 1:5 slides exhibit hyper infection (> 5 vacuoles / host) in both the continuous and pulse-chase infection methods (Figure 8). Overlays without phase contrast clearly show the number of vacuoles, and indicate that some of HFFs got infected with more than 10 parasites initially. Some host cells have vacuoles with only one or two *T. gondii*, and this indicates that these parasites may have lysed other hosts and re-infected the host cell. For the 1:2 MOI experiment, four infection methods: continuous, pulse-chase, spin seeding + continuous, and spin seeding + pulse-chase: were applied and compared as to their infection effectiveness. All methods effectively infected host cells, yet spin seeding + pulse-chase displayed hyper infection (Figure 9, Table 2). Pulse-chase had a 100% infection rate without hyper infection, and the parasites replicated 3-4 times in 24 hours. Continuous infection method showed virtually identical results (Figure10). Based upon the results of these MOI experiments, it was determined that pulse-chase was the best method of infection. The pulse-chase method of infection was thus used thereafter for infection with Me49 and CTG and all other experiments with RH.
Me49 Strain. HFF’s were infected with Me49, type II strain by the pulse-chase method with MOI’s of 1:2 and 1:5. Me49 strain had a drastically lower infection rate and growth rate as compared to the RH strain (figure 11). Yet, 1:5 MOI successfully reached nearly 90% infection, with 2 vacuoles with 2~4 parasites seen in infected host cells at 24 hours p.i. (Table 3). Even though the number of the parasites per host were smaller than what was expected, the % infection and the number of vacuoles formed by MOI of 1:5 were ideal for proteomic sample collection.

CTG Strain. The type III strain has lowest virulence, so MOI of 1:2, 1:5, and 1:10 were initially tried with the pulse-chase infection method. However, MOI of 1:10 showed hyper infections, and surprisingly even an MOI of 1:2 an infection rate greater than that for Me49 with MOI of 1:5 was obtained (Figure 12). An MOI of 1:2 successfully infected 90% of cells with more than 2 parasites infecting host cells on average. The number of parasites per vacuole was typically between 4~8, meaning 2~3 replication cycles in 24 hours. Interestingly, there was no statistical difference between MOIs of 1:2 and 1:5 in all aspects of infection measured; i.e. the % infection, the number of vacuoles per host cell, or the number of parasites per vacuole (Table 4). Based on the results, for sample collection for proteomic study, MOI of 1:2 was used.

A comparison of the three strains of T. gondii with the optimal MOI’s of 1:2 for RH, 1:5 for Me49 and 1:2 for CTG is shown in Figure 14. As is evident from the Figure, these MOI’s resulted in high rates of infection for each strain with none of these infection rates resulting in hyper-infections. Also, as is evident from Figure 13, at these infection rates, the monolayer was intact and cell integrity maintained throughout the 24 hour
infection period, thus confirming that these infection rates were adequate for collection of samples for proteomic analysis. A comparison of the % infection achieved for each strain at the different MOI determinations are shown in Figure 13 A, and a comparison of the different growth rates between the three parasite strains at different MOI’s is shown in Figure 13 B.

Discussion

MOI determination clearly showed differences in virulence among three genotypes, RH, Me49, and CTG. RH is highly virulent and replicates faster than other strains. Vacuoles of RH had an average of 10 parasites at 24 hours p.i., indicating it replicates 3-4 times in 24 hours. This means RH starts replication immediately after infection and replicate every 6-7 hours. Even though it was 1:2 MOI infection, more than two vacuoles were found in a host, in some cases as many as 5 vacuoles. There are two possibilities why this happened. One is that the number of host cells was incorrect. This could have occurred because the number of host cells was not counted. It is also possible that the parasite count was inaccurate because more parasites were loaded onto cell monolayers thus resulting in more than 1:2 MOI. Another possibility is that some of the infected cells were lysed within 24 hours, and then re-invasion happened. In this case, the number of parasites per vacuole has to be a smaller number compared to others. Yet, statistically the infection method did not affect the growth rate except when using spinseeding + pulse-chase. If the experiment is performed again, it is better to count host cells before infection to avoid over loading of parasite than certain MOI.
Me49 had slowest growth rate and lowest infection rate which was not expect. Because type II has a higher virulence than type III in mice, it was expected that Me49 have a higher infection rate and growth rate. Interestingly, when we infect Me49 by quick and dirty medium transfer in a culture flask, Me49 starts lysing the host almost at the same time as CTG strain. This MOI determination results showed that within 24 hours, Me49 had slow growth or started replication long after infection time point zero. Another type II strain, Prugniaud (Pru), behave also similar to Me49 (data not shown). These results imply that type II have unique infection and initiation of replication process compared to type I and II.

CTG strain infection was exactly what was expected, with lower virulence and slower replication time, doubling time of 8 – 9 hours. At 24 hours p.i., the averages of 12 parasites were found in each host cell with 1:2 MOI. Based on those MOI results, for RH and CTG strain infections, 1:2 MOI was applied to collect samples for 2D-DIGE experiment, and MOI of 1:5 was applied Me49 strain infection.

One-Dimensional Gel Electrophoresis and Western Blotting

Introduction

One-dimensional (1D) gel electrophoresis is a method used to separate proteins based on molecular weight and is an extremely simple and quick method as compared to two-dimensional (2D) gels. However, each protein band on the gel contains many proteins, unlike protein spots on 2D gel, where each spot represents a single protein. Because of this, 1D gels are not the best method for comparing individual protein
expression changes between untreated and treated samples and for subsequent protein identification. However, 1D gels are useful to check the sample quality, consistency amongst the samples and possibly can detect dramatic protein expression level changes that occur between two samples. Thus, 1D gels were used to obtain an idea of how different and/or similar the uninfected vs. infected protein samples were, to assess the consistency amongst the sample replicates and to assess the sample quality, before performing 2D-DIGE analysis. Additionally, dyes that detect protein post-translationally modifications, such as phosphorylation, can be easily used on 1D gels, to quickly assess specific types of changes between untreated and treated samples. Thus we also used Pro-Q® Diamond Phosphoprotein Gel Staining, which stains phosphorylated proteins, to determine if there were significant differences in this type of post-translational modification between the uninfected vs. infected cells. Based upon previous studies, indicating parasite kinases and phosphatases differ between virulent vs. avirulent strains, we hypothesized that significant changes in phosphorylated proteins would be observable. Finally, western blots were done for evaluating the ability of membrane protein extraction by lysis buffer and checking the level of parasite protein existence with in infected host protein extracts.
Materials and Methods

Cell Culture and Cell Harvest. To harvest cells for electrophoresis, quadruplicates of uninfected samples and infected samples were prepared with each strain. Aliquots of these samples were first run via 1D gel electrophoresis to test the sample quality and then if samples of sufficient quality, they were used for 2D-DIGE analysis (see Chapter 3). HFFs were grown and infected as described above. To infect HFFs, for RH and CTG strain *T. gondii*, an MOI of 1:2 was used, and for ME49, an MOI of 1:5 was used to obtain comparable percent infections amongst the three strains. The number of parasites needed was calculated based on the estimated number of cells in the culture dish (Table 1). Then cells were harvested at 24 hours p.i. by washing them once with 0.05 % trypsin/0.53 mM EDTA (CellGro®) and incubated in a small amount of trypsin solution for less than 5 minutes. Once cells were removed from the flask surface by trypsin, four times the volume of growth medium was added to stop trypsin activity. The cells were centrifuged at 1200 rpm (~180 xg) for 5 minutes, the cell pellet washed with sterile phosphate buffered saline (PBS, CellGro®) three times by repetition of centrifugation and resuspension. After the final wash, cell pellets were resuspended 1 ml of PBS and transferred to a microfuge tube. Then the tube containing the cells was rinsed with 0.5 ml of PBS to ensure all the cells had been collected, and transferred into the microfuge tube. Cells were then centrifuged, the PBS was removed and the cell pellet stored at -80 °C.
Protein Extraction and Protein Concentration Determination. Urea-CHAPS buffer was made by dissolving 7 M urea, 2 M thiourea, 4 % (w/v) or 0.065 M of 3-[3-Cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS, Fisher), and 1.4 % (w/v) or 0.317 M of 3-[N,N-Dimethyl(3-myristoylaminopropyl)ammonio] propanesulfonate (ASB-14) in 30 mM Tris-HCl at pH 8.5 with a trace of bromophenol blue. Urea-CHAPS buffer was aliquoted into 900 µl and stored at -80 °C for future use. Just before use, urea-CHAPS buffer was mixed with 100 µl of protease inhibitor (Roche) and nuclease mix (GE Healthcare). Protease inhibitor was dissolved in 1 ml of filtered water, aliquoted into 110 µl, and stored at -20°C. Nuclease mix was aliquoted into 12 µl, and stored at -20 °C until future use.

