DEVELOPMENT OF DNA AND RECOMBINANT VACCINES AGAINST *TOXOPLASMA GONDII* INFECTION

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ABSTRACT

Toxoplasma gondii is an obligate intracellular protozoan parasite, infecting a broad range of warm-blooded hosts, including humans. *T. gondii* infection is a relapsing infection and causes encephalitis especially in immunocompromised patients. Miscarriage is another form of severe sequela resulting from primary *T. gondii* infection in pregnant women during early pregnancy. *T. gondii* infection occurs in livestock as well, contributing to great economic loss in the food industry. As such, it is essential to develop a vaccine to confer long-term protection from the infection. The *T. gondii* dense granule antigen 2 and 5 (GRA2 and GRA5) have been targeted in this study because these proteins are essential to the development of parasitophorous vacuole (PV), a specialized compartment formed within the infected host cell. PV is resistance to host cell endosomes and lysosomes thereby protecting the invaded parasite. In this study, recombinant GRA2 (rGRA2) and GRA5 (rGRA5) were produced in prokaryotic and eukaryotic expression systems and evaluated in serodiagnosis and vaccination tests. Gene fragments encoding GRA2 and GRA5 were amplified and cloned into pRSET B (prokaryotic) and pcDNA 3.1C (eukaryotic) expression vectors. Expression of recombinant GRAs-pRSET B was achieved in *Escherichia coli* BL21 (DE3) pLysS followed by purification through Probond^{TM} Purification System. Sensitivity and specificity of the purified rGRA2 and rGRA5 were assessed in western blot assays against *Toxoplasma*-infected human serum samples. Their sensitivity towards acute infection is 100% for rGRA2 and 46.8% for rGRA5 respectively. Almost similar sensitivity was obtained towards chronic infection (≈61%). rGRA2 and rGRA5 showed high specificity of 90% and 100% respectively when tested against *Toxoplasma*-negative human serum samples. Meanwhile, expression of recombinant GRAs-pcDNA 3.1C was attained in Chinese hamster ovary (CHO) cells. The recombinant vectors pcGRA2 and pcGRA5- cells produced antigenic proteins with the molecular weight in

transfected CHO cells. Both the *E. coli*-expressed subunit and DNA vaccines were evaluated against lethal challenge of the virulent *T. gondii* RH strain in BALB/c mice. rGRA2 and rGRA5 elicited humoral and cellular-mediated immunity in the mice. High level of IgG antibody was produced with the isotype IgG2a/IgG1 ratio of ≈0.87 ($p<0.001$). Significant increase ($p<0.05$) in the level of four cytokines (IFN-γ, IL-2, IL-4 and IL-10) was obtained. The antibody and cytokine results suggest that a mix mode of Th1/Th2-immunity was elicited with predominant Th1-immune response inducing partial protection against *T. gondii* infection. On the other hand, the DNA vaccines pcGRA2 and pcGRA5 elicited cellular-mediated immune response with significantly higher levels of IFN-γ, IL-2, IL-4 and IL-10 ($p<0.05$). A mix Th1/Th2-immunity was also obtained with predominantly Th1-immune response which slightly prolonged the survival days of the immunized BALB/c mice. In conclusion, GRA2 and GRA5 have been shown to be potential candidates for diagnostic and vaccine development against *T. gondii* infection.

ABSTRAK

Toxoplasma gondii merupakan parasit protozoa intrasel yang obligat dan menjangkiti pelbagai jenis hos berdarah panas, termasuk manusia. Jangkitan *T. gondii* ialah jangkitan berulang yang boleh menyebabkan ensefalitis, terutamanya dalam pesakit yang sistem imunnya terjejas. Keguguran sering berlaku di kalangan wanita mengandung yang pertama kali dijangkiti *T. gondii* waktu peringkat awal kehamilan mereka. Jangkitan *T. gondii* turut berlaku kepada ternakan, mengakibatkan kerugian ekonomi yang besar dalam industri pemakanan. Oleh demikian, usaha penghasilan vaksin amat penting untuk memberi perlindungan jangka panjang daripada jangkitan tersebut. Antigen Dense Granule 2 dan 5 (GRA2 dan GRA5) *T. gondii* disasarkan dalam kajian ini kerana antigen-antigen ini penting untuk pembangunan vakuol parasitoforus (PV), iaitu kompartmen khusus yang dibentuk dalam sel yang terjangkit. PV rintang terhadap endosom dan lisosom sel terjangkit, dengan itu melindungi parasit daripada lisis sel. Rekombinan GRA2 (rGRA2) dan GRA5 (rGRA5) telah dihasilkan dalam sistem pengekspresan prokariot dan eukariot dan dinilai dalam serodiagnosis dan kajian vaksinasi. Fragmen gen pengekodan GRA2 dan GRA5 telah diamplifikasikan dan diklonkan ke dalam pengkespresan vektor pRSET B (prokariot) dan pcDNA 3.1C (eukariot). Pengekspresan rekombinan GRAs-pRSET B telah dilakukan dalam *Escherichia coli* BL21 (DE3) pLysS diikuti dengan penulenan protein melalui Sistem Penulenan Probond™. Sensitiviti dan spesifikasi rGRA2 dan rGRA5 dinilai melalui analisis Western Blot terhadap sampel serum pesakit terjangkit dengan *Toxoplasma*. Sensitiviti rGRA2 dan rGRA5 terhadap jangkitan akut adalah 100% dan 46.8% masing-masing. Sensitiviti rGRA2 dan rGRA5 terhadap jangkitan kronik adalah lebih kurang sama (≈61%). rGRA2 dan rGRA5 memaparkan spesifikasi setinggi 90% dan 100% masing-masing terhadap sampel serum manusia yang bersifat negatif toxoplasmosis. Sementara itu, pengkekspresan rekombinan GRAs-pcDNA 3.1C dilakukan dalam sel-

sel mamalia CHO. Sel-sel CHO tertransfek dengan pcGRA2 dan pcGRA5 menghasilkan protein-protein bersifat antigenik dengan saiz jangkaan masing-masing. Kedua-dua jenis vaksin subunit dan vaksin DNA terhadap cabaran maut *T. gondii* tachyzoites berstrain RH dinilai dalam mencit BALB/c melalui kajian imunisasi. rGRA2 dan rGRA5 mencetuskan imuniti humoral dan selular. Tahap tinggi antibodi IgG telah dihasilkan dengan nisbah isotip IgG2a/IgG1 ≈0.87 ($p<0.001$). Pencirian sitokin menunjukkan peningkatan yang ketara dalam keempat-empat sitokin (IFN-γ, IL-2, IL-4 dan IL-10) ($p<0.05$). Profil antibodi dan sitokin yang diperolehi menunjukkan bahawa mod campuran imuniti Th1/Th2 tercetus dengan kehadiran predominan Th1 yang memberi perlindungan separa terhadap jangkitan *T. gondii*. Sementara itu, vaksin DNA pcGRA2 dan pcGRA5 mencetuskan tindak balas imuniti selular dengan tahap IFN-γ, IL-2, IL-4 dan IL-10 yang lebih tinggi ($p<0.05$). Campuran imuniti Th1/Th2 juga tercetus dengan kehadiran predominan imuniti Th1 yang memanjangkan hayat mencit BALB/c yang diimunisasi. Sebagai kesimpulan, keputusan kami menunjukkan bahawa GRA2 dan GRA5 merupakan calon-calon berpotensi untuk pembangunan diagnostik dan vaksin terhadap jangkitan *T. gondii*.

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There were times that I had almost come to the state of giving up my post-graduate study after years of ups and downs. However, I reminded myself with two quotes; 'what doesn't kill you makes you stronger' and 'when you feel like quitting, think about why you started' which eventually lead me to this point of thesis completion. All the best to those who are considering post-graduate study and thank you.

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LIST OF SYMBOLS AND ABBREVIATIONS

:	ratio
%	percent
pg	picogram
ng	nanogram
μg	microgram
mg	milligram
g	gram
μl	microliter
ml	milliliter
g/l	gram per liter
w/v	weight per volume
v/v	volume per volume
μM	micromolar
mM	millimolar
M	molar
nm	nanometer
μm	micrometer
cm^3	cubic centimeter
mm^3	cubic millimeter
˚C	degree Celsius
rpm	revolutions per minute
x g	gravitational force (gravity)
s	second
min	minute
h	hour

pmoles	picomoles
V	volt
bp	basepair
kDa	kilodalton
et al	et alia (and others)
ACN	acetonitrile
APS	ammonium persulphate
ConA	concanavalin A
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TEMED	N,N,N',N'-tetramethylenediamine
WB	western blot

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CHAPTER 1: INTRODUCTION

Toxoplasma gondii (*T. gondii*) is a ubiquitous and obligate intracellular protozoan parasite. It is capable of infecting a broad range of warm-blooded hosts (Dubey, 2010) causing a disease known as toxoplasmosis. In spite of the fact that toxoplasmosis is an old disease, it should not be neglected as it is still a common infection which is globally distributed affecting up to one-third of the world's human population (Jackson & Hutchison, 1989). It also poses danger especially to the AIDS patients and pregnant women where fatality and abortions can result, respectively. Two of the main aspects that play important roles in preventing or controlling the disease are diagnosis and vaccination.

Rapid diagnosis technique with high sensitivity, specificity and accuracy is required for the detection of *Toxoplasma* infection so that immediate and appropriate action, either early treatment or prevention (especially infection of fetus), can be done before worsening of the clinical conditions. Serological assay is the most commonly used diagnostic test. Such assays usually rely on *Toxoplasma* lysate antigens (TLAs) from tachyzoites propagated *in vivo* through peritoneal cavities of mice or *in vitro* cultures. However, there are several disadvantages pertaining to the usage of antigens originating from tachyzoites; high cost, time-consuming, inconsistent quality, batch-to-batch variations, contamination with host proteins or extra-parasitic components as well as exposing the staff to the harmful living parasites (Aubert *et al.*, 2000; Beghetto *et al.*, 2006; Gatkowska *et al.*, 2006; Golkar *et al.*, 2008). In order to overcome or to at least reduce these problems, recombinant DNA technology such as heterologous expression of *T. gondii* antigenic proteins in either prokaryotic or eukaryotic expression system can be an alternative. This technology contributes to the production of large quantity of recombinant antigens in a safer manner, with lower cost of production and purification

as well as reducing variation of quality, thereby enabling the development of a more specific and standardized serological assay (Holec-Gasior & Kur, 2010).

On the other hand, the available drugs alone are not reliable in treating the infection. Possibility of pathogen recrudescence, impossibility of parasites eradication from the infected host (Innes, 2010) and side effects caused by the available drugs greatly hinder the efficacy of drug treatments. This is when the development of an effective vaccine becomes one of the promising approaches for providing cost effective interventions to complement currently available control strategies for toxoplasmosis. Effective vaccines against toxoplasmosis are needed to stimulate the recipient's immune system for developing protective adaptive immunity to fight against the parasite. To date, there is only one vaccine available in the market which is for the prevention of toxoplasmosis in domestic animals especially goats and sheep, known as Toxovax. This vaccine is not widely acceptable for human use mainly due to the high possibility of regaining the parasite's pathogenicity (Chen *et al.*, 2009), side effects and high cost of production (Ismael *et al.*, 2003). However, production of safe recombinant vaccines (naked DNA or recombinant antigens) is made possible through recombinant DNA technology.

T. gondii infection begins when the parasites in the active stage of tachyzoites invade host cells, followed by uncontrolled replication and rupturing of the infected cells followed by dissemination of new parasites to invade the neighboring cells. These processes are mediated by various antigens originating from the tachyzoites which could be exploited as diagnosis and vaccination candidates. Two essential antigens have been selected as the target subjects, namely dense granular antigen (GRA) 2 and 5. GRA2 contributes to the formation of intravacuolar network in the parasitophorous vacuole (PV), allowing nutrients transportation to nourish the parasites while GRA5

helps to inhibit apoptosis of the infected cells thereby protecting the parasites during cell invasion.