Cell pellets were removed from -80 °C, and thawed at the room temperature. The cell pellets were resuspended in urea-CHAPS buffer, frozen in liquid nitrogen for 1 minute and then thawed. The freeze and thaw cycle was repeated two more times to disrupt cells. Tubes were centrifuged at 14,000 rpm (21,000 xg) for 15 minutes and supernatant transferred to a new tube. The pellet was mixed with a small amount of urea-CHAPS buffer and then disrupted with three freeze and thaw cycles. These were centrifuged again, and the supernatant combined with the supernatant harvested from the previous step. To remove salt in the protein extract, four times the volume of ice cold acetone was added to the extract, and then immediately stored at -80 °C overnight. To recover proteins, the tube was centrifuged at 14,000 rpm (21,000 xg) for 30 minutes after thawing, and dissolved in a urea-CHAPS buffer that was mixed with protease inhibitor
and nuclease mix. To avoid protein degradation due to the freeze and thawing, the concentration of the protein extract was performed immediately.

For the determination of solubilized protein concentration, Bio-Rad Protein Assay Kit II, the Bradford protein assay based method, was used. The dye in this kit, coomassie bind to proteins and color changes in the dye. Originally the dye is blue color and as protein concentration higher, the solution color becomes more like brown/ red. The dye reagent concentrate from the kit was diluted 1:5 with Milli-Q water and filtered with Whatman #1 filter to remove particles. The filtered dye reagents were then aliquoted in 1.0 ml in tubes, and using 2 µg/µl BSA, a blank and four standards from 2 µg/µl to 8µg/µl were used to make the standard curve. For the protein samples, 1 µl was added to 1.0 ml dye reagent aliquot and triplicates were made for each sample to get a reliable protein concentration. All the tubes were mixed, allowed to rest for 5 minutes and then the absorbance measured at 595 nm with a spectrophotometer using the standard curve used to determine the protein concentration. All of the absorbance measurements were done within an hour after the protein sample was added to the dye. Sample concentrations were calculated by taking the average of three readings. Total protein harvest was also calculated for each sample. To avoid freeze and thawing of the protein samples, samples were aliquoted into three sets of 50 µg and 25 µg in 0.5 ml microfuge tubes.

**1D Gel Electrophoresis.** 25 µg of protein extract dissolved in urea-CHAPS was acetone precipitated and stored at -80 °C for at least a few hours or overnight. The samples were then centrifuged at 14,000 rpm (21,000 xg) for 20 minutes. The supernatant was carefully removed and the pellet was dissolved in 20 µl of Laemmli sample buffer
(Bio-Rad) in which DTT (Sigma-Aldrich) was added to the final concentration of 50 mM DTT. The sample was denatured at 95 °C incubation for 5 minutes. To avoid loading undissolved proteins, samples were spun at 14,000 rpm (21,000 xg) for 10 minutes. During that time, a well comb and seal at the bottom of a 4–20 % Mini-PROTEAN® TGX™ precast polyacrylamide gels (Bio-Rad) were removed and set in electrophoresis apparatus. SDS running buffer contained 192mM glycine (Omnipur®), 24.8 mM tris-base (Fisher), and 3.47 mM SDS (Fisher) was used to fill the buffer dam and half-way in buffer tank. Then, wells of the gel, were rinsed with running buffer by pipetting several times in wells. Samples were carefully transferred to wells, taking care to avoid cross contamination between wells. Then, 25 mA was constantly applied to the gel for 50 minutes to an hour.

Gel Staining with Pro-Q® Diamond Phosphoprotein Gel Stain and GelCode Blue Safe-Stain. Gels were stained with Pro-Q® Diamond Phosphoprotein Gel Stain (Life Technologies) first, and then stained with GelCode Blue Safe Protein Stain (Thermo-Scientific). The Pro-Q® Diamond Phosphoprotein stains phosphorylated proteins with a detection limit of 1-16 ng, while and the GelCode Blue Safe-stain stains all proteins with a detections limit of 7 ng. GelCode Blue Safe-stain is similar to Commassie stain, but has a higher sensitivity and is less time consuming requiring a few hours.

To stain the gels, the gel cassette was taken from an electrophoresis apparatus, and rinsed with Milli-Q water. Then the gel was separated from the cassette and immersed in fixing solution containing 50 % methanol and 10 % acetic acid for 30
minutes to fix proteins in the gel. During 30 minutes of incubation, a container with gel
was gently agitated on a shaker at room temperature, and all incubations done on a shaker
with gentle agitation. The fixing solution was changed with fresh solution and again
incubated for half an hour. The gels were then washed with Milli-Q water for 10 minutes
for three times. During fixation, some shrinkage of the gel occurs, but after washing three
times, the gel returns to the initial size. Once the wash was done, the gel was immersed in
Pro-Q Diamond Stain overnight in the dark. The stain was removed carefully with a
pipette and kept in a clean bottle for reuse. The gels were destained with 20 %
acetonitrile (Fisher) in 50 mM sodium acetate (Fisher) at pH4 for 30 minutes. Then, a
destaining solution was changed to a fresh solution and destaining was repeated two more
times. During the destaining, the gels were kept in the dark. Gels were then washed with
Milli-Q water two times for 5 minutes each. To avoid breaking the gel, the gel was
immersed in Milli-Q water for at least an hour in the dark after washing. The picture of
the gel was obtained on Typhoon Trio with 532 nm excitation laser and 580 nm emission
filter.

The Pro-Q Diamond Phosphoprotein stained gel was directly immersed in
GelCode Blue Safe Protein Stain for at least 2 hours. Then, the gel was destained in
Milli-Q water. During destaining, a kimwipe was soaked at the corner of the container to
absorb stains. The water and the kimwipe were changed at least every 30 minutes until
the gel exhibited a fair quality of protein bands. The gel picture was taken on an Epson
Expression 1680 scanner.
**Western Blot.** All materials and reagents were purchased from Bio-Rad. A gel with cassettes was taken from the electrophoresis apparatus and quickly rinsed with Milli-Q water. Then the gel was separated from the cassettes and immersed in filtered water first, then in Towbin buffer containing 25 mM tris, 192 mM glycine, 20 % (v/v) methanol to equilibrate the gel for 15 minutes. Also, two filter papers and two foam pads were soaked in Towbin buffer. A PVDF membrane was soaked in 100 % methanol for one minute and then transferred to Towbin buffer. The gel was assembled with foam pads and filter papers in a gel holder. A magnetic stir bar was placed at the bottom of transfer buffer tank, and the tank was filled half way with Towbin buffer (transfer buffer). The gel holder was set in blotting module and placed in the tank. The buffer tank was filled with transfer buffer to the fill line. To avoid heating buffer up during electroblotting, the assembled tank was transferred in cold room at 4 °C. The tank was placed on a stirring/heating plate, and a stirring bar used to mix buffer during electroblotting to keep buffer temperature low. Proteins on the gel were transferred to PVDF membrane by electrobolot with 70 V and a limit of 350mA for 16 hours.

After electroblotting, the apparatus was disassembled and the PVDF membrane was immersed for an hour in a blocking buffer contained 3 % gelatin dissolved in tris buffered saline (TBS). To dissolve the gelatin, the solution was heated to 50 °C, with care taken not to heat above 65 °C, and then cooled to room temperature for use. During the one-hour incubation, the container with the membrane was gently agitated on shaker. Then, the membrane was washed with 0.05 % (v/v) Tween-20 in TBS solution (TTBS) for 10 minutes. The primary antibody used was mouse-anti-p30
monoclonal antibody (Meridian Life Science, Inc.), diluted 1: 10,000 with 0.05 % (v/v) Tween-20 and 1 % gelatin in TBS solution. The diluent was made first and cooled to 4 °C before mixing with antibodies. The membrane was immersed in primary antibody buffer for two hours with gentle agitation. To remove unbound antibodies, the membrane was washed with TTBS for 10 minutes on shaker. The secondary antibody used was goat-anti mouse IgG (H+L) conjugated with alkaline phosphatase (AP), diluted 1:30,000 with 1 % gelatin in TTBS. AP color development concentrate was 1: 25 Milli-Q with filtered water and mixed with color reagents, nitroblue tetrazolium in aqueous DMF and 5-bromo-4-chloro-3-indolylphosphohate in aqueous DMF just before the use. The membrane was immersed in the color development buffer for 10-30 minutes. The detection limit of the enzyme was 10-100 pg of proteins. When bands were shown on the membrane, it was transferred into Milli-Q water to stop color development, and air-dried.

Results

Comparison of Three Strains via 1D Gel Electrophoresis. Protein samples harvested for 2D-DIGE analysis were first analyzed via 1D gel electrophoresis to assess sample quality and consistency amongst the replicates. The samples were collected in quadruplicates and replicates run on 1D-gel as a set of uninfected and infected for each strain (Figure 15). These gels were stained with Pro-Q Diamond stain to detect phosphorylated proteins and then stained with GelCode Blue Safe Protein Stain to see whole proteins.
Sample sets of all three parasite strains (RH, Me49 and CTG) showed good consistency amongst the biological quadruplicates in both total protein and phosphorylated protein (Figure 15). Even though 1D is not as sensitive as 2D, changes were observable in both total protein and phosphorylated proteins (Figure 15). Since there was good consistency amongst the replicates, one sample from each sample set was chosen and acetone precipitated to obtain cleaner bands on a gel (Figure 16). Some differences in protein banding patterns were seen between RH and Me49 infected cells vs. uninfected cells in both the total protein staining and the phosphorylated stains (Figure 16A and B; yellow arrows). Both RH and Me49 infected cells had evidence of increased and decreased proteins in comparison to the uninfected cells (Figure 16A). Changes in phosphorylation patterns were also observed in both RH infected cells and Me49 infected cells (Figure 16B). Interestingly, the banding patterns of both total protein and phosphorylated proteins, between RH-infected cells and Me49-infected cells were distinct, indicating the two strains exert differential responses in HFF host cells (i.e. yellow arrows in lanes 2 vs. 4; Figure 16A and 16B). No changes were found on CTG infected lysate in either total protein stain or phosphorylated protein stain (Figure 16). In general, more changes were seen in virulent RH strain infected sample in both total protein and phosphorylated proteins than in either Me49 infected or CTG infected cells. This indicates that the host cell changes induced by RH strain are more significant than the avirulent, CTG strain, both in total the proteins as well as phosphorylated host cell proteins.
1D Gel of *T. gondii* Proteins vs. *Parasite Infected and Uninfected Proteins*. To compare, the banding pattern of the parasite vs. the host cells, freshly harvested parasite lysates from each strain were acetone precipitated. Then, all samples were dissolved in sample buffer and denatured at 95 °C for 5 minutes. Host cell lysates and parasite lysate samples were run on one gel, and the gel was stained the same as other runs. The 1D gel showed distinct protein band patterns between human and *T. gondii* though both of them are eukaryotic cells (Figure 17). Further gel analysis showed that there were similarities in *T. gondii* RH strain and CTG strains, and Me49 has unique band patterns (Figure 18).

A Western blot was performed to detect *T. gondii* major surface protein p30, also known as surface antigen 1, in infected and *T. gondii* lysate. Protein p30 on electroboltted membrane was tagged with mouse anti-p30 antibodies, and then the primary antibodies were tagged with goat-anti mouse IgG conjugated with alkaline phosohatase (AP) which develops a bluish color once the substrate is added. The parasite protein p30 is an abundant surface and expected to be in the lysate though urea-CHAPS mainly harvest cytosolic proteins. The results showed the presence of p30 in all protein lysate of *T. gondii* strains and infected samples (Figure 19). The parasite lysates exhibit clear bands, and infected samples had faint bands. However, RH infected samples had stronger bands than the other two strains infected samples. This likely due to the fact that there are more RH parasites in these infected cells than the Me49 and CTG-infected cells at 24 hours p.i. as is expected from the MOI determination experiments.
Discussion

1D gel results were useful and more meaningful than expected. 1D gel results confirmed that there are no inconsistencies in quadruplicates for each sample set, and that sample qualities are sufficient for 2D-DIGE analysis. On the 1D gels, RH-infected cells showed the most changes, and as the virulence got lower (type II vs. type III avirulent strains), the host expression changes are less in both total proteins and phosphorylated proteins.

Interestingly, it was found that among three strains of *T. gondii*, Me49 had unique band patterns compared to the other two strains. This result is very interesting because RH and CTG have the greatest degree of difference in virulence phenotype and replication time, yet the CTG expressed proteins are more similar to RH than to ME49 which have more in common. Additionally, Me49 was found to be distinctly different than type I and type III strains in the rate of growth in the first 24h post-infection. My other studies indicate another type II strain, Prugniad, also has a slower rate of growth in the first 24h post-infection (data not shown). The reason for this difference in type II strain vs. type I and type III strains, was unexpected and is not understood. It would be interesting to compare *T. gondii* strains on 2D-DIGE to explore this further.
Figure 7. Determination of MOI workflow for RH strain

Figure 8. Microscopic observations of RH infected cells at 24 hours p.i. with 1:5 MOI and different infection methods
Figure 9. Microscopic observations of RH infected cells at 24 hours p.i. with 1:2 MOI and different infection methods.
Figure 10. Infection method comparison with RH infection

Figure 11. Microscopic observations of Me49 infected cells at 24 hours p.i. with 1:2 and 1:5 MOI
Figure 12. Microscopic observations of CTG infected cells at 24 hours p.i. with multiple MOI
Figure 13. MOI determination statistical results. A) Percent infection of each strain with different MOI and infection method. B) Parasite growth at 24 hours p.i. was compared between strains and MOI at 24 hours after pulse-chase infection.
Figure 14. Microscopic observations of three strains at 24 hours p.i. with the best MOI
Figure 15. Gels with quadruplicate samples of uninfected and infected for three different *T. gondii* strains comparing total protein and phosphorylated proteins. Samples in red boxes are uninfected, and samples in blue boxes are infected cell lysate.
Figure 16. Comparison of 1D gel band pattern between uninfected and infected samples in all three strains. A) Total proteins. B) Phosphorylated proteins. UI means uninfected. Yellow arrows indicate the bands are more expressed than uninfected or infected sample.
Figure 17. Comparison of 1D gel band patterns between *T. gondii*, uninfected, and infected samples. A) Total proteins. B) Phosphorylated proteins.
Figure 18. Gel band intensity analysis on three *T. gondii* strains. The gel band is RH strain whole protein sample. Red: RH, Green: Me49, Blue: CTG.

Figure 19. Western blot detecting *T. gondii* surface protein p30 in *T. gondii*, uninfected and infected samples.
Table 1. Estimated number of cells in different cell culture container

<table>
<thead>
<tr>
<th></th>
<th>Growth area (cm²)</th>
<th>Volume of medium (ml)</th>
<th>The number of host cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-well slide</td>
<td>4</td>
<td>1</td>
<td>5 x 10⁵</td>
</tr>
<tr>
<td>(per well)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T25 flask</td>
<td>25</td>
<td>5</td>
<td>3 x 10⁶</td>
</tr>
<tr>
<td>T75 flask</td>
<td>75</td>
<td>10~15</td>
<td>1 x 10⁷</td>
</tr>
<tr>
<td>T175 flask</td>
<td>175</td>
<td>20~30</td>
<td>2.5 x 10⁷</td>
</tr>
</tbody>
</table>

Table 2. Statistical difference between infection methods.
HFFs were infected with RH at 1:2 MOI by four different infection methods.

<table>
<thead>
<tr>
<th>Infection method</th>
<th>% infection</th>
<th>Vacuole/ host</th>
<th>Parasite/ vacuole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous</td>
<td>85.11 ± 9.51</td>
<td>2.09 ± 0.35</td>
<td>8.53 ± 1.57</td>
</tr>
<tr>
<td>Pulse-chase</td>
<td>100.0 ± 0.00</td>
<td>3.28 ± 0.81</td>
<td>9.94 ± 1.54</td>
</tr>
<tr>
<td>Spin seeding + continuous</td>
<td>91.62 ±9.43</td>
<td>3.41 ± 0.47</td>
<td>9.10 ± 0.59</td>
</tr>
<tr>
<td>Spin seeding + pulse-chase</td>
<td>100.0 ± 0.00</td>
<td>5.34 ± 1.48</td>
<td>7.42 ± 1.05</td>
</tr>
</tbody>
</table>

Table 3. Statistical analysis of Me49 infection with two MOIs on HFFs

<table>
<thead>
<tr>
<th>MOI</th>
<th>Infection method</th>
<th>% infection</th>
<th>Vacuole/ host</th>
<th>Parasite/ vacuole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>Pulse-chase</td>
<td>53.34 ± 18.59</td>
<td>2.10 ± 0.47</td>
<td>2.24 ± 0.51</td>
</tr>
<tr>
<td>1:5</td>
<td>Pulse-chase</td>
<td>86.02 ± 6.86</td>
<td>2.52 ± 0.50</td>
<td>2.43 ± 0.35</td>
</tr>
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Table 4. Statistical analysis of CTG infection with two MOIs on HFFs

<table>
<thead>
<tr>
<th>MOI</th>
<th>Infection method</th>
<th>% infection</th>
<th>Vacuole/ host</th>
<th>Parasite/ vacuole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>Pulse-chase</td>
<td>90.95 ± 10.16</td>
<td>2.21 ± 0.54</td>
<td>6.17 ± 0.70</td>
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<tr>
<td>1:5</td>
<td>Pulse-chase</td>
<td>92.25 ± 8.59</td>
<td>2.39 ± 0.69</td>
<td>5.58 ± 0.54</td>
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CHAPTER THREE

TWO-DIMENSIONAL DIFFERENTIAL GEL ELECTROPHORESIS (2D-DIGE) OF *TOXOPLASMA GONDII* TYPE I, II, AND III STRAINS IN HUMAN FORESKIN FIBROBLASTS (HFFS)

**Introduction**

Two dimensional (2D) gel electrophoresis is a very powerful tool to separate and visualize complex protein mixtures such as cell lysates according to isoelectric point (1\textsuperscript{st} dimension, horizontal axis) and molecular weight (2\textsuperscript{nd} dimension, vertical axis). This technique was first reported in 1975 by Patrick H. O’Farrell in The Journal of Biological Chemistry\textsuperscript{33}. The significance of the paper was that his 2D gel method increased the resolution and sensitivity dramatically (theoretically 7000 spots detectable) from other 2D procedures that detected only around 50 spots. Also, his protocol made it possible to separate proteins uniformly across both vertical and horizontal directions, unlike other methods in which protein spots tend to show up diagonally on gels\textsuperscript{33}.