Previous study had evaluated the immunoreactivities of rGRA2 and rGRA5 against *Toxoplasma*-infected patients' sera merely based on ELISA assay (Golkar *et al.*, 2007a; Holec-Gasior & Kur, 2010; Holec-Gasior *et al.*, 2009). Diagnosis evaluation of the same recombinant proteins through western blot assay has not been reported yet. On the other hand, several earlier studies had been conducted on the evaluation of multi-component vaccine candidate incorporating GRA2 or GRA5 with other potential genes against toxoplasmosis (Cao *et al.*, 2015; Igarashi *et al.*, 2008a; Liu *et al.*, 2009; Naserifar *et al.*, 2015; Xue *et al.*, 2008; Zhou *et al.*, 2007). However, limited number of study had been performed on these two target genes as single antigen vaccine especially GRA5. The only report on rGRA2 expressed in *E. coli* as single subunit vaccine candidate investigated its efficacy against chronic toxoplasmosis based on the *T. gondii* brain cysts counts (Golkar *et al.*, 2007b).

In this study, recombinant expression of GRA2 and GRA5 were carried out through prokaryotic (bacteria: *Escherichia coli*) system. Large quantity of the two proteins was produced in the bacteria expression system. Immunoreactivities of the proteins against toxoplasmosis-positive human serum samples were evaluated through western blot assay. Also, the antigens were subjected to mice immunization study as single antigen vaccine candidate in two forms; DNA plasmid vaccination and recombinant proteins against acute *T. gondii* infection in BALB/c mice. Results obtained indicated that the sensitivity of both recombinant GRA2 and GRA5 proteins towards acute infection is 100% and 46.8% respectively and shared almost similar sensitivity towards chronic infection (≈61%). They are also highly specific for the analysis of toxoplasmosis-negative human sera (90% and 100% respectively). On top of that, it was determined that these two target genes could actually trigger Th1/Th2-

immunity with predominant Th1-directed responses conferring partial protection against lethal challenge with *T. gondii* tachyzoites (acute infection) in BALB/c mice by prolonging the survival days.

Generally, study of the selected proteins was successfully carried out. The results and information gained from this study serve as a source of reference especially on the characteristics of the proteins. The findings of this study can contribute to the development of better serological diagnostic tools and vaccines against *Toxoplasma* infection.

1.1 Project objectives

The objectives of this study were to:

a) clone the GRA2 and GRA5 gene fragments into prokaryotic vector, pRSET B and eukaryotic vector, pcDNA 3.1C followed by recombinant protein expression using prokaryotic system (*Escherichia coli*) and eukaryotic system (CHO cells) respectively.

b) evaluate immunoreactivities of the recombinant proteins (rGRA2 and rGRA5) against human toxoplasmosis serum samples.

c) conduct immunization study using recombinant proteins (rGRA2 and rGRA5) and recombinant plasmids (pcGRA2 and pcGRA5) to characterize the immune responses elicited via IgG subclass, splenocytes proliferation, interferon-gamma (IFN-γ), interleukin 2 (IL-2), IL-4 and IL-10 assays.

d) determine protective properties of the recombinant proteins and plasmids in mice challenging assay.

CHAPTER 2: LITERATURE REVIEW

2.1 *Toxoplasma gondii* (*T. gondii)*

T. gondii is a member of Apicomplexan parasite (Mercier *et al.*, 2005) with a complex life cycle with two main stages: the sexual and asexual (Black & Boothroyd, 2000). It was discovered more than 100 years ago, in 1908 by Nicolle and Manceaux in a North African rodent known as *Ctenodactylus gundi* and at the same time by Splendore (Brazil) in rabbit tissues. The parasite derives its species name from *Ctenodactylus gundi*, the host from which it was isolated from. *Toxoplasma* comes from the Greek word; *toxon* means bow, indicating the crescent shape of the parasite and *plasma* represents life or creature (Black & Boothroyd, 2000; Ferguson, 2009; Lambert & Barragan, 2010; Weiss & Dubey, 2009). *T. gondii* is capable of infecting a broad range of warm-blooded hosts (Dubey, 2010) causing a disease known as toxoplasmosis. The first fatal case of toxoplasmosis (ocular) was reported in an 11 month-old baby in 1923 by Janku but *T. gondii* was only confirmed and accepted as a pathogen in 1939 when Wolf and colleagues identified it as a cause of human disease (Wolf *et al.*, 1939).

T. gondii is an obligate intracellular parasite which only survives and multiplies inside parasitophorous vacuole (PV) after host cell invasion. Zoites are responsible for the invasion, either within or between the hosts (Mercier *et al.*, 2005). The three infectious stages of *T. gondii* are the tachyzoites (free living), bradyzoites (encysted in tissue cysts) and sporozoites (contained in oocysts) (Dubey *et al.*, 1998).

Investigation of the clonal population structure of *T. gondii* indicated that there are actually very few strains worldwide. Isoenzyme, restriction fragment length polymorphism (RFLP) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses have been used to genotype *T. gondii* (Cristina *et al.*, 1995; Howe & Sibley, 1995). A study of 6 independent loci from *Toxoplasma* isolates through PCR-RFLP successfully categorized these isolates into three major

clonal lineages, namely type I, II and III which were mainly from Europe and North America. However, there are several isolates resulting from natural combination of type II with III and type I with III. The study also demonstrated that type I and II strains were closely related to congenital toxoplasmosis and reactivation of chronic infections (AIDS patients) in humans, respectively. Type III strains are common in animals (rodent, bear, deer, turkey, dove, pig) (Howe & Sibley, 1995).

Recently, sequencing analysis showed up to 12 haplogroups of the parasite including the previously identified three major lineages, type I, II and III. These newly discovered haplogroups are not completely homogenous (Robert-Gangneux & Darde, 2012).

Correlation between the genotypes and virulence of the parasite has been investigated using mouse model. Type I strains are highly virulent, while type II and III isolates are avirulent (Robert-Gangneux & Darde, 2012). Nevertheless, the display of virulence in human beings is usually much more complex compared to mice especially in immunocompromised hosts whereby similar strain of *T. gondii* may cause different clinical manifestations. This phenomenon can be clearly seen in three different genotyping studies, revealing high prevalence of type II in ocular toxoplasmosis (immunocompetent and immunocompromised patients) (Fekkar *et al.*, 2011) and congenital toxoplasmosis (Ajzenberg *et al.*, 2002) as well as acquired *T. gondii* infection among the immunocompromised patients (Ajzenberg *et al.*, 2009). Such complexity is mainly due to the immunity status of the patients (Maubon *et al.*, 2008).

2.2 Life cycle of *T. gondii*

The complete life cycle of the parasite was only known in the late 1960s, describing the sexual stage in the intestinal epithelial cells of a cat with the infectious

oocysts being excreted in the infected cat's feces. It was also concluded that *T. gondii* is a coccidian parasite (Bruna-Romero *et al.*, 2012; Hutchison *et al.*, 1969).

As previously mentioned, the parasite has a complex life cycle with alternating sexual and asexual phases. The definitive host is members of the cat family Felidae (domestic and wild). The parasite is capable of infecting any kind of nucleated cells of humans and a wide range of warm-blooded vertebrates (Dubey, 2010) as its intermediate hosts such as pigs, chickens, dogs, cattle, goats and sheep (Buxton, 1998; Chandrawathani *et al.*, 2008; Dubey *et al.*, 1993; Dubey & Urban, 1990).

The life cycle of *T. gondii* is illustrated in Figure 2.1. The sexual cycle begins and occurs only in the cat's intestine, when it consumes an infected rodent. Ingestion of tissue cysts (infected meat) results in the destruction of cyst wall by gastric enzymes thereby releasing bradyzoites followed by invasion into the intestinal epithelial cells. Within the epithelium, bradyzoites grow and differentiate through schizogony before undergoing gametogony to produce gametocytes. Fusion between macrogametes and motile microgametes leads to the formation of oocysts. The immature oocysts are then excreted in the cat feces.

The period between ingestion of the parasites and formation of oocysts in the cats' feces greatly depends on the stage of the parasites at which they are being ingested. It is short, usually about 3 to 10 days if cysts are ingested by cats from a chronic infection and will be longer, about 21 to 24 days if the infection is initiated with oocysts. Oocysts are as effective in producing a generalized infection as are cysts in other animals (David & William, 2006). Oocysts are ovoid in shape (Bhopale, 2003) and measuring approximately 9 to 11 µm in width and 11 to 14 µm in length (David & William, 2006). Upon excretion, they undergo sporogony to form mature and highly infectious oocysts in 1 to 5 days (Viqar & Loh, 1995). During sporogony, two

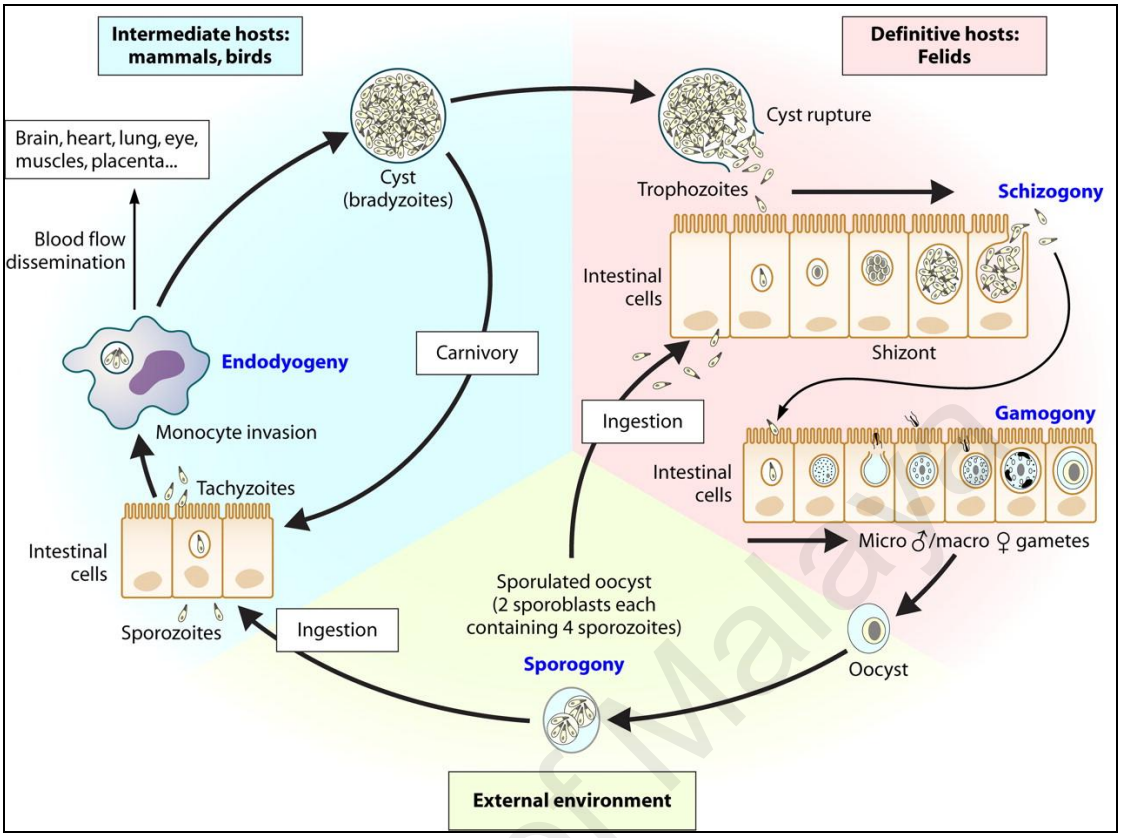

Figure 2.1: Life cycle of *T. gondii* [adapted from Robert-Gangneux & Darde (2012)]. The parasite has a complex life cycle with alternating sexual (felids) and asexual phases (warm-blooded vertebrates). Briefly, sexual phase begins with liberation of bradyzoites from the ruptured tissue cyst followed by schizogony, gametogony and ended with oocyst formation. On the other hand, asexual phase is initiated when the bradyzoites or sporozoites are differentiated into tachyzoites. Tachyzoites undergo endodyogeny in the infected host cells. The cycle ends with encystation of bradyzoites in the brain or muscle tissues.

sporoblasts are formed initially and transform into two sporocysts after cyst wall development. Each sporocyst contains four sporozoites. These mature oocysts are resistant to the environmental stress for more than a year until the ingestion by intermediate hosts. Besides, they are also resistant to acids, alkalis, and common laboratory detergents but can be killed by drying or heating up to 55℃ for 30 minutes (David & William, 2006).

Cats shed oocysts for about 2 weeks following a primary infection, but excrete fewer oocysts for a shorter period, or none at all on re-infection due to their developing immunity. Kittens that are fed with bradyzoites in cysts develop greatest immunity (measured by oocysts production) as compared to kittens fed with tachyzoites and sporozoites. Natural toxoplasmosis infection happens primarily through the consumption of bradyzoites in prey animals (David & William, 2006).

One the other hand, the asexual cycle of the parasite is initiated when either the mature sporulated oocysts or tissue cysts are ingested by intermediate hosts such as human through uptake of contaminated water or food (containing oocysts or tissue cysts). Both sporozoites and bradyzoites liberated from oocysts and tissue cysts respectively penetrate human intestinal epithelial cells. The zoites undergo differentiation to form tachyzoites which will invade monocyte cells before being circulated through blood stream throughout the whole body to infect other tissues such as brain, heart, lung, eye and muscles. Tachyzoites may also cross the placenta to infect the fetus if primary infection takes place during pregnancy.

Tachyzoites are crescentic in shape, varying in length from 4 to 6 μm and from 2 to 3 μm in breadth (David & William, 2006). Tachyzoite has a specialized structure with a collection of organelles in order to maintain its structural integrity, move around and recognize and attach to the host cells. This is followed by invasion via active penetration as well as forming a PV. The morphology of *T. gondii* tachyzoite is

illustrated in Figure 2.2. The tachyzoite is surrounded by plasma membrane and consists of a nucleus, mitochondrion, Golgi complex and endoplasmic reticulum (ER). It has an elongated shape with anterior and posterior pole. However, the three unique features of a tachyzoite are the inner membrane complex (IMC) just below plasma membrane, apical complex and apicoplast. An apicoplast is a chloroplast-like organelle attained by secondary endosymbiosis of an ancestral green alga (Kohler *et al.*, 1997; Striepen *et al.*, 2000) which is essential for the parasite's survival (Fichera & Roos, 1997).

An apical complex is made up of an apical polar ring, conoid and specific secretory organelles (Morrissette & Sibley, 2002). The apical complex is located at the anterior end of the tachyzoite (Figure 2.2). The apical polar ring is one of the microtubule-organizing centers (MTOC) for ensuring correct shape and polarity of the parasite by controlling the formation and organization of the cytoskeleton (Russell & Burns, 1984). The conoid is a small and hollow cone-like structure composed of tubulin polymer which plays an essential mechanical role in host cell invasion through protrusion and retraction from and into the apical polar ring respectively (Hu *et al.*, 2002; Scholtyseck *et al.*, 1970). The three types of secretory organelles are micronemes, rhoptries and dense granules (DG) (Mercier *et al.*, 2005; Morrissette & Sibley, 2002), and they are found within the cytoplasm (Nam, 2009). Micronemes are small and apical organelles with cigar-shaped, rhoptries are organelles with club-shaped, while dense granules are small organelles with round-shaped.

Various tissues, especially lung, heart, lymphoid organs and the cells of the central nervous system (CNS) may be parasitized. Multiplication of the tachyzoites by endodyogeny occurs within a host's cell brings about rupturing and death of the infected cell, especially after accumulation of 64 to 128 tachyzoites in each cell which

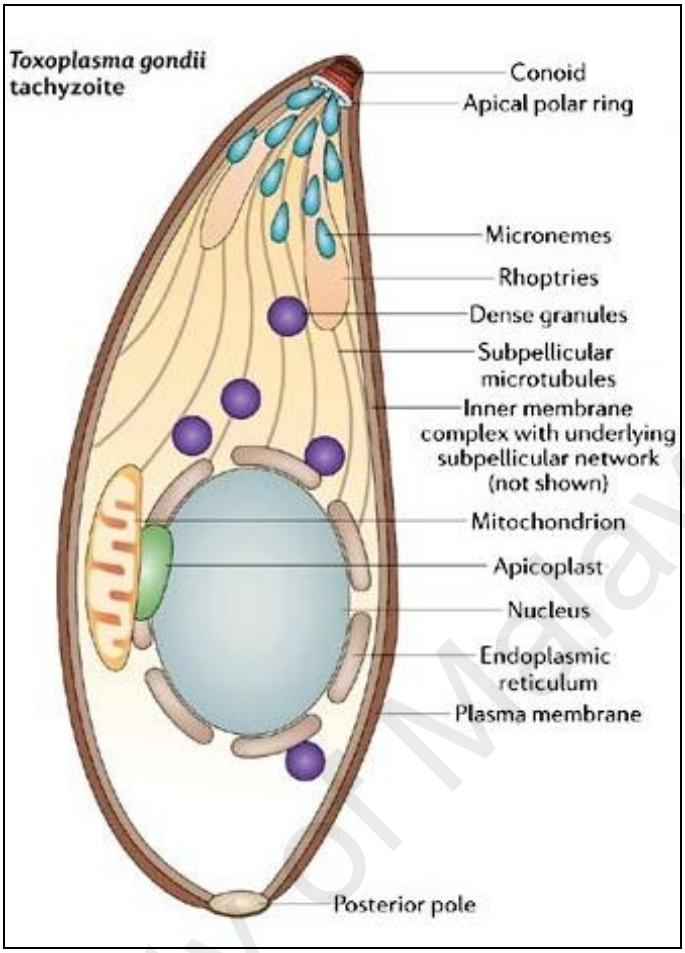

Figure 2.2: The morphology of *T. gondii* tachyzoite [adapted from Baum *et al.* (2006)]. Tachyzoite is surrounded by plasma membrane and inner membrane complex (IMC). It contains a nucleus, mitochondrion and endoplasmic reticulum (ER) as well as specialized apical complex and apicoplast. The apical complex composed of apical polar ring, conoid and secretory organelles (micronemes, rhoptries and dense granules).

takes about 6 to 8 hours (Radke & White, 1998), thereby freeing more tachyzoites to spread the infection to the neighboring cells. Endodyogeny is an asexual reproduction process that involves division of the mother cell through single internal budding resulting in the formation of two daughter cells.

The rapid-growing tachyzoites in the infected host will differentiate to the slower-growing bradyzoites forming tissue cysts especially in brain and muscle tissues. Each cyst encloses hundreds of bradyzoites and is retained for years. Such conversion will usually be seen about 10 to 14 days after infection, thereby ending the asexual stage (Bhopale, 2003; Black & Boothroyd, 2000; David & William, 2006; Lyons *et al.*, 2002). Tissue cysts can be killed either by freezing for at least 3 days at a temperature of -12 ℃ or lower or heating at 67 ℃ (Robert-Gangneux & Darde, 2012).

2.3 Host cell invasion by *T. gondii*

A complete host cell invasion by the parasite through active penetration is a very rapid process taking only approximately 25-40 seconds (Morisaki *et al.*, 1995). However, it requires several factors in order to achieve success in invasion. The most crucial factors are the parasite's calcium reservoir, motility, surface antigens (SAGs) and three specific secretory organelles. These three secretory organelles are the micronemes (MICs), rhoptries (ROPs) and dense granules (GRAs).

SAGs are found on the surface of the parasite and appear to be the first group of antigens interacting with the host cell surfaces before invasion. SAGs are the crucial attachment ligand for the host cell (Robinson *et al.*, 2004). Within the parasite, MICs, ROPs and GRAs release their respective proteins in a sequential manner during the invasion process (Carruthers & Sibley, 1997). Micronemes recognize host cell surfaces and drive the attachment of the parasite to the host cell (Huynh *et al.*, 2003). After the attachment, rhoptries will be liberated to participate in the formation of parasitophorous

vacuole (PV) during invasion (Ngo *et al.*, 2004). This is followed by the release of dense granular proteins into the PV formed during and after invasion. These antigens are involved in the maturation as well as modification of both PV and PV membrane within which the parasite survives and replicates (Nam, 2009). These proteins will remain in the PV in soluble form, associate with the PV membrane (PVM) or the intravacuolar network within PV (Mercier *et al.*, 2002).

T. gondii in non-feline hosts does not have structures like cilia or flagella for its locomotion, it moves around by gliding, depending on a microfilament system involving actin-myosin interactions (Dobrowolski *et al.*, 1997; Dobrowolski & Sibley, 1996). Host cell invasion by *T. gondii* is shown in Figure 2.3. When the parasite comes into contact with a host cell, it glides and re-orientates, so that the conoid is facing and touching the cell surface where the attachment process is driven by SAGs and proteins secreted from MICs. This is followed by the formation of a tight junction at the site of penetration which will move from the apical end towards posterior end of *T. gondii* as the parasite glides in to complete the invasion. Several MICs and rhoptry neck proteins (RONs) contribute in the tight junction development. At the same time, PV (tight-fitting vacuole) is formed from the invagination of host cell plasma membrane, enclosing the whole parasite. PV is capable of preventing acidification (Sibley *et al.*, 1985) and lysosome fusion (Jones & Hirsch, 1972) leading to the protection of the parasite. Resistant to endocytic fusion could be due to internalization of certain required host cell proteins during PV formation and their rapid removal from the mature PV when invasion is completed (de Carvalho & de Souza, 1989).

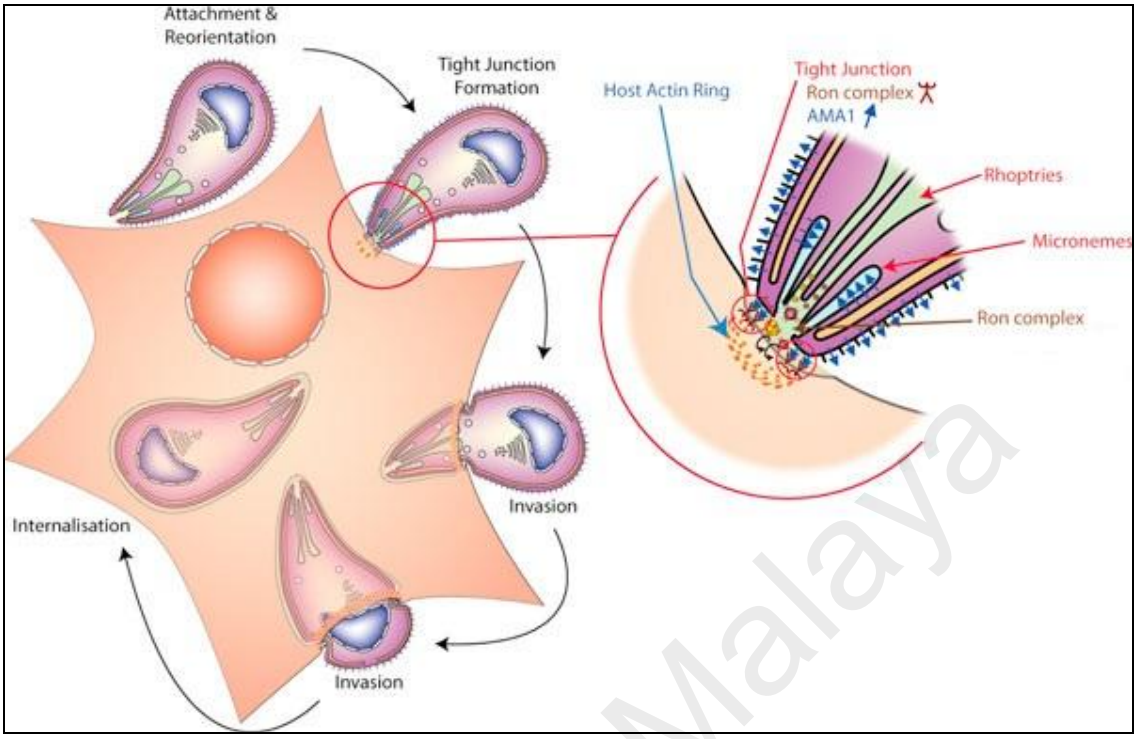

Figure 2.3: Host cell invasions by *T. gondii* [adapted from Gilson & Crabb (2009)]. *T. gondii* glides and re-orientates before attaching to the host cell surface. A tight junction is formed at the site of penetration, and it moves from the apical end towards posterior end of the parasite as it glides in. The invasion is completed when PV is formed via internalization.