The bacterium, *Escherichia coli* was used for these experiment. They were cultured in the medium and then labeled with mixed $^{14}$C-amino-acids in order to obtain a gel picture by autoradiography. For the first dimension, isoelectric focus (IEF), O’Farrell added ampholines to the 1\textsuperscript{st} dimensional gel, and an electric field was applied to it to create a pH gradient. Ampholine is a mixture of hundreds of chemical entities with thousands of isoforms called “carrier ampholytes (CA)” which was first synthesized by Olof Alfred Yngve Vesterberg accidentally in 1964\textsuperscript{34}. Once the pH gradient was set up,
the protein extract was applied on the 1st dimension gel, and then electrophoresis was run. Through the experiments, the author found 1100 spots on a gel and also found that the protein concentration correlated with spot size.

This method has since been widely used, but low reproducibility has been a problem in comparing protein expression between two different conditions. The main reason for the low reproducibility was CA. CA are synthesized chemicals at the end of this complicated procedure and there is low reproducibility in batch-to-batch, even in commercially available products. Also, pH draft (on cathode) occurs often due to electroendosmosis during IEF.

To avoid those problems, a new IEF method that does not require CA, called immobilized pH gradient (IPG), was established. The method was introduced in 1982 by Bjellgivist, and then Görg developed the protocol as 1st dimensional IEF by 1988. The biggest difference is that the pH gradient already exists in the gel without the application of an electric field. This is because acidic and basic derivatives, called immobile, are covalently bound to the gel matrix in the polyacrylamide gel. Also, there is no charge on the IEF gel, so electroendosmosis cannot occur, which means the cathodic draft would not happen after the longer period of time of electric field application. Other advantages of IPG are: 1) higher resolution (0.01pH/cm), 2) insensitivity to salt contaminations, 3) bigger loading capacity, and 4) higher reproducibility. However, there are two main drawbacks. One is that IPG requires longer focusing time. The other is the complexity of the gel preparation. The second disadvantage was solved by the development of the first commercially available product, IPG strip, by GE healthcare.
Another problem was the difficulty in gel-to-gel comparison, because each 2D gel runs differently and multiple gels cannot simply be superposed to compare protein expression changes among multiple conditions. This problem was solved by developing florescent dyes that tag proteins. The dyes were named “CyDyes” and were developed by Jonathan Minden in 1991 with his chemist colleague, Alen Waggoner\textsuperscript{38}. To develop the dyes, several criteria must be met for 2D-DIGE use: 1) dye molecules must be of similar mass, 2) dyes must be pH insensitive, 3) dyes must not change the charge on a protein, and 4) dyes must attach to most reactive amino acids, lysine or cysteine. Cy3 and Cy 5 were synthesized which meet these criterion. These dyes react with lysine residues on proteins, have one positive charge, and the molecular masses are only 2 Da different. An $N$-hydroxysuccinimide ester active group on the dyes covalently binds to lysine residue at pH 8.5 (Figure 20)\textsuperscript{38}. During the chemical reaction, lysine residue loss one positive charge, yet the one positive charge on dye remained. This allows the protein to maintain its charge after being tagged with dyes. After development of this method in 1997, pictured in Figure 20, the first 2D-DIGE paper was published\textsuperscript{39}.

The development of CyDyes was a huge breakthrough to the application of 2D gel research because of the high sensitivities and the ease of analysis compared to the past method. Yet, the dyes are structurally non-polar and affect the solubility of tagged proteins in aqueous solution. To overcome that, zwitterionic CyDyes (Z-CyDyes) were developed by the Paul Grieco lab in the Chemistry and Biochemistry department at Montana State University\textsuperscript{40}. By modifying the linkage between NHS-ester reactive group and dyes to have zwitterionic feature, Z-CyDyes dyes were successfully synthesized,
which do not affect the excitation and emission wavelength, and at the same time have increased sensitivity (Figure 21)\textsuperscript{40}. Those features of Z-CyDyes allow the detection of more proteins than CyDyes tagged proteins on 2D-DIGE. Because of these features, Z-CyDyes were chosen for use in this study.

In the past, three studies of host proteomic with \textit{T. gondii} infection have been done. 2D-DIGE was used partially in one of the studies, and the other study used conventional 2D gels. The study with 2D-DIGE found 162 protein spots with significant protein expression level changes induced by infection and in the study using conventional 2D gels, 60 protein spots had significant expression level changes\textsuperscript{30,31,32}. Even though there were some differences in samples and sample collection methods between the three studies such as \textit{in vitro vs. in vivo} conditions, HFFs, macrophage, or brain cells as host cells, this clearly showed that CyDyes were very sensitive and a useful tool in proteomics and that proteomics could be used to analyze the host cell responses to infection\textsuperscript{40}.

In this study, we compared the host cell proteomic responses to strains of distinct virulence phenotypes utilizing 2D-DIGE with Z-CyDyes, and RH, a type I virulent strain, and CTG, a type III avirulent strain, were used. Also, infected cells were sampled at 2 hours, 12 hours, and 24 hours post infection (p.i.) to compare host-parasite interaction at different points after infection for RH infection. At 2 hours p.i, \textit{T. gondii} are have completed invasion with establishment of parasite vacuole (PV) in host cells, and are able to begin replication. At 12 hours p.i., parasites have begun replicating in a vacuole and should have between 2-4 parasites in a vacuole, depending upon the strain. At the last time point, 24 hours p.i., parasites are still actively replicating, with a vacuole containing
8 - 16 parasites for type I strains and 4-8 parasites for type III strains, yet host cell lysis will not yet have occurred. Initially, type II, Me49 strain was intended to be analyzed on 2D-DIGE, but due to results from MOI experiments (see Chapter 2), indicating type II strain was distinctly different in growth characteristics than type I and type III strain, it was decided not to use type II strain in the 2D-DIGE analysis.

**Materials and Methods**

**Cell Culture and Cell Harvest**

To harvest cells for electrophoresis, quadruplicates of uninfected samples and infected samples were prepared with each strain. Aliquots of these samples were first run via 1D gel electrophoresis to ensure the sample quality (see Chapter 2) and they then were used for 2D-DIGE analysis. HFFs were grown and infected as described above in Chapter 2. To infect HFFs, for 2D-DIGE analysis of RH and CTG strain infection with *T. gondii*, an MOI of 1:2 was used as this MOI was found to obtain comparable percent infections between type I vs. type III infection. The number of parasites needed was calculated based on the estimated number of cells in the culture dish (See Chapter 2). Then cells were harvested at 24 hours p.i. by washing them once with 0.05 % trypsin/0.53 mM EDTA (CellGro®) and incubated in a small amount of trypsin solution for less than 5 minutes. Once cells were removed from the flask surface by trypsin, four times the volume of growth medium was added to stop trypsin activity. The cells were centrifuged at 1200 rpm (~180 xg) for 5 minutes and the cell pellet washed with sterile phosphate buffered saline (PBS, CellGro®) three times by repetition of centrifugations
and resuspension. When the cell pellets were resuspended for the last wash, they were
first suspended in 1 ml of PBS and transferred to a microfuge tube. Then the Falcon tube
was rinsed with 0.5ml of PBS and transferred into the microfuge tube. After the third
wash the PBS was removed and the cell pellet stored at -80 °C. To
avoid protein degradation due to the freeze and thaw steps, concentration of protein
extract was performed immediately.

Protein Extraction and Protein
Concentration Determination

Urea-CHAPS buffer was made by dissolving 7 M urea, 2 M thiourea, 4 % (w/v)
or 0.065 M of 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS,
Fisher), and 1.4 % (w/v) or 0.317 M of 3-[N,N-Dimethyl(3-myristoylaminopropyl)
ammonio] propanesulfonate (ASB-14) in 30 mM Tris-HCl at pH 8.5 with a trace of
bromophenol blue. Then, the urea-CHAPS buffer was aliquoted with 900 µl and stored at
-80 °C. Just before use, urea-CHAPS buffer was mixed with 100 µl of protease inhibitor
(Roche) and nuclease mix (GE Healthcare). Protease inhibitor came as a small pill, so it
was dissolved in 1 ml of filtered water, aliquoted 110 µl, and stored at -20 °C. Nuclease
mix was aliquoted 12 µl, and stored at -20 °C upon arrival.

Cell pellets were removed from -80 °C, and thawed at the room temperature. The
cell pellets were resuspended in urea-CHAPS buffer, frozen in liquid nitrogen for 1
minute and then thawed. The freeze and thaw cycle was repeated two more times to
disrupt cells. Tubes were centrifuged at 14,000 rpm (21,000 xg) for 15 minutes and
supernatant transferred to a new tube. The pellet was mixed with a small amount of urea-
centrifuged again, and the supernatant combined with the supernatant harvested from the previous step. To remove salt in the protein extract, four times the volume of ice cold acetone was added to the extract, and then immediately stored at -80 °C overnight. To recover proteins, the tube was centrifuged at 14,000 rpm (21,000 xg) for 30 minutes after thawing, and dissolved in a urea-CHAPS buffer that was mixed with protease inhibitor and nuclease mix. To avoid protein degradation due to the freeze and thaw steps, concentration of protein extract was performed immediately.