Previous investigation indicated that calcium reservoir of the parasite is essential for host cell invasion where an increase of cytosolic calcium is necessary only for regulating or controlling parasite's motility and adhesins secretion especially MICs proteins but not to complete cell entry. Meanwhile, host cell calcium remains unaltered throughout the whole process (Lovett & Sibley, 2003). The invasion process does not interrupt or interfere host cell structure as membrane ruffling, cytoskeleton rearrangement and tyrosine phosphorylation of the host cell are not detected (Morisaki *et al.*, 1995).

2.4 Toxoplasmosis

Infection of *T. gondii* leads to a disease known as toxoplasmosis. Humans can become infected via horizontal or vertical transmission (Figure 2.4), the latter happens between mother and fetus. Transmission of the disease involves three different forms of the parasite; mature oocysts, tissue cysts (bradyzoites) and tachyzoites.

The most direct horizontal transmission is through contact with cats' feces contaminated with sporulated oocysts (Black, 2004). Feces from the infected cats may contaminate water and soil used in cultivation of crops such as vegetables and fruits. Humans and animals (herbivores, carnivores or omnivores) may get infected when they consume the contaminated water, vegetables and fruits containing the oocysts. Another possible source of infection for humans is associated with oyster consumption due to their filter-feeding activity where they concentrate *T. gondii* oocysts from seawater (Lindsay *et al.*, 2004).

Infection also occurs through transmission by means of tissue cysts consisting of bradyzoites by eating contaminated raw or undercooked meat, such as pork, mutton, beef and poultry. Study showed that populations consuming large amounts of steak

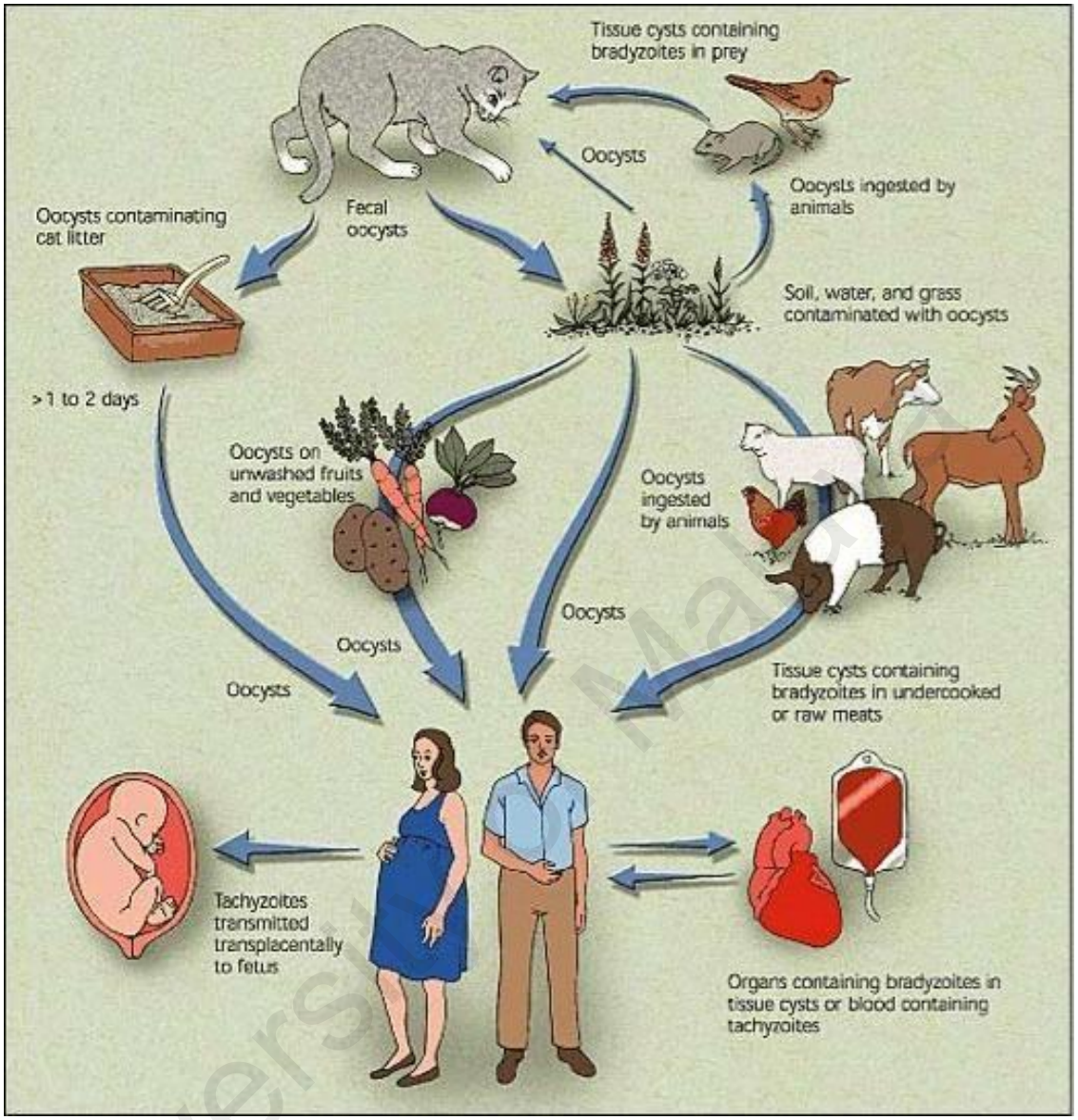

Figure 2.4: Mode of toxoplasmosis transmissions in humans [adapted from Jones *et al.* (2003)]. Humans get infected through ingestion of raw or undercooked meat containing tissue cysts with bradyzoites or water contaminated with oocysts from cat feces. Newborns or children are usually infected congenitally.

tartare (raw ground beef), especially among the French, have the highest incidence of infection in the world (Black, 2004).

Organ transplantation is another possible way of transmitting *T. gondii* infection, especially in heart transplant since bradyzoites encystment is more common in muscle tissue compared to other organs such as lung, liver and kidney (Robert-Gangneux & Darde, 2012). Infection by tachyzoites is also possible through infected blood transfusions or other body fluids, but with little importance in comparison with that by means of cysts and oocysts (David & William, 2006). Meanwhile, there are rare cases where humans especially personnel handling tachyzoites in the laboratory may get infected through exposure or accidental injection of the parasites.

Vertical transmission refers to transplacental infection or more commonly known as congenital toxoplasmosis. The disseminated tachyzoites within the mother's body cross the placenta to infect the fetus during pregnancy (Dubey, 1996). Experimental studies have indicated that infected animals are able to transmit tachyzoites to their suckling young through milk. Therefore, consuming raw milk from infected goats may be the cause of acute toxoplasmosis (David & William, 2006).

Acute toxoplasmosis is often correlated with intracellular growth of the rapidly replicating tachyzoites, causing the death of infected host cell by bursting or rupturing to liberate more tachyzoites to continue invading neighboring cells (Bhopale, 2003). Chronic toxoplasmosis is related to the formation of tissue cysts containing bradyzoites which occurs as the parasites responses to the development of humoral immune response of the infected host (Lyons *et al.*, 2002). Tissue cysts are found predominantly in the brain and skeletal muscle of the host. The cysts will not trigger any inflammation but remain dormant throughout the entire life of the host (Black & Boothroyd, 2000). Encystation of bradyzoites protects them from being detected by the host's immune system.

2.5 Pathogenesis and immunity against *T. gondii* infection

Dissemination of the pathogenic *T. gondii* to mesenteric lymph nodes as well as other distant organs and tissues of the infected host occurs through lymphatics and blood circulation. Invasion, intracellular multiplication and finally disruption of these infected cells by tachyzoites are responsible for creating focal area of necrosis (surrounded by lymphocytes, monocytes and plasma cells, which arise from the death of the infected cells) since the parasites do not produce toxins (Dubey, 1996).

Active infection of *T. gondii* may persist longer in the CNS, including the eye. Retinochoroiditis is caused by either hypersensitivity response to cyst rupture or a chronic progressive effect of the tachyzoites proliferation in the retina, an immunologically deficient tissue. Active retinochoroiditis is characterized by an inflammatory process consisting of a zonal granuloma with intense central necrosis. It is surrounded by successive layers of lymphocytes, macrophages, plasma cells and sometimes epithelioid cells. Plasma cells secrete antibodies to destroy the parasites in the extracellular space besides inducing cyst formation. The retinal pigment epithelial cells proliferate from the epithelium behave as phagocytes (Tabbara, 1995).

Toxoplasmosis infection is often benign in patients with strong protective immunity as the presence of extracellular antibody and intracellular T-cell factors often trigger the conversion of tachyzoites into dormant bradyzoites. Endogenous interferon gamma is another important mediator of host resistance to the infection. The weak immunity of immunosuppressed individuals such as AIDS patients and newborns/children infected congenitally will eventually succumb to the disease. Acute infection acquired by the mother at different stages of pregnancy determines the severity of congenital toxoplasmosis (Black & Boothroyd, 2000).

The key element involved in the success of fighting against *T. gondii* infection is the ability of the infected host to trigger T helper-1 (Th1) cellular mediated immune

response by production of pro-inflammatory cytokines such as interleukin-12 (IL-12), tumor necrosis factor-alpha (TNF-α) and most importantly interferon-gamma (IFN-γ) through a series of immunological pathways of the innate immunity. However, overwhelming production of such cytokines leads to severe inflammation at the infected sites causing severe tissue damages which may cause fatality of susceptible host. Therefore, anti-inflammatory cytokines including interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β) will be secreted at the same time to achieve equilibrium.

Generally, *T. gondii* invasion of monocyte cells in intestinal lamina propria induces chemokine secretion, attracting phagocytic cells to the infected area, thereby awakening the innate immune response of the infected host including dendritic cells (DC), macrophages and neutrophils. DC and macrophages appear to be the most crucial cell populations of innate immunity as they are capable of presenting parasite antigens for T cell priming. Two major roles of DC as an antigen presenting cell (APC) and IL-12–producing activator of Th1 immunity are clearly demonstrated (Reis e Sousa *et al.*, 1997). Another study has also reported on the priming of $CD8^+$ T cells against *T. gondii* by the infected DC (Dzierszinski *et al.*, 2007). $CD4^+$ T lymphocytes secrete IL-2 and IFN-γ for the development of $CD8^+$ lymphocytes which in turn produce more IFN-γ and exert protection through cytotoxic killing of the infected cells (Bhopale, 2003; Montoya *et al.*, 1996). DC and macrophages also stimulate activation of natural killer (NK) cells by secreting IL-12 and IL-18, leading to IFN-γ secretion (French *et al.*, 2006). A recent review describes that different sets of IFN-γ-producing cells participate in host protection against different stages of *Toxoplasma* infection; NK and $CD4^+$ T cells against acute infection whereas $CD8^+$ T and $CD4^+$ T cells against chronic infection (Yarovinsky, 2014). Activated macrophages produce TNF-α (Robert-Gangneux & Darde, 2012). Collectively, IFN-γ and TNF-α further regulate the killing of parasite by

the macrophages (Gazzinelli *et al.*, 1996a). Neutrophils are involved in phagocytosis of parasites and production of IFN-γ, their important role in fighting against intracellular pathogens have been studied (Bliss *et al.*, 2001; Sturge *et al.*, 2013).