For the determination of protein concentration, Bio-Rad Protein Assay Kit II was used. Dye reagent concentrate from the kit was diluted 1:5 with distilled water and filtered with Whatman #1 filter to remove particles. Filtered dye reagents were aliquoted in 1.0 ml in tubes, and using 2 µg/µl BSA, a blank and four standards from 2 µg µl to 8µg/µl were used to make the standard curve. For the protein samples, 1 µl was added to 1.0 ml dye reagent aliquot and triplicates were made for each sample to get a reliable protein concentration. All the tubes were mixed, allowed to rest for 5 minutes and then the absorbance measured at 595 nm with a spectrophotometer using the standard curve used to determine the protein concentration. All of the absorbance measurements were done within an hour after the protein sample was added to the dye. Sample concentrations were calculated by taking the average of three readings. Total protein harvest was also calculated for each sample. To avoid freeze and thaw of protein samples, samples were aliquoted into three sets of 50 µg and 25 µg in 0.5 ml microfuge tubes.
Z-CyDye Labeling and Isoelectric Focusing (IEF)

Z-CyDyes from Grieco lab were aliquoted into 8 nmol and stored in -80 °C. The dyes were dissolved in 8 µl dimethyl sulfoxide (DMSO, Fisher) or dimethylformamide (DMF, Sigma-Aldrich) to get 1 nmol/µl aliquots and stored at -80 °C for up to several months. For labeling use, the dyes were diluted 1:1.5 to achieve 400 pmol/µl, which was stored at -80 °C for less than two weeks. Uninfected samples were labeled with Z-Cy3, infected samples were labeled with Z-Cy5, and 1:1 mixtures of uninfected and infected samples were labeled with Z-Cy2, as an internal standard. Aliquots of 50 µg and 25 µg samples were thawed and centrifuged. The 25 µg uninfected and infected samples were combined to make an internal standard sample (Table 5). For all samples, about 10~20 µl samples, about 10~20 samples were a protein inhibitor and nuclease mix, and added to keep a pH of 8.5 in samples for Z-CyDye labeling. Then, 400pmol of corresponding Z-CyDyes were added to each sample and incubated at room temperature in the dark for 30~45 minutes. The Z-CyDye attachment reaction was terminated by adding 5 µl of 1M lysine that was pre-made and stored at -20°C. After adding lysine, samples were mixed well and incubated on the ice and keep in the dark for a minimum of 15 minutes. Then, all the samples were briefly centrifuged, with uninfected, infected, and standard samples combined together. To remove salts, four times the volume of ice cold acetone was added to each sample and stored at -80 °C at least 2 hours or overnight. To recover proteins, the tube was centrifuged at 14,000rpm (21,000 xg) for 30 minutes after thawing, and the pellet was dissolved completely in fresh 450 µl of urea-CHAPS buffer that was mixed with protease inhibitor and nuclease mix.
19.2 µl of 1M dithiothreitol (DTT, Sigma-Aldrich), a strong reducing agent, was pre-made and stored at -20 °C, and 4 µl of ampholytes, IPG buffer pH3-11NL (GE Healthcare) were added and mixed in the dark for 15 minutes. The samples were centrifuged at 14,000 rpm (21,000 xg) for 15 minutes to spin down insolubilized particles. Then, 450 µl of samples were transferred to an IPG strip holder, avoiding the introduction of bubbles and addition of insolubilized particles at the bottom of the tube. pH3-11NL IPG strips (GE Healthcare) were taken out from -80 °C and thawed. A plastic cover was removed from the IPG strip using forceps, and the strip was placed into the buffer with gel side down (so the strip number could be read in the right direction) and the acidic side of the strip on the anode side of the holder (numbers and barcodes were on cathode side). Finally the buffer and strip were covered with ~3 ml of pure, clean, proteomic grade mineral oil by adding it drop by drop from the cathode side of the holder. The strip holder was covered with its plastic lid and loaded onto the IEF instrument, IPGphor (Pharmacia Biotech) in the correct orientation. The instrument was covered with a black sheet to prevent light during the run to avoid quenching of Z-CyDyes. The IEF focusing progress was monitored by the movement of the bromophenol blue (BPB) dye toward the anode side. The blue dye should be at the anode end of the strip by the end of the focusing. These focusing conditions were as follows: 50 V for 12 hours to hydrate the strip, 500 V for 1 hours, Gradient to 1000 V for 1 hours, Gradient to 3000 V for 1 hours, Gradient to 5000 V for 1 hours, gradient to 8000 V for 1 hour, and 8000 V until the BPB dye reached to the end of the strip or reached to 40,000-
45,000Vhr total. After the run, the strips were stored in a plastic strip holder upside down (the strip number was inverted) and stored at -80°C until the 2nd dimension run.

**SDS-PAGE Casting and Running**

For the second dimension run, SDS-PAGE gels were freshly made. In this experiment, 14 % SDS-PAGE was used. To cast the gel, glass cassettes and casting system were rinsed with DI water and dried completely. Unscratched and spotlessly clean glass cassettes were used to ensure a good picture quality. Then, the glass cassettes were placed with a plastic panel between each set of glass cassettes in a casting system. In the system, 13 gels could be made at once, so if fewer than 13 gels were made, spacers were used to fill up the space in the system. The casting system was completely sealed by tightening all eight screws equally. Then, before making the gel solution, a displacing solution was made by mixing 25 ml 1.5 M Tris-HCl, pH 8.8, 25 ml double distilled deionized water, 50ml glycerol, and a few drops of BPB. Because of highly viscose glycerol, it took time to homogenize the displacing solution. 1.5 M Tris-HCl, pH 8.8 and 10 % (w/v) SDS were made as stock solution. These solutions were filtered with 0.45 µm pore filter (Millipore), and stored at room temperature. To make one SDS-PAGE gel, 35 ml of 40 % Polyacrylamide (Omnipur®mni25 ml 1.5 M Tris-HCl, pH 8.8, 38.5 ml double distilled deionized water, and 1 ml 10% SDS were mixed and filtered with a 0.45 µm pore filter. A stirring bar was placed in the mixture and 0.5 ml of freshly made 10 % (w/v) APS (Fisher) and 0.05 ml TEMED (Fisher) were added as the solution was stirred. Once TEMED is added to the solution, gel polymerization starts. Thus, after ~ 30 seconds of stirring, the gel solution was poured into the casting system. To avoid wasting
gel solution and clogging the casting system, displacing solution was added following the gel solution. The gel was sealed with water or mixture of butanol and water to prevent evaporation from the top of the gel. The gel was left overnight to complete polymerization. Then, the casting system was disassembled and the gel with glass cassettes was rinsed with DI water.

An IEF strip stored at -80 °C was thawed and equilibrated in the dark to resolubilize the proteins and to reduce –S-S- bonds. The equilibration buffer was made by dissolving 6 M urea, 30% glycerol (v/v), 2 % SDS (w/v) in 50 mM of Tris-HCl pH 8.8 with a trace of Bromophenol Blue. The buffer was stored at -20°C until use, and 1 % DTT (w/v) or 2.5 % iodoacetamide (IAA, w/v) were added just before use. The IEF strip was equilibrated with > 3ml of DTT equilibration buffer for 30 minutes to 1 hour in the dark. Then the DTT containing equilibration buffer was discarded, and the strip was incubated in > 3 ml of IAA equilibration buffer for 30 minutes to an hour. Then the strip was placed on top of the SDS-PAGE gel and sealed with sealing gel made by heating the 1: 200 w/ v ratio mixture of agarose and 1X SDS running buffer. The running buffer was stored as a 10X stock solution which was made by dissolving 521mM glycine (Omnipur®) 247.6 mM Tris-base (Fisher), and 34.7 mM Sodium dodecyl sulfate (SDS, Fisher) in filtered water. To avoid contamination, the10X buffer was filtered with 0.45 μm pore filter, and diluted with filtered water for use. The gel was run with 1 W/gel for the first hour, then increased to 2~ 3 W/gel for the overnight run. Then, in the morning, it could be increased gradually, avoiding dramatic increases such as from 3 W/gel to 15 W/gel as this would cause BPB to remain in the gel when the voltage quickly increased.
Gel Imaging and Analysis

Gels were scanned on Typhoon Trio™ Variable Mode Imager (GE Healthcare). For each Z-CyDye, a different laser and emission filter was used to scan the gel image (Table 5). Then, image was obtained with a pixel size of 100 microns. Laser intensities for each dye were adjusted to optimize the dynamic range and available intensity levels on Progenesis™ software (Nonlinear Dynamics). Then, quadruplicates of gels were analyzed on Progenesis™, and protein spots were considered as significant expression level changes when there were 1.2 fold changes with p < 0.05.

Results

RH Strain, Type I Strain

Preliminary 2D-DIGE and Mass Spectrometry Experiment. The first set of 2D-DIGE were run with one RH infected sample harvested at 24 hours p.i. against biological triplicates of the uninfected sample treated and harvested at the same time point as the infected sample. For this preliminary run, a definitive MOI was not used for *T. gondii* RH strain infection, but rather a large inoculum of parasite was added (MOI > 5) to achieve high percent infection. Additionally, CyDyes were used in this gels as opposed to Z-CyDyes, which was used for the subsequent 2D-DIGE gels. Because of these reasons, this preliminary data is not necessarily statistically accurate and comparable to other 2D-DIGE results presented in this chapter, yet could be provided as reference data for further experiments. 1203 protein spots were commonly found on all three gels, and 273 spots had significant change in expression level when a threshold was set at 1.4 fold changes.
with \( p < 0.05 \). Among 273 protein spots, 143 proteins spots had increased expression levels of 1.4 -23 fold. The remainder of the protein spots (130 spots) were decreased, and fold changes calculated against the uninfected sample was between 0.167 and 0.714 (= 6 to 1.4 fold of decrease). In figure 23, protein spots with high \( p \)-values \( (p< 0.01) \) were shown with spot numbers given, based on fold change rankings provided by Progenesis software. About 20 proteins spots were isolated and digested by trypsin in gel to run on mass spectrometry, yet only seven proteins were identified due to heavy keratin contamination (Table 6). Three proteins were proteins from \( T. gondii \), and four proteins were human proteins. Because there are no parasite proteins in the uninfected sample, all the proteins from \( T. gondii \) increased their expression levels, and interestingly, all the identified human proteins were decreased in their expression levels (Table 6). Protein spots with a higher rank on Progenesis analysis tended to have increased expression levels and these are suspected to be mostly parasite proteins. In contrast, decreased protein spots tend to be in the lower rank and these were found to be human proteins (Figure 24).

**Kinetic Analysis of 2, 12 and 24 Hours p.i.** Since the first 2D- DIGE was successfully done, HFFs and \( T. gondii \) cultures were generated specifically for proteomic study using an MOI of 1:2, with pulse-chase infection. All biological quadruplicates for each time point (2 hours, 12 hours, and 24 hours) were cultured and harvested at each time point by trypsinization (Figure 25). At 2 hours p.i, \( T. gondii \) should have established the parasite vacuole (PV) in host cells, although they will not have replicated. At 12 hours p.i., parasites are actively replicating in a vacuole and expected to have 2-4 parasites per vacuole for RH type I strain. At point, 24 hours p.i., parasites are actively
replicating, with vacuoles of 8 - 16 parasites, but host cell lysis will not yet have occurred. One 2D-DIGE gel picture was successfully obtained at each time point (Figure 26). The overlay pictures on Figure 26 were made with the uninfected sample with green color and the infected sample with red color. When the protein spots are commonly expressed in both uninfected and infected samples, the protein spots are yellow in color.

A green spot on the overlay means that the protein spot was expressed only in the uninfected sample, while red spots on the overlay indicate that the protein is seen only in the infected sample. When the overlays between three time courses were compared, the number of red protein spots increased as time goes on. As samples for the kinetic analysis were not run as quadruplicates of 2D-DIGE at each time point, Progenesis could not analyze the protein spots statistically. However, just based on the overlay pictures, it is clear that as 24 hour p.i., the most expression changes (many red and some green protein spots) occur. On the other hand, the fewest changes were observed in 2 hours p.i.. Also, taking into account that one of the brightest red spots identified was the parasite surface protein p30 precursor in preliminary study (Figure 23, Table 6), some of the spots exhibiting the greatest degree of up-regulation on 2D-DIGE gels are likely parasite proteins. Also, as more spots (both red and green) were seen at 24 hours p.i. than 2 or 12 hours p.i.. This indicates that as parasite replication increases, host protein expression is increasingly affected more in both up-regulating and down-regulating proteins. However, as this kinetic analysis indicated only a few protein changes were observed at 2 and 12 hours p.i.. It was decided to only analyze the 24 hours p.i. samples via 2D-DIGE of parasite RH (typeI) and CTG (typeIII) strains in further 2D-DIGE analysis.
2D-DIGE at 24 Hours p.i. Quadruplicate samples of RH infected HFFs were collected, and three of those were run and imaged on 2D-DIGE (Figure 27). The qualities of the gels were exceptionally good, leading to ~1900 spots found on the gels, via Progenesis analysis. A threshold of 1.2 fold change with a minimum p-value of 0.05 was used to analyze the differentially expressed proteins using Progenesis. A total of 434 spots were recognized as having significant expression changes. Among them, 237 spots increased expression upon *T. gondii* RH strain infection (Figure 28A), and 187 spots decreased expression upon RH strain infection (Figure 28B). The fold changes were also significant. The largest fold changes calculated among increased spots was 13.1 fold, and in decreased spots, it was 0.16 fold (= 6.23 fold change). Log2 analysis which illustrates the degree of change of both increased and decreased proteins equally, was applied to normalized expression level data obtained from Progenesis (Figure 31). This analysis suggests that more significant expression changes were found in the increased protein spots, yet ~2/3 of spots with increased expression had smaller changes (< 2 fold changes) as compared to spots with decreased expression where most of the decreased proteins had < 2-fold changes (Figure 31). Since *T. gondii* is present in the infected samples, some increased protein is likely due to parasite protein and the proteins with the highest fold change increase are likely parasite proteins. Some of the increased proteins on the gel however may be due to modulation of host cell protein, as a result of infection.
CTG Strain, Type III Strain

Quadruplicates of CTG strain samples at 24 hours p.i. were run and imaged on 2D-DIGE (Figure 29). Progenesis gel analysis found 955 spots on all four gels, with 175 spots significantly differentially expressed levels as a result of infection (fold change > 1.2, p-value < 0.05). Of these differentially regulated proteins, 48 spots were increased and 127 spots exhibited decreased expression levels upon infection with *T. gondii* CTG type III strain. The maximum increased change was 2.413 fold, and the maximum decreased change was 0.663 fold (= 1.5 fold change) (Figure 30). Log2 analysis of expression level changes shows there are larger changes in increased proteins than decreased proteins (Figure 31). Increased protein spots tend to have better p-values compared to decreased spots and are spread over the entire pH gradient (from lower pH to higher pH). As for RH infection some of those increased spots likely are due to parasite proteins with the proteins with highest fold increase, most likely due to the parasite. The higher fold changes seen with RH vs. CTG infection may reflect with greater number of parasite in RH-infected cells. The decreased spots are due to the parasite effect on the host cell and in both RH and CTG infected cells, a large number of proteins were decreased (Figure 31). Interestingly, for CTG, many of the decreased protein spots were clustered around a lower pH with higher molecular weight and formed train like spots (Figure 30B). Thus, those proteins might be the same proteins that are post-translationally modified. Also, it was noticed that other decreased protein spots tend to form small clusters.
Discussion

From the preliminary 2D-DIGE experiments with RH infection, seven proteins were identified, with three of these proteins derived from *T. gondii*. These results suggest that many other parasite proteins may be found among the spots with increased expression and high fold changes. One of the identified parasite proteins was major surface antigen P30 precursor. This result indicates that protein extraction method using urea-CHAPS have the ability to extract some membrane proteins even though it is used mainly for harvesting cytosolic proteins. This conclusion was also supported by Western blots where p30 was also detected amongst the infected cells lysates by electroblotting (see Chapter 2). Some host cell proteins were also identified included calmenin, vimentin, porin and tropomyosin 1 alpha chain isoform 5, with all of them decreasing their expression upon RH strain infection. These preliminary data indicate proteomic analysis in combination with MS analysis can be used to identify host cell proteomic response to infection. Additionally, we found we could identify parasite proteins via 2D-DIGE gel analysis, in combination with protein identification via MS. Thus although this was not the focus of the current study, future studies could use this approach to study both the host proteomic response as well as the parasite proteomic response to infection.

2D-DIGE results from the RH strain kinetic analysis at 2, 12 and 24 hours p.i. found an increasing number of spots over the course of infection. More red spots, indicative of proteins only expressed in the infected cells were found with increasing time after infection. Some of these changes may be due to the increase of *T. gondii* proteins due to the replication of the parasite throughout the 24 hours of infection, although this
cannot be concluded definitively until protein spots are identified. Additionally, these results also indicate 2D-DIGE analysis in combination with MS, can be used to identify changes in parasite protein expression at specific time points after infection, thus for example, enabling the identification of host cell proteins that are affected during establishment of infection vs. in the active growth phase of infection. For the current study however, as only a few protein spots were found on 2D-DIGE overlay pictures at 2 and 12h p.i., only 24h p.i. was selected for analysis for further experiments comparing the host proteomic response to infection with RH type I strain and with CTG, type III strain.