Suzuki and colleagues reported the importance of IFN-γ as a major mediator of resistance against acute *T. gondii* infection in mice model. It was reported that IFN-γ has the ability to activate macrophages to inhibit the growth or kill the parasite (Suzuki *et al.*, 1988). Besides being an activator, IFN-γ also appears as an inducer triggering the expression of indoleamine 2,3-dioxygenase (IDO) enzyme in the infected host, catalyzing the degradation of tryptophan to N-formylkynurenine (Pfefferkorn, 1984; Pfefferkorn *et al.*, 1986). The growth of parasite is then restricted without the supply of that particular essential amino acid. These two IFN-γ-mediated mechanisms of growth suppression and elimination of *T. gondii* are more likely to happen in humans.

2.6 Symptoms

Toxoplasmosis may exhibit different clinical presentations or manifestations ranging from asymptomatic, mild and severe symptoms, depending on the category of hosts being infected. The four most common categories are the immunocompetent patients, immunocompromised patients, pregnant women and animals.

2.6.1 *T. gondii* infection in immunocompetent patients

T. gondii infection in immunocompetent patient (adults and children) is usually asymptomatic, self-limiting or developing mild illness during the first few weeks (Chen *et al.*, 2009). This is because of the protection of the patient's own immune system, where the proliferation of tachyzoites is controlled, leading to bradyzoite (dormant) stage formation (Golkar *et al.*, 2007a; Holec-Gasior *et al.*, 2009). Life-long immune

protection will be induced as well against toxoplasmosis re-infection (Daryani *et al.*, 2003).

Most commonly, clinical presentation is the inflammation and enlargement of lymph node, known as lymphadenopathy which is associated with extreme fatigue, low-grade fever, headache and myalgia (Ismael *et al.*, 2003). Enlargement of lymph node lasts from 4 to 6 weeks in cervical lymphadenopathy and may prolong for months in chronic lymphadenopathy (Montoya & Liesenfeld, 2004). More severe but rare symptoms such as pneumonitis, polymyositis and myocarditis may be presented as well (Robert-Gangneux & Darde, 2012). Several studies have also pointed out that such infection may contribute to neurological and pshychiatric symptoms even though it is generally thought to be asymptomatic in immunocompetent patients (Gulinello *et al.*, 2010).

2.6.2 *T. gondii* infection in immunocompromised patients

When *T. gondii* infection occurs in patients with severe immunosuppression, it can be very serious. Patients in this category include those on prolonged immunosuppressive therapies or patients suffering from acquired immune deficiency syndrome (AIDS), neoplastic disease, Hodgkin's disease, bone marrow or organ transplant recipients (Chen *et al.*, 2009; Holec-Gasior *et al.*, 2009).

AIDS patients' cellular immunity is suppressed due to the depletion of the helper T ($CD4^+$) lymphocytes. This may lead to reactivation of chronic toxoplasmosis, especially when the $CD4^+$ T lymphocyte counts drop to less than 100/µl. Reactivation of chronic infection in such patients can cause intracerebral focal lesions resulting in toxoplasmic encephalitis (Mamidi *et al.*, 2002; Wong & Remington, 1993) with symptoms such as headache, hallucination, drowsiness, hemiparesis, reflex changes and convulsion. It may also cause the patients to go into coma state or cause fatality if it is

not treated at the early stage of infection (Dubey, 1996; Luft & Remington, 1992). Conversion of the dormant bradyzoite stage to the active and rapidly replicating tachyzoite stage explains the medical condition of toxoplasmic encephalitis (Weiss & Kim, 2000). Tachyzoites are therefore found predominantly in immunocompromised patients. Approximately 10-50% seropositive AIDS patients develop toxoplasmic encephalitis (Wong *et al.*, 1995). An estimated 10% and 30% of AIDS patients succumb to *Toxoplasma* infection in USA and Europe respectively (Luft & Remington, 1992). Other than toxoplasmic encephalitis, AIDS patients may develop toxoplasmic orchitis, toxoplasmic myocarditis, spinal cord toxoplasmosis as well as pulmonary toxoplasmosis.

Besides AIDS patients, organ transplant recipients may also experience severe clinical outcome from toxoplasmosis. Thus, screening of potential organ donors for toxoplasmosis is essential prior to organ transplantation to avoid transmission of the disease to the recipients. Acute disseminated *Toxoplasma* infection in the recipient, may affect many other organs including the transplanted organ. Toxoplasmosis in the immunocompromised patients exhibits several other severe symptoms, such as hepatitis, splenomegaly, dermatomyosis, pneumonitis, myocarditis and multisystem organ failure (Ismael *et al.*, 2003).

2.6.3 *T. gondii* infection in pregnant women

Another high risk group of people who exhibit severe clinical conditions when infected with *T. gondii* is pregnant women. Placenta is one of the target sites for *T. gondii* invasion and infection can trigger inflammation and necrosis (Robert-Gangneux & Darde, 2012). The parasites are able to cross the placenta barrier of the infected mother to infect the developing fetus (Black, 2004). Transplacental transmission usually occurs in the course of an acute but unapparent or undiagnosed maternal infection.

Toxoplasmosis acquired during pregnancy of first trimester may be responsible for stillbirths and spontaneous abortions (Black, 2004). However, intrauterine infections of infants occurring in the last trimester are usually normal during birth but may later on develop retinochoroiditis with 20% are symptomatic. Besides through congenital infection, toxoplasmic retinochoroiditis may be acquired postnatally as well but it is somehow difficult to identify the origin of infection (Montoya & Liesenfeld, 2004). Congenital infection is relapsing and is able to cause serious congenital defects in the newborns, including the accumulation of cerebrospinal fluid (hydrocephalus) (Figure 2.5), abnormally small head (microcephaly), convulsions, intracerebral calcifications, pneumonitis, hepatosplenomegaly, severe cognitive impairment, disorders of movement, severe neonatal malformations, neurological and ocular complications, and other fetal abnormalities (Black, 2004; Martin *et al.*, 2004). Hydrocephalus and ocular disease are the most dramatic damage and common sequela of congenital toxoplasmosis respectively (Dubey, 1996). However, only half of the infected newborns show symptoms at birth, whereas severe symptoms may occur at the age of three months to early adulthood, especially blindness and mental retardation. Similar symptoms are seen if the infection happens after birth, but with less severity than those in fetuses.

A recent study indicated a possible relation between toxoplasmosis and schizophrenia where more than 50% of schizophrenics and their mothers tested positive for toxoplasmosis, a value far more than the general population does. Schizophrenia has a definite biological basis. If a person with no history of schizophrenia receives a unit of blood from a person exhibiting schizophrenia, the recipient will also exhibit symptoms for several hours (Black, 2004). It has also been proven that toxoplasmosis infection can actually alter one's behavior as has been observed in infected mice which lose fear of cats (Vyas *et al.*, 2007).

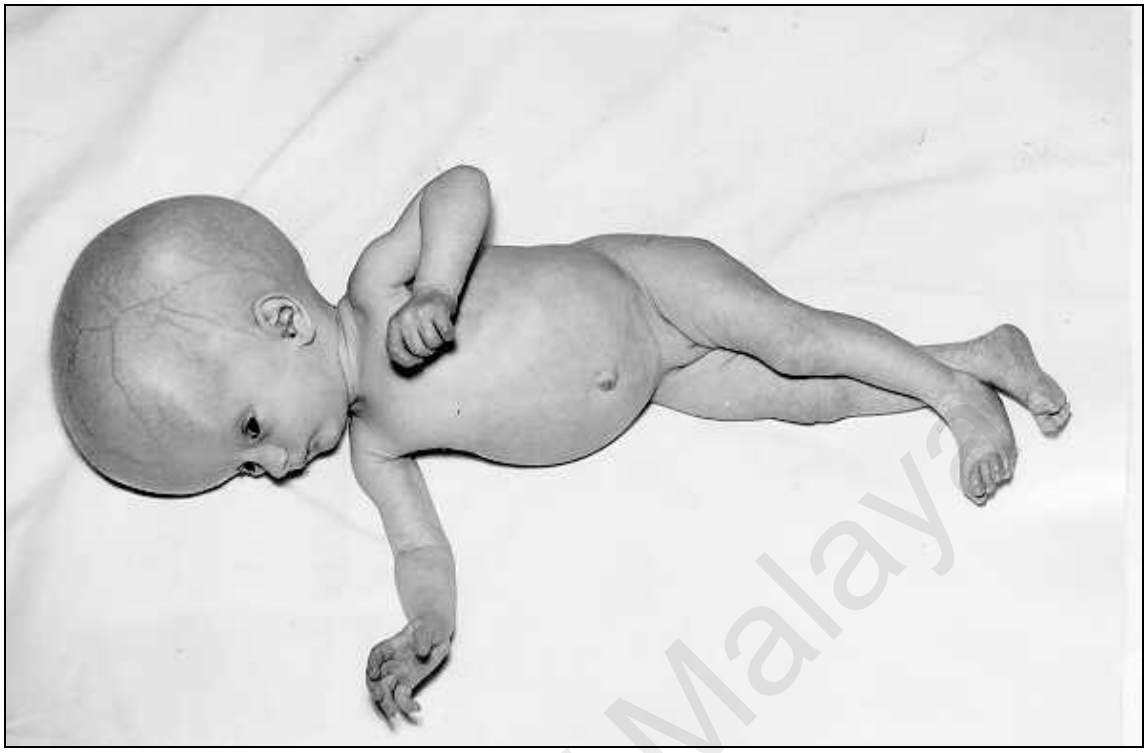

Figure 2.5: Baby girl with hydrocephalus [adapted from Dubey & Beattie (1988)]. It is a medical condition of an abnormal accumulation of cerebrospinal fluid (CSF) in the cavities of the brain as a result from congenital toxoplasmosis.

2.6.4 *T. gondii* infection in animals

Besides being a dreadful threat to human's health, toxoplasmosis is also of veterinary and economic importance. *T. gondii* infection in livestock contributes to abortions, stillbirth and neonatal loss, especially when the infection occurs during pregnancy in sheep and goats, leading to great economic losses in livestock and food industry (Buxton, 1998). Infected livestock harboring parasite tissue cysts may transmit the parasite to human through undercooked meat consumption. Toxoplasmosis in dogs is often correlated with concurrent distemper virus infection which causes fatality due to pneumonia, hepatitis and encephalitis. Certain species of marsupials and New World monkeys are prone to *T. gondii* infection (Dubey, 1996).

2.7 Epidemiology

Toxoplasmosis is a worldwide disease with 30-50% human population being infected chronically (Tenter *et al.*, 2000). However, less than 0.1% of the infection is via congenital transmission (Dubey, 1996). Congenital infection may re-occur in rodents without external sources but not in immunocompetent mothers as they can protect their infants from successive congenital transmission.

Prevalence of human toxoplasmosis differs across the countries worldwide, ranging from 10-80%. Three ranges of seroprevalence have been noted: 10-30% in North America (low), North Europe and South East Asia (middle), 30-50% in Central and South Europe, 60-80% in Latin America (high) (Robert-Gangneux & Darde, 2012). Seroprevalence in Malaysia fall into the range of 20-30% (Nissapatorn *et al.*, 2002).

In Malaysia, the prevalence of the disease in goats, cats, dogs and cattle were 35.5%, 14.6%, 9.6% and 6.3% respectively. However, anti-*T. gondii* antibodies are not found in pigs (Chandrawathani *et al.*, 2008). In other countries, the seroprevalences of *T. gondii* infections in chickens and domestic sheep were found to be 6.9% and 15.1%

respectively (Alvarado-Esquivel *et al.*, 2011a; Alvarado-Esquivel *et al.*, 2011b). In China, seroprevalence in pet dogs as well as household and stray cats is 10.8% as well as 15.6% and 45.2% respectively (Wu *et al.*, 2011a; Wu *et al.*, 2011b).

Excretions of millions of resistant oocysts by domestic cats cause widespread natural infection. Possible factors contributing to the increased risk and rate of infection are environmental conditions, individual lifestyles (such as eating habit, hygiene and cooking style), sosio-economic factors, animal species and the presence of invertebrates. Oocysts are more viable in warm and humid environment, thus countries with such weather will have a much higher rate of toxoplasmosis (Robert-Gangneux & Darde, 2012). Invertebrates such as flies, cockroaches and earthworms may act as 'transporters', aiding in dispersion of the oocysts.