On the overlay of 2D-DIGE pictures with RH infected samples at 24 hours p.i., many increased and decreased protein spots were observed. Through the Progenesis analysis found a total of 434 protein spots with significant expression changes. Among those protein spots, 247 proteins increased and 187 proteins had decreased expression as a result of the RH parasite infection. Protein identification via MS, of the most significantly differentially regulated spots (highest p value), could be done to identify the host proteins differentially regulated by type I strain.

CTG infection did not have as many obvious differences on the gels as did the RH, type I infection. Only a few red spots were found on the sample collected at 24 hours p.i.. Progenesis gel analysis found less dramatic significant protein expression changes due to infection of CTG compared to RH strain. The lower number of proteins affected by type III infection vs. type I infection might be due in part because CTG replicates slower than RH and fewer parasites were in host cells compared to RH strain at 24 hours p.i. For example, there were total of 12 parasites in a CTG infected host in average at 24h
p.i., while 30 parasites were present in RH infected host at 24 hours p.i. (See Chapter 2, MOI experiment). However, some of the differences at 24 hours p.i. may be due to differences in the host cell proteomic response to type I vs. type III infection. Protein identification via MS of the most significantly differentially regulated spots (highest p value) could be done to identify the host proteins differentially regulated by type III strains.

By comparing RH infected and CTG infected results, Progenesis found almost 2.5 times more protein spots with expression changes in RH infected sample. However, the number of protein spots with decreased expression was only about 1.5 times more, and the number of protein spots with increased expression was approximately 5.5 times more than CTG infected sample. The highest increasing proteins may be due to the greater replication rate of RH strain compared to CTG strain. However, other differences may be due to host cell response to infection. At the same time, the similar number of protein spots with decreased expression indicates there might be some “core” proteins affected by all *T. gondii* infections regardless of the virulence phenotypes. It is interesting that so many proteins were down-regulated by both type I and type III infection. This suggests that successful infection may be due to suppression of specific host cell functions. Identification of the decreased proteins, may yield important insights into the host cell proteins and pathways that are down-regulated by parasite infection. Finally, the differences found between RH and CTG infection roughly agrees with transcriptomic studies which has found a lower transcriptomic response to type III strains vs. type I
strains. A comprehensive study of transcriptomic and proteomic data with protein identification could be done in future.

Comparing the overlay of 2D-DIGE pictures between RH and CTG infected samples, very distinct two red protein spots are present on all gels. One of those on RH sample was identified as p30 precursor, and it is expected that on CTG infected sample, one of those red spots is from parasite because major surface protein p30, s detected on the Western blot. However their pH seems very different based on the DIGE pictures. It might be the one found on RH was precursor and the one on CTG is not precursor. Another possibility is their p30 structure and pI is different.

When comparing these results to past studies while taking into account the differences in those studies (Table 8), they provide some direction for future studies. The present study found more protein spots via 2D- DIGE analysis than did previously 2-DIGE studies (Table 8). This indicates in part, the increased sensitivity of the Z-CyeDyes used in this study and that upon protein identification, new host cell proteins modulated by RH infection may likely be identified. Although protein identification of this study was limited, some human proteins identified in our study were also found in past studies (Table 9). The first protein calumenin, was found in the study done by D.H. Zhou et al.. Both this study and the previous study found decreased expression in infected samples. The protein is calcium-binding protein, and localized at the lumen of the ER. Calumenin works as a universal second messenger by binding to Ca$^{+}$ involved in ER functions, including protein folding & sorting, gene expression, and signal transduction. The other protein vimentin, was found in all three studies. In the study done by M.M.
Nelson et al., the protein was up-regulated while in the D.H. Zhou et al. study and in this study, it was decreased. Vimentin is a very abundant intermediate filament and has a spiral structure. It is a cytoskeletal protein and has a structural role, but in addition vimentin also plays a role in cell cycles and mitosis, lipid metabolism, transportation system, and mRNA regulation. According to M.M. Nelson et al, phosphorylation on vimentin controls the complex formation between vimentin molecules and it might be associated with physiological reorganization upon infection. Thus, this protein would be one candidate to study further to see how vimentin is associated with the infection of *T. gondii*. 
Figure 20. Chemical reaction of CyDye labeling
From http://www.gelifesciences.co.jp/newsletter/protein_sciences/dige/dige2.html

Figure 21. Structural comparison between commercially available CyDyes and Z-CyDyes
Figure 22. Outline of 2D-DIGE based proteomic

Figure 23. Gel image with protein spot numbers provided by Progenesis analysis on uninfected vs. RH infected sample. Red circled spots with numbers are the spots with high p-values. Red circles indicate that those proteins were successfully identified.
Figure 24. Frequency of number of increased and decreased proteins from highest rank (1-10) to lower rank (261-270)

Figure 25. Culturing schedule for proteomic sample collections
Figure 26. Uninfected vs. RH infected samples at three different time points. On the overlay pictures, uninfected sample is green and infected sample is red. When the spots are in common between uninfected and infected, the spots get yellow.
<table>
<thead>
<tr>
<th></th>
<th>Uninfected (Z-Cy3)</th>
<th>Infected (Z-Cy5)</th>
<th>Standard (Z-Cy2)</th>
<th>Overlay</th>
</tr>
</thead>
</table>

Figure 27. Uninfected and RH infected triplicates samples on 2D-DIGE
Figure 28. Gel image with protein spot numbers provided by Progenesis analysis on uninfected vs. RH infected sample.  A) Increased expression level protein spots.  B) Decreased expression level protein spots.
Figure 29. Uninfected and CTG infected quadruplicates samples on 2D-DIGE
Figure 30. Gel image with protein spot numbers provided by Progenesis analysis of uninfected vs. CTG infected. A). Increased expression level protein spots. Spots with yellow circles have very high p-values (p < 0.001) B). Decreased expression level protein spots.
**Table 5. Z-CyDye/sample combination and gel scanning settings**

<table>
<thead>
<tr>
<th>Dyes</th>
<th>Samples</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\text{Em}_{\text{max}}$ (nm)</th>
<th>Laser (nm)</th>
<th>Emission filter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Cy3</td>
<td>50 µg of uninfected</td>
<td>548</td>
<td>564</td>
<td>532</td>
<td>580</td>
</tr>
<tr>
<td>Z-Cy5</td>
<td>50 µg of infected</td>
<td>642</td>
<td>663</td>
<td>633</td>
<td>670</td>
</tr>
<tr>
<td>Z-Cy2</td>
<td>Mixture of 25 µg of uninfected and 25 µg of infected</td>
<td>485</td>
<td>500</td>
<td>488</td>
<td>520</td>
</tr>
</tbody>
</table>

Figure 31. Log 2 analysis of the fold changes of differentially regulated proteins in RH vs. CTG infected host cells.

Log2 analysis of expression level changes (log2 (I/UI))

Log2 analysis of the fold changes of differentially regulated proteins in RH vs. CTG infected host cells.
Table 6. Identified proteins by preliminary experiment with RH infected sample at 24 hours p.i.

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Fold (I/U)</th>
<th>p-value</th>
<th>Protein</th>
<th>MASCOT score</th>
<th>Protein functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>12.1</td>
<td>8.224 x $10^{-4}$</td>
<td>major surface antigen P30 precursor [Toxoplasma gondii]</td>
<td>92</td>
<td>319aa length abundant protein, conserved in most strains, found both in the vesicular network of the parasitophorous vacuole and on the parasite surface, cell adhesion, proteolysis, receptor-ligand interactions</td>
</tr>
<tr>
<td>20</td>
<td>3.7</td>
<td>1.371 x $10^{-4}$</td>
<td>enolase 1 [Toxoplasma gondii ME49]</td>
<td>32</td>
<td>444aa length enzyme. Catalyze the ninth and penultimate step of glycolysis which is the conversion of 2-phosphoglycerate to phosphoenolpyruvate. Expressed preferentially in the bradyzoite stage.</td>
</tr>
<tr>
<td>40</td>
<td>2.8</td>
<td>0.004</td>
<td>protein disulfide isomerase [Toxoplasma gondii ME49]</td>
<td>148</td>
<td>471aa length enzyme in the endoplasmic reticulum in eukaryotes that catalyzes the formation and breakage of disulfide bonds between cysteine residues within proteins as they fold</td>
</tr>
<tr>
<td>97</td>
<td>0.56</td>
<td>0.003</td>
<td>calumenin [Homo sapiens]</td>
<td>188</td>
<td>315 aa length calcium-binding protein localized at the lumen of the ER, involved in ER functions, protein folding &amp; sorting</td>
</tr>
<tr>
<td>160</td>
<td>0.62</td>
<td>0.013</td>
<td>vimentin [Homo sapiens]</td>
<td>283</td>
<td>466aa length intermediate filamentous. Structural roles, cell cycle and mitosis, lipid metabolism</td>
</tr>
<tr>
<td>195</td>
<td>0.67</td>
<td>0.006</td>
<td>porin, partial [Homo sapiens]</td>
<td>89</td>
<td>Only on mitochondria in human, channel on membrane for passive diffusion of small metabolites like sugars, ions, and amino acids.</td>
</tr>
<tr>
<td>235</td>
<td>0.71</td>
<td>0.006</td>
<td>tropomyosin 1 alpha chain isoform 5 [Homo sapiens]</td>
<td>128</td>
<td>284aa length actin-binding proteins involved in the contractile system of striated and smooth muscles and the cytoskeleton of non-muscle cells</td>
</tr>
</tbody>
</table>
Table 7. 2D-DIGE results summary