2.8 Diagnosis of toxoplasmosis

Toxoplasmosis is one of the most common parasitic infections in humans especially in countries with temperate climate. Patients infected with toxoplasmosis often developed unspecific symptoms which are indistinguishable from other infectious diseases especially those showing similar symptoms. As a result, diagnostic tests which are simple, rapid, accurate, reliable and affordable are needed to confirm the disease so that proper treatments can be given to the patients to prevent worsening of the medical conditions.

Laboratory diagnosis methods for toxoplasmosis include histologic examination (finding the parasites in the blood, CSF or tissues), isolation of the parasite from biopsy tissue and blood/body fluids of the infected patients followed by inoculation into peritoneal cavities of mice (*in vivo*) or tissue cultures (*in vitro*), serological assays (antibody detection) and polymerase chain reaction (PCR; specific gene detection) (Liu

et al., 2015; Montoya, 2002). These techniques can be applied in combination for confirmation purposes.

For example, several techniques employed to diagnose prenatal congenital infection include detection of IgM antibodies in the fetus serum, parasite isolation from fetal blood or amniotic fluid by animal or cell culture inoculation, ultrasound scanning of the fetus for enlargement of cerebral ventricles and *Toxoplasma* specific gene sequence detection through PCR (David & William, 2006). Infection is usually shown by a significant increase in the specific anti-*Toxoplasma* IgG titres between acute and convalescent patient's serum (Viqar & Loh, 1995).

2.8.1 Histologic examination of biopsy specimens

Diagnosis can be done through examinations of biopsy specimens, for example infected lymph node. Detection of the tachyzoites is necessary to prove active toxoplasmosis within the tissue biopsy specimens as the tissue cysts may reside there for years. A peroxidase-antiperoxidase method which enables localization of the *T. gondii* is thus useful.

2.8.2 Isolation of *Toxoplasma* parasite

This method is less commonly applied due to the high cost, time-consuming as well as hazard of handling live parasites. The infected specimens from the patients such as bone marrow, lymph gland, blood or CSF are inoculated either into the laboratory mice intraperitoneally or cell cultures (Montoya, 2002). The infected mice are then tested two to three weeks later for anti-*Toxoplasma* antibodies. If positive results are obtained, the respective mice can be sacrificed for examination of the brains (tissue cysts) and peritoneal cavities (tachyzoites).

2.8.3 Serological assays

Serological tests for the diagnosis of *T. gondii* infection include Sabin-Feldman dye test (SF), immunofluorescence test (IF), indirect haemagglutination test (IHA), complement fixation test (CF) and enzyme-linked immunosorbent assay (ELISA). Upon infection, IgM will be detected much earlier than IgG, within the first week of infection and may persist in the infected patient from months to two years after acute infection (Viqar & Loh, 1995). IgG is produced in 1 to 2 weeks post-infection and remains for a lifetime (Montoya & Liesenfeld, 2004).

2.8.3.1 Sabin-Feldman dye test (SF)

Sabin-Feldman dye test is the gold standard test for toxoplasmosis diagnosis. It is based on the cytoplasmic lysis of the parasites when they are exposed to anti-*Toxoplasma* antibodies in the infected patient's serum. Positive results indicate either previous infection or active disease but not necessarily a current infection. The disadvantages of this dye test include the requirement for live parasites harvested from the peritoneal fluid of infected mice, high cost and also difficulties in standardization (Reiter-Owona *et al.*, 1999).

2.8.3.2 Immunofluorescence test (IF)

Immunofluorescence test is a sensitive and safer test compared to the SF dye test as it does not involve live parasites. There is no passive transport of antibody IgM from the mother to fetus, IF test can therefore diagnose *Toxoplasma* infection in any newborn using fluorescein-labelled anti-IgM. High IgM titre but low titre of indirect haemagglutination reaction suggests acute infection in patients with acquired toxoplasmosis.

2.8.3.3 Indirect haemagglutination test (IHA)

Indirect haemagglutination test is a very sensitive test but it needs a longer time to show positive compared to SF and IF. The positive reaction obtained can remain for many years, therefore IHA is more suitable and useful for antibody surveys compared to diagnosis of acute toxoplasmosis.

2.8.3.4 Complement fixation test (CF)

Complement fixation test also requires a long time to show positive, usually one month after infection. Therefore, it is unable to detect acute toxoplasmosis. The positive reaction will start to decline and disappear eventually after several years. Although CF test requires less cost, it is time consuming, has low sensitivity and often not specific.

2.8.3.5 Enzyme-linked immunosorbent assay (ELISA)

Generally, the abovementioned tests such as SF, IF, IHA and CF are not easily automated for large-scale screening. However, ELISA can be automated for large scale screening (Wisdom, 1976). Other advantages of ELISA include good sensitivity, ease of performance, safe and low cost per reaction due to the need of small volume of specimens (Ruitenberg *et al.*, 1977a; Ruitenberg *et al.*, 1977b). ELISA is the commonly used assay in most clinical laboratories.

Currently, there are many commercial ELISA kits available. The two types of IgM assays are the double-sandwich IgM enzyme-linked immunosorbent assay (DS-IgM-ELISA) and IgM immunosorbent assay (IgM-ISA). Due to its persistence up to years in the infected patient, IgM no longer serves as appropriate indicator of recently acquired infection. However, negative result of IgM detection somehow plays an important role in ruling out the possibility of acute infection (Montoya & Liesenfeld, 2004). IgG avidity assay which measures the binding affinity between IgG from the

infected serum samples with corresponding antigens is a better indicator of acute toxoplasmosis in which low avidity indicates acute while high avidity indicates chronic infection (Hedman *et al.*, 1989).

Serological examinations are not useful in cases of immunosuppressed patients such as AIDS patients as there will not be any significant increase in their antibody titres. Thus, other approaches should be carried out such as histological diagnosis of the parasites in the body fluids or tissues. Direct agglutination study using *Toxoplasma* tachyzoites seems to be more sensitive in AIDS patients as compared to other tests. Molecular direct detection can be an alternative method of diagnosis.

2.8.4 Molecular direct detection

Several techniques discussed earlier are either time-consuming or require the presence of specific anti-*Toxoplasma* antibodies and are therefore inefficient in cases of pregnant women and their developing fetus as well as AIDS patients. Delayed production of antibodies in infected pregnant women especially during recent infection may show negative results with serological assays at a particular check-up moment. AIDS patients with suppressed immune system may also fail to develop detectable antibodies. Molecular detection assay has thus appeared as alternative to overcome the abovementioned problems.

Several PCR amplification techniques have been applied for different types of body fluids and tissues such as amniotic fluid, blood, CSF and aqueous humor. The 35-fold repetitive B1 gene is a good target for PCR to detect different genotypes of *T. gondii*. Nested PCR and real-time quantitative PCR of B1 gene had been reported (Burg *et al.*, 1989; Jones *et al.*, 2000; Lebech *et al.*, 1992; Lin *et al.*, 2000). Despite the advantages such as high sensitivity and rapidity, PCR does have several disadvantages

such as high contamination risk, problems in shipping and storage method of reagents as well as high cost especially in real-time PCR.

2.9 Treatment

Treatment for toxoplasmosis infections can be problematic due to the variation of the parasites in terms of their virulence. Nevertheless, infected patients are commonly treated with pyrimethamine, sulphadiazine and spiramycin (during pregnancy) even though these drugs are unable to eliminate the parasites from the patients completely (Hill & Dubey, 2002; Montoya & Liesenfeld, 2004).

Drugs combination of pyrimethamine-sulphadiazine/other sulphonamides helps to kill the parasites by acting together to inhibit the production of dihydrofolate reductase, an enzyme required for DNA, RNA and protein synthesis in the parasites (David & William, 2006). Patients treated with pyrimethamine are usually given leucovorin (folinic acid) as the drug is a folic acid antagonist which may depress the bone marrow, resulting in thrombocytopenia, leucopenia and anemia (Viqar & Loh, 1995). On the other hand, spiramycin is a macrolide antibiotic derived from *Streptomyces ambodaciens*. This antibiotic is mainly used in continental Europe for treating *Toxoplasma* infected pregnant women since spiramycin is not teratogenic (Montoya & Remington, 2008).

2.9.1 Treatment for immunocompetent patients

Treatment for immunocompetent patient is not required unless severe symptoms occur. A combination of pyrimethamine, sulfadiazine and leucovorin (folinic acid) for a period of 4 to 6 weeks' time is usually recommended (Montoya & Liesenfeld, 2004).

2.9.2 Treatment for immunocompromised patients

Infected organ transplantation recipients may be given trimethoprim-sulfamethoxazole. This drug combination has been demonstrated as an alternative to the commonly administrated combination of pyrimethamine, sulfadiazine and leucovorin in treating toxoplasmic encephalitis (TE) in AIDS patients (Torre *et al.*, 1998). Clindamycin can be used in place of sulfadiazine especially in patients who are intolerance to sulfonamides. A combination of atovaquone-pyrimethamine or atovaquone-sulfadiazine can be used to overcome sulfonamides or pyrimethamine intolerance respectively (Chirgwin *et al.*, 2002).

2.9.3 Treatment for pregnant women

Infected pregnant mother during the first and early of second trimester of gestation is advised to follow a regime of consuming spiramycin until about 4 months of (18^{th} weeks) of pregnancy, followed by pyrimethamine plus sulfadiazine for a month especially when the PCR result of the amniotic fluid appears to be negative (Remington *et al.*, 2006). Spiramycin has been shown to reduce the risk of transplacental transmission (Couvreur *et al.*, 1988b). However, it does not cross the placenta (Montoya & Remington, 2008). Pyrimethamine-sulfadiazine-leucovorin is given to infected pregnant women during second and third trimester of gestation (after 18^{th} weeks) with positive PCR result of amniotic fluid indicating high risk of infection of the developing fetus (Remington *et al.*, 2006). Meanwhile, patients with retinochoroiditis are either given pyrimethamine-sulfadiazine and prednisone or clindamycin/trimethoprim-sulfamethoxazole.

2.10 Prevention and control

Prevention of toxoplasmosis infection is necessary especially when the efficacy of the available drugs for the treatment of such parasitic infection is still uncertain. Since modes of transmissions of the disease are known, prevention and control can be easily carried out.

A cat may shed up to 100 million oocysts per day in its feces and may become infectious in 1 to 5 days. Therefore, human, especially pregnant women should avoid contact with cat feces. Someone other than the pregnant woman should change the cat's litter pan daily and disinfect the pan with boiling water to prevent accumulation of infectious oocysts. Cleaning should be done with disposable gloves. Pregnant women are advised to avoid direct contact with stray cats. Hands are to be washed thoroughly after touching any cats. Pet cats should be given dry, canned and cooked food instead of letting them hunt for food especially rodents.

Infectious oocysts may be found in soil and water as well; therefore hand washing is essential especially after outdoor activities. Disposable gloves are also needed during gardening. Habit of drinking tap water should be prevented. Washing of vegetables and fruits are necessary before consumption. Oysters should not be taken raw. Stringent pest control especially against rodents should be carried out to prevent the spread of toxoplasmosis to cats and others animals. Public health awareness campaign and education on the risks and dangers of *Toxoplasma* infection should be carried out.

Proper handling and cooking of the meat is another crucial precaution. Thorough washing and cleaning of hands or any cooking utensils which have contact with the uncooked meat should be carried out with water and soap as such practice is able to kill the parasites (Dubey & Beattie, 1988). Meat can be frozen to -12˚C or heated up to 67˚C before consumption in order to kill the tissue cysts.