<table>
<thead>
<tr>
<th></th>
<th>RH (Type I)</th>
<th>CTG (Type III)</th>
</tr>
</thead>
<tbody>
<tr>
<td># of proteins spots with significant</td>
<td>434</td>
<td>175</td>
</tr>
<tr>
<td>expression change</td>
<td></td>
<td></td>
</tr>
<tr>
<td># of Increased protein spots</td>
<td>247 (13.1)</td>
<td>45 (2.41)</td>
</tr>
<tr>
<td>(Fold change)</td>
<td></td>
<td></td>
</tr>
<tr>
<td># of decreased protein spots</td>
<td>187 (0.16)</td>
<td>127 (0.66)</td>
</tr>
<tr>
<td>(Fold change)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel overlay pictures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green-uninfected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red-infected</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 8. Comparison of materials and methods of past similar studies and my study

<table>
<thead>
<tr>
<th></th>
<th>Host cells</th>
<th>strain</th>
<th>Method</th>
<th>pH range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M.M. Nelson et al. (2008)</strong></td>
<td>In vitro</td>
<td>HFFs</td>
<td>RH 2D-DIGE</td>
<td>pH 4-7</td>
</tr>
<tr>
<td><strong>D.H. Zhou et al. (2011)</strong></td>
<td>In vivo (mouse)</td>
<td>Macrophage</td>
<td>RH Conventional 2D gel</td>
<td>pH 4-7</td>
</tr>
<tr>
<td><strong>D.H. Zhou et al. (2013)</strong></td>
<td>In vivo (mouse)</td>
<td>Brain cells</td>
<td>Pru Conventional 2D gel</td>
<td>pH 4-7</td>
</tr>
<tr>
<td><strong>This study</strong></td>
<td>In vitro</td>
<td>HFFs</td>
<td>RH CTG 2D-DIGE</td>
<td>pH 3-11</td>
</tr>
</tbody>
</table>

Table 9. Comparison of the results of past similar studies and my study

<table>
<thead>
<tr>
<th></th>
<th>Protein spots with expression changes (p&gt; 0.05)</th>
<th>Fold change threshold</th>
<th># of host proteins</th>
<th>Increased proteins (largest fold change)</th>
<th>Decreased proteins (largest fold change)</th>
<th>Modified proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M.M. Nelson et al. (2008)</strong></td>
<td>162</td>
<td>1.3</td>
<td>147</td>
<td>59 (37.2)</td>
<td>56 (0.075)</td>
<td>32</td>
</tr>
<tr>
<td><strong>D.H. Zhou et al.(2011)</strong></td>
<td>60</td>
<td>2</td>
<td>52</td>
<td>15 (N/A)</td>
<td>23 (N/A)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>D.H. Zhou et al.(2013)</strong></td>
<td>60</td>
<td>1.5</td>
<td>45</td>
<td>25 (∞)</td>
<td>20(∞)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>This study with RH</strong></td>
<td>434</td>
<td>1.2</td>
<td>N/A</td>
<td>247 (13.1)</td>
<td>187 (0.16)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>This study with CTG</strong></td>
<td>175</td>
<td>1.2</td>
<td>N/A</td>
<td>48 (2.41)</td>
<td>127 (0.66)</td>
<td>N/A</td>
</tr>
</tbody>
</table>
CHAPTER FOUR

CONCLUSION

This study successfully illustrated the virulence and growth rate difference between three distinct virulence phenotypes, RH from Type I, Me49 from type II, and CTG from type III with MOI determination experiments. The virulence of type II was expected to be between type I and type III. However, the results showed the slowest replication rate for type II strain, which contradict the facts we know about phenotypes of type I, II and III strains. The MOI experiments were done to determine the optimal MOI for 2D-DIGE analysis. However, results from these MOI experiments, highlighted the uniqueness in replication cycle in type II strains. To instigate this unique feature in type II, it would be useful to observe infected host cells every 3 - 4 hours for 48-72 hours to make a growth curve. The unique replication rate, exemplified by a lag in replication through the first 24h post-infection, may be related to its avirulence. Interestingly, the avirulent type III strain, did not exhibit this lag in growth indicating the mechanisms determining avirulence in type II and type III strains may be due to distinct mechanisms. This is an interesting and unexpected idea, that remains to be investigated further.

1D gel was a very efficient way to check the qualities of protein samples and confirmed that all quadruplicates of samples are consistent. Because of use of the two different stains, Pro-Q® Diamond Phosphoprotein Gel Stain and GelCode Blue Safe Protein Stain, both phosphorylated proteins and total protein expressions could be compared between uninfected and infected samples. In both phosphorylated and total protein band pattern on 1D gels, the most protein expression changes were found in RH
infected HFFs, less in ME49 infected sample, and no noticeable changes were found in CTG infected host. The 1D gel results were confirmed via 2D-DIGE analysis, where type I strains caused over 400 proteins to be modulated by infection as compared to only about 130 proteins were modulated by CTG strain.

2D-DIGE gels of *T. gondii* infected samples visually described dramatic differences between virulent and avilurent strains. The degree of expression level changes and the number of proteins expressed differently upon infections were distinct between two strains, RH and CTG. The identification of the host cell proteins differentially regulated by virulent type I vs. avirulent type III strain, will provide insight into understanding of parasite virulence for further research. For example, the preliminary data of RH strain infected HFFs at 24 hours p.i. identified two proteins, vimentin and calumenin, were differentially modulated by type I RH infection. These proteins were also found in past studies. Vimentin showed a decreased expression upon infection in this experiment and in the macrophage study, while up-regulation was found in HFFs host proteomic study by M.M. Nelson et al.. The protein is not only a structural protein, but functions in the cell cycle and mitosis, lipid metabolism, transportation system, and gene regulations by utilizing their spiral structure. Those functions were regulated by phosphorylation and de-phosphorylations. It was suggested that phosphorylation of the protein is closely related to physiological changes upon infection. Based on the results, vimentin would be an interesting protein to focus on for further study due to the wide range of functions and the possibility of high interaction with *T. gondii* and other infectious agents.
2D-DIGE analysis on RH infected and CTG infected samples were performed independently in this study, and a direct comparison of infected host cells of RH vs. CTG infected cells was done in this study. For further studies, analysis of the ME49 infected sample on 2D-DIGE could be done. Given the different rate of growth, collecting the Me49 sample at 36-48 hours p.i., where vacuoles contain comparable number of parasites as in the RH-infected cells at 24 hours p.i. could be normalize the differential rates of infections of type I vs. type II strain. Once all 2D-DIGE pictures are collected, quadruplicates of samples could be analyzed together on Progenesis to compare host responses between three different phenotypes. Due to differences in gel qualities, it might not be easy to compare the 2D-DIGE gel run between uninfected and infected cells directly, so running 2D-DIGE gel with only infected samples as a reference gel on Progenesis could be done. Progenesis is largely depended on gel qualities, so better quality gels result in more protein spots and better statistical data in each protein spots. When unique protein changes are seen between the three strains, these spots could represent the key host proteins related to virulence in *T. gondii*, and these would be the proteins preferentially identified. Also p-values calculated by Progenesis analysis is a good indicator to rank the priorities for identification. Protein spots with decreased protein expressions are surely HFF modified proteins, yet increased expression spots would be *T. gondii* proteins. Because of these reasons, uniquely commonly found proteins among three strains with decreased expression upon infection with lowest p-values would be prioritized to be identified. At the same time, the commonly found
protein spots with decreased expression and low p-value could be valuable to be identified to find core proteins which affected by any strains of *T. gondii*.

To promote protein identification process, running mass spectrometry with the 1D gel bands provides a rough idea of what kind of human proteins would be expected on 2D-DIGE. Since about 450 spots were found differentially expressed upon RH infection, it is not practical to identify every single protein spots. To eliminate *T. gondii* derived protein spots, it might be valuable to run another 2D-DIGE with infected sample and parasite lysate. Also, it could be helpful to find phosphorylated proteins by staining conventional 2D gel with Pro-Q diamond since it is known that kinases and phosphatase secreted from *T. gondii* somehow modify protein expression in host.

Lastly, in this study samples were collected at one time point for comparison, but it might be better to, collect host cells with vacuoles containing the same number of parasites within each vacuole. Thus for RH infected cells, samples would be collected at 18h post-infection while CTG which replicates slower could be collected at 24h p.i. with vacuoles in each containing 4-8 parasites. This might result in fewer differences on 2D-DIGE due simply to quantity of parasite proteins and allow host proteomic changes to *T. gondii* infection to be more easily seen. However, it is also expected that many host cell proteins differentially modified by virulent vs. avirulent strains, are reflective of pathways differentially modulated by parasite strains and not just simply a matter of parasite number. Thus protein identification of differentially regulated spots needs to be done to determine differential host proteomic response to virulent vs. avirulent strains.
REFERENCES CITED