2.11 Vaccination approaches

Interconversion between tachyzoites and bradyzoites is a reversible process. Suppressed level of nitric oxide, T lymphocytes, IFN-γ, IL-12 and TNF-α especially in the immunocompromised patients will cause reactivation of *T. gondii* infection through rupture of tissue cysts, releasing bradyzoites which will then convert into active tachyzoites (Lyons *et al.*, 2002) and vice versa. The phenomenon of disease reactivation, toxic effects and possibility of emerging drug resistance in parasites makes drug treatment unreliable for long term (Bhopale, 2003; Kur *et al.*, 2009). As a result, there is a need to develop vaccines against toxoplasmosis which are capable of eliciting similar to the immune response triggered during natural *Toxoplasma* infection. Vaccines are thought to be safer, environmental friendly and are able to confer life-long protection to the recipients against either primary infection (during pregnancy), reactivation (immunocompromised patients) or re-infection at any time of their life (Kur *et al.*, 2009). Different types of vaccines had been experimented involving live attenuated whole parasites, soluble total lysate antigens of the parasite, recombinant purified proteins, recombinant plasmid DNA and recombinant live vectors (bacterial or viral).

2.11.1 Live attenuated whole parasite vaccine

The first commercialized and licensed live attenuated *T. gondii* vaccine, known as Tovovax, is a tissue culture grown vaccine utilizing live and incomplete strain of S48 tachyzoite in sheep (Buxton, 1993; O'Connell *et al.*, 1988; Wilkins *et al.*, 1988). S48 was initially isolated from the fetal membranes of an aborted lamb in New Zealand, and recovered from the peritoneal fluid of the mice at second passage. It then lost its ability to induce the formation of tissue cysts and oocysts after undergoing more than 3000 passages in mice. It was reported that S48 replicated in the lymph node nearest to the site of injection and caused feverish response when injected into sheep which were

initially seronegative for *T. gondii* S48 strain. The antibody titres of the injected sheep peaked at the 6^{th} weeks and dropped by the 20^{th} weeks. The protection conferred by the vaccine lasted up to 18 months even after single jab (Buxton, 1993) through the induction of $CD4^+$ and $CD8^+$ T lymphocytes which produces IFN-γ (Buxton & Innes, 1995).

However, this vaccine is not widely accepted and not used in humans mainly due to the high possibility of regaining the parasite's pathogenicity, side effects, high cost of production and short shelf life (2 to 3 weeks only) (Buxton, 1993; Chen *et al.*, 2009; Ismael *et al.*, 2003; Kur *et al.*, 2009). The need for developing safer vaccines has thus encouraged more efforts in identifying immunogenic and immunoprotective antigens of the parasite. These vaccination candidates can be produced in a safer manner through recombinant DNA technology giving rise to protein- or DNA-based vaccines. Nevertheless, selection of potential antigens is difficult as there are many vaccine candidates, different forms or stages of the parasite, and different parasite strains (Bruna-Romero *et al.*, 2012).

2.11.2 Protein-based vaccine

Three basic concerns ought to be taken into consideration in the vaccination development strategy: determination of immunogenic and immunoprotective antigens, induction of strong protective antigen-specific immune responses and combinations of antigens.

Development of protein-based vaccine involves antigen production in either prokaryotic (bacteria) or eukaryotic (yeast) expression systems. They are basically safer and more specific in boosting the immune response of the recipients by presenting only selected immunogenic antigens instead of the whole parasite (Schaap *et al.*, 2007).

One of the common routes of injection of the purified recombinant protein is via subcutaneous tissue. Upon injection, the proteins will most likely be taken up by circulating antigen presenting cell (APC) such as macrophage. The proteins will be processed into peptide-MHC class II complex within APC before being presented on the cell surface to $CD4^+$ helper T cells, stimulating humoral-mediated immunity (Th2) resulting in antibody production. Difficulties in generating Th1 immunity can be overcome by formulating the recombinant proteins with appropriate adjuvants as they play important role in directing the desired Th1/Th2 profiles (Bruna-Romero *et al.*, 2012; Kur *et al.*, 2009). For example, formulation of alum (Th2 inducer) and IL-12 (Th1 inducer) result in a strong Th1 activity (Schaap *et al.*, 2007). Other adjuvants that are commonly used in subcutaneous injection are Freund's complete adjuvant (CFA), Freund's incomplete adjuvant (FIA), liposomes and IL-12.

2.11.3 DNA-based vaccine

There are several advantages in developing DNA-based vaccine. First and foremost, DNA vaccination is a novel modality for immunization and is promising because it can induce long-lasting protective immunity. Secondly, DNA-based vaccine allows expressions of antigens encoded by the gene fragments inside the cells of vaccine recipients. Proper expression and post-translational modifications yield antigens that are similar to the native antigens (Encke *et al.*, 1999). Thirdly, manipulation of coding sequence can be done easily through recombinant DNA technology in which regions coding for different epitopes of interest are selected and combine in the plasmid DNA. Furthermore, plasmid DNA is thermostable and can be easily stored and shipped. At the same time, it can be generated in large quantity with much lower cost (Encke *et al.*, 1999).

Plasmid DNA encoding the protein of interest is taken up by muscle cells in intramuscular injection. Protein expression is based on the host-cell machinery. Expressed protein will be degraded by host proteases into smaller peptides before being transported into endoplasmic reticulum and binding to major histocompatibility complex (MHC) class I molecules followed by presentation on the cell surface for recognition by $CD8^+$ cytotoxic T cells thereby inducing cellular-mediated immunity. Some of the expressed proteins may be delivered out from the muscle cell via exocytosis and taken up by APC such as macrophage. The protein will be processed into peptide-MHC class II complex within APC before being presented on cell surface to $CD4^+$ helper T cells, stimulating humoral-mediated immunity (Corr & Tighe, 1997). Plasmid DNA vaccine elicits the same mechanism of immune responses triggered when the host cells are exposed and infected by *T. gondii*. Therefore, it is crucial to choose potential vaccine candidates based on their immunogenicity and roles or functions in the pathogenicity of the parasite.

2.12 Antigenic proteins of *T. gondii*

Basically, selection of potential vaccine candidates should be directed to the antigens that are involved in host cell invasion and parasite survival, especially the excreted/secreted antigens (ESA) as they are most likely responsible for triggering host immunity during natural *Toxoplasma* infection. Four major antigenic protein groups of *T. gondii* are the surface antigens (SAGs), micronemes (MICs), rhoptries (ROPs) and dense granules (GRAs).

2.12.1 Surface antigen (SAG)

There are five families of surface antigens, namely SAG1, SAG2, SAG3, SAG4 and SAG5. Among this, SAG1 is a superfamily, having a total of 20 homologous

proteins. SAG1 and SAG2 are distantly related. SAG1 which is expressed only on tachyzoites (Lyons *et al.*, 2002) has always been a vaccine candidate for toxoplasmosis (Couvreur *et al.*, 1988a; Khan *et al.*, 1991). Protective immune response elicited by DNA vaccines encoding SAG1 and 14-3-3 in BALB/c mice model has been reported (Meng *et al.*, 2012). Elevated levels of IgG2a subclass and gamma-interferon were demonstrated, indicating Th1 type immune response which partially protected vaccinated mice against *T. gondii*. Meanwhile, pSAG1/14-3-3-immunized mice showed longer survival time than both pSAG1- or p14-3-3-immunized mice, thus multi-antigenic DNA vaccine gave better protection than single gene vaccine.

2.12.2 Microneme (MIC)

Discharge of micronemal proteins is associated with successful host cell invasion by the parasite. Inhibition of the discharge is capable of preventing the parasite from attaching to the host cell (Carruthers *et al.*, 1999). So far, 11 microneme proteins have been investigated through phage-display technology, namely MIC1 (Lourenco *et al.*, 2001), MIC2 (Wan *et al.*, 1997), MIC2-associated protein (M2AP) (Rabenau *et al.*, 2001), MIC4 (Brecht *et al.*, 2001), MIC6 (Reiss *et al.*, 2001), MIC7, MIC8, MIC9 (Meissner *et al.*, 2002), MIC10 (Hoff *et al.*, 2001), MIC11 (Harper *et al.*, 2004) and AMA1 (apical membrane antigen 1) (Donahue *et al.*, 2000). A recent study determined the protective effect of DNA vaccine encoding MIC11 against acute toxoplasmosis in mice model (Tao *et al.*, 2013), demonstrating that MIC11 is immunogenic and capable of inducing both humoral (high levels of anti-TLA antibodies were detected) and cell-mediated immunity (increased production of IFN-γ, IL-12, and IL-2). The vaccinated mice had long survival time against acute toxoplasmosis.

2.12.3 Rhoptry (ROP)

Generally there are 8 to 16 rhoptries that are passed on to the daughter cells during multiplication. Rhoptry proteins 1-8 play major roles in host cell invasion and formation of PV, namely ROP1, 2, 4, 6, 8, 16, 17 and 18. ROP2 is the prototype of a large protein family. The ROP2 family of proteins (ROP2, ROP3, ROP4, ROP8, and ROP18) was originally identified by cross-reacting monoclonal antibodies (MAbs) produced against a rhoptry enriched fraction of tachyzoites (Sadak *et al.*, 1988). ROP1 is expressed in tachyzoites and is secreted during the early stage of invasion (Bradley & Boothroyd, 2001) and it plays an important role in host cell penetration. ROP8 is a member of ROP2 family (Sadak *et al.*, 1988) and is expressed in tachyzoites and bradyzoites (Bhopale, 2003; Vercammen *et al.*, 2000). ROP16 has been tested for protection in mice against *T. gondii* infection via DNA immunization (Yuan *et al.*, 2011a). It was shown that IFN-γ and IL-2 were produced in much larger quantity whereas low levels of IL-4 and IL-10 were generated but still significantly higher than the control mice ($p<0.05$). It was postulated that both Th1- and Th2-type immune responses were stimulated to protect the vaccinated mice against parasite (virulent RH strain) challenge. The survival time was prolonged up to 21 days compared to 7 days in negative control mice.

2.12.4 Dense granule (GRA)

A total of 12 GRA proteins (GRA1-10, GRA12 and GRA14) with molecular weight range of 21 to 41 kDa have been identified (Ahn *et al.*, 2005; Cesbron-Delauw, 1994; Mercier *et al.*, 2005; Michelin *et al.*, 2008; Rome *et al.*, 2008). GRAs are the major components of both vacuole surrounding tachyzoites and encysted bradyzoites (Capron & Dessaint, 1988; Cesbron-Delauw & Capron, 1993). Some of the GRAs are important to ensure and maintain survival of the parasite after host cell invasion as they

are involved in the formation of the intravacuolar network (Labruyere *et al.*, 1999; Nam, 2009) in PV which allows proteins and nutrients transportation from the invaded host cell (Nam, 2009) as well as regulating intracellular calcium concentration to inhibit apoptosis (Ahn *et al.*, 2006; Feng *et al.*, 2002). GRAs have been identified as potential vaccines (Hiszczynska-Sawicka *et al.*, 2011; Scorza *et al.*, 2003; Sun *et al.*, 2011) and diagnosis candidates (Ching *et al.*, 2013; Golkar *et al.*, 2007a; Jacobs *et al.*, 1999; Redlich & Muller, 1998). Efficacy of pcDNA3.1-HisGRA6 as a DNA vaccine candidate was determined (Sun *et al.*, 2011). Both humoral and cell-mediated immune responses ($CD8^+$ T cell response) were induced, giving partial protection to challenged mice by significantly increasing the survival rate by 53% and 40% with and without adjuvant respectively.

2.12.4.1 GRA2

Dense granule antigen 2 (GRA2) is a 28 kDa hydrophobic protein containing the N-terminal hydrophobic signal peptide, which enables secretion into the PV post-invasion of the infected host cell (Nam, 2009). Within the PV, GRA2 tends to form a multimeric complex with both GRA4 and GRA6 (Labruyere *et al.*, 1999), followed by association with the intravacuolar network due to the present of two internal amphipathic alpha-helices of GRA2 (Bittame *et al.*, 2015; Nam, 2009). Formation of such network in the PV allows proteins and nutrients transportation and further support the survival of the parasites (Nam, 2009). Generally, GRA2 is not a stage-specific antigen but is expressed throughout the whole intermediate host life cycle of *T. gondii* instead (Zhou *et al.*, 2007) making it an interesting target subject of study as it would not lead to stage-limited protection against toxoplasmosis. It has been reported that GRA2 contributes to the virulence of *T. gondii* acute infection in mice (Mercier *et al.*,

1998) and is highly immunogenic during infection in both human as well as experimental models (Golkar *et al.*, 2007a; Murray *et al.*, 1993).

Previous studies had shown that recombinant GRA2 antigen is a good marker for acute infection as it has higher sensitivity (90-100%) towards *Toxoplasma*-infected serum samples compared to other GRAs recombinant antigens (80-90%) through ELISA assay (Beghetto *et al.*, 2003; Ferrandiz *et al.*, 2004; Golkar *et al.*, 2007a; Holec-Gasior *et al.*, 2009; Jacobs *et al.*, 1999; Lecordier *et al.*, 2000; Murray *et al.*, 1993; Redlich & Muller, 1998). On the other hand, it was also reported that GRA2 had induced specific $CD4^+$ T cells with long term immune response against *T. gondii* in chronically infected humans (Prigione *et al.*, 2000). This was supported by another study which demonstrated the significant reduction of brain cysts formation observed in the vaccinated mice (Golkar *et al.*, 2007b). As such, it would be interesting to assess the immunoreactivity of GRA2 through another technique such as WB as well as evaluating the immunoprotectivity against *T. gondii* acute infection.

2.12.4.2 GRA5

Dense granule antigen 5 (GRA5) is a 21 kDa hydrophobic protein consisting of a N-terminal hydrophobic signal peptide and a hydrophobic transmembrane domain (Nam, 2009). GRA5 appears in both soluble and hydrophobic forms (Lecordier *et al.*, 1999). It is secreted into the PV as a soluble form during host cell invasion (Lecordier *et al.*, 1993) followed by transmembrane insertion into the PVM with its N-terminal projecting into the host cell cytoplasm while the C-terminus remains in the vacuole lumen (Lecordier *et al.*, 1999). A yeast two-hybrid analysis with GRA5 (Ahn *et al.*, 2006) showed that this antigen bound to calcium modulating ligand (CALMG) for regulation of intracellular calcium concentration which helped to inhibit apoptosis (Feng *et al.*, 2002) and further allowed long-term survival of *T. gondii*. Besides playing an

important role in host cell invasion, maintenance of the PV and long-term survival of the parasite, GRA5 also exists in all life stages of the parasite (Tilley *et al.*, 1997) which is identical to GRA2.

Although GRA5 did not contribute to the virulence of *T. gondii* acute infection (Mercier *et al.*, 2001), but it was previously shown that GRA5 in combination with other antigens could assist in reducing brain cyst formation in the vaccinated mice (Igarashi *et al.*, 2008a) and also enhanced the sensitivity and specificity of the antigen cocktails in the serodiagnosis study (Holec-Gasior & Kur, 2010). Therefore, GRA5 will be investigated individually in both serodiagnosis and vaccination against lethal tachyzoites challenge study hoping to retrieve more information on its characteristics and roles played in the parasitic infection.

2.13 Expression system

Rapid development of recombinant DNA technology together with the increasing demand for therapeutic recombinant protein production (Jana & Deb, 2005) are encouraging the development of various molecular cloning vectors for heterologous protein productions through either prokaryotic or eukaryotic expression system (Fuerst *et al.*, 1986). These host cell systems have advantages and disadvantages. Several factors have to be considered before choosing an expression system in order to obtain high-level expression. These include the aim of production, physical, chemical properties and biological activity of the proteins of interest, preference for intracellular or extracellular protein expression, preference for post-translational modifications of the protein produced, cell growth characteristics, cost of production, ease of usage and safety level of the system (Jana & Deb, 2005; Yin *et al.*, 2007).

2.13.1 Prokaryotic expression system: Bacteria (*Escherichia coli*)

The gram-negative bacteria *E. coli* has been the top choice for heterologous protein expression due to the multiple advantages it can offer (Baneyx, 1999).

The fast growing *E. coli* is easy to manage and control which makes the heterologous protein production possible to be scaled up. Expression usually can be achieved within one day (Yin *et al.*, 2007). Economical cost of protein production is made possible with this system as the amount of protein produced is much higher compared to other systems. Availability of various cloning vectors and mutant expression host strains allow manipulation of the system for high-level recombinant production (Baneyx, 1999; Hewitt & McDonnell, 2004). Recombinant insulin and bovine growth hormone are examples of success in using this expression system (Jana & Deb, 2005).

Despite of the advantages *E. coli* possess, there are still some limitations especially when expressing eukaryotic proteins. *E. coli* does not have post-translational modification mechanisms such as protein folding, glycosylation, proteolytic processing and secretion out of the cells (Fuerst *et al.*, 1986). Without these modifications, eukaryotic proteins expressed may not be functional or may show different characteristics.

Some genes are not suitable for expression in *E. coli*. Expression depends on structural features of the gene sequence, stability and translational efficiency of mRNA generated as well as the existence of rare codons which inhibit the expression in *E. coli*. Problems such as toxicity of the proteins should be taken into considerations as well (Jana & Deb, 2005).

In fact, *E. coli* has been vastly used as an expression host for the production of recombinant *Toxoplasma* antigens especially for evaluation as potential candidates in both the study of diagnosis (Hiszczynska-Sawicka *et al.*, 2003; Holec-Gasior *et al.*,

2012; Holec *et al.*, 2007; Sonaimuthu *et al.*, 2014) and vaccination (Dziadek *et al.*, 2011; Petersen *et al.*, 1998; Wang *et al.*, 2013; Zheng *et al.*, 2013).

2.13.2 Eukaryotic expression system: Mammalian cells (CHO)

Besides prokaryotic system, heterologous protein expression of the foreign genes could be performed in mammalian cells employing eukaryotic system as well. In contrast to the bacterial expression system, the latter possess highest level of post-translational modification activity producing precisely folded proteins mimicking the native form. However, the protein yields are very much lower with higher production cost (Kaufman, 2000; Yin *et al.*, 2007). As such, mammalian cells are less commonly chosen especially for large scale protein expression compared to bacterial cells unless native form is required for further study.

In spite of the disadvantages it offer, mammalian cells such as CHO cells do play an important role in numerous studies such as the expression of *Toxoplasma* recombinant protein (Kim *et al.*, 1994), functional validation of *Toxoplasma* recombinant DNA plasmid (Abdizadeh *et al.*, 2015; Parthasarathy *et al.*, 2013) as well as *T. gondii* parasitic infection for its growth investigation (Ihara & Nishikawa, 2014).

In this study, CHO cell was involved to validate the expression of target protein directed by the transfected recombinant DNA construct prior to DNA vaccination study, in other words to ensure the DNA plasmid is functional. Such validation requires transient expression whereby protein expression only persists over a limited time. Three major factors determining the expression efficiency of a particular gene are the plasmid copy number, expression level of the target gene and the rate of successful transfection (Kaufman, 2000).

CHAPTER 3: MATERIALS AND METHODS

3.1 Overview

Two major approaches that were utilised in this research project were the productions of recombinant proteins (rGRA2 and rGRA5) and recombinant DNA plasmids (pcGRA2 and pcGRA5) prior to the evaluation of their protective efficacy as vaccination candidates against *T. gondii* infection in mice model.

Briefly, genes encoding *T. gondii* dense granular antigens GRA2 and GRA5 were amplified and cloned into two sets of expression vectors: pRSET B (prokaryotic system), and pcDNA 3.1C (eukaryotic system), respectively. Both the pRSET B-GRA2 and pRSET B-GRA5 constructs were subjected to protein expression through *E. coli* BL21 pLysS (DE3) strain as the prokaryotic host followed by affinity purification. The purified recombinant proteins were then evaluated on their immunoreactivities against *Toxoplasma*-infected human sera via western blot analysis and their protective potential against *T. gondii* infection via immunization and tachyzoites challenging studies involving mice model.

On the other hand, positive recombinant pcDNA 3.1C-GRA2 and pcDNA 3.1C-GRA5 constructs were propagated in *E. coli* TOP10F'. DNA plasmids purification was performed and their concentration and purity were determined. The extracted recombinant DNA plasmids were transfected into eukaryotic cell culture to confirm the expression of the encoded proteins before being injected into mice model for the protective study.

Purified total proteins of pRSET B and plasmid of pcDNA 3.1C were employed as the negative controls for the respective experiments. The overall workflows were depicted in Figures 3.1 and 3.2.

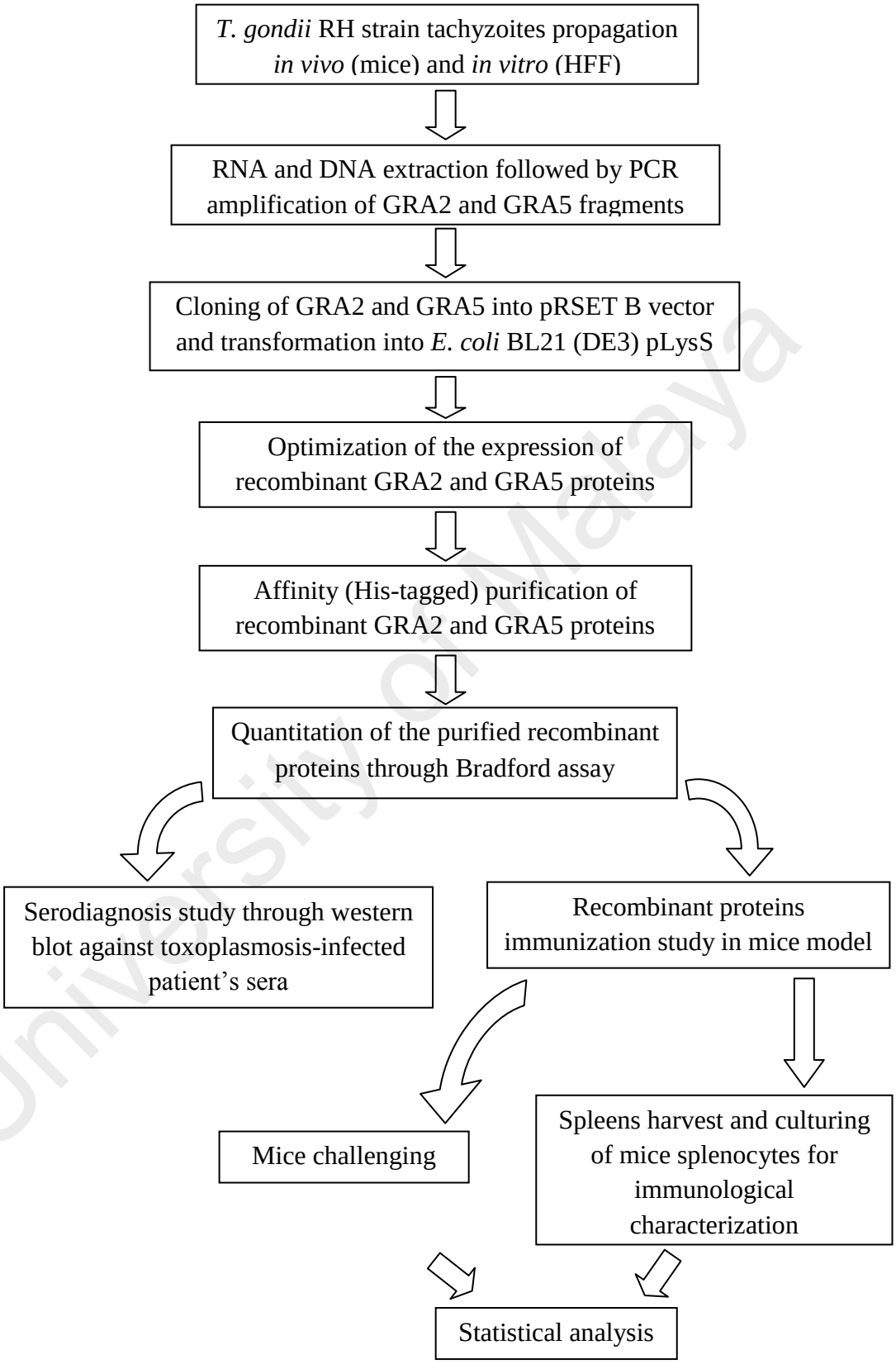

Figure 3.1: Overall approaches in recombinant proteins immunization study

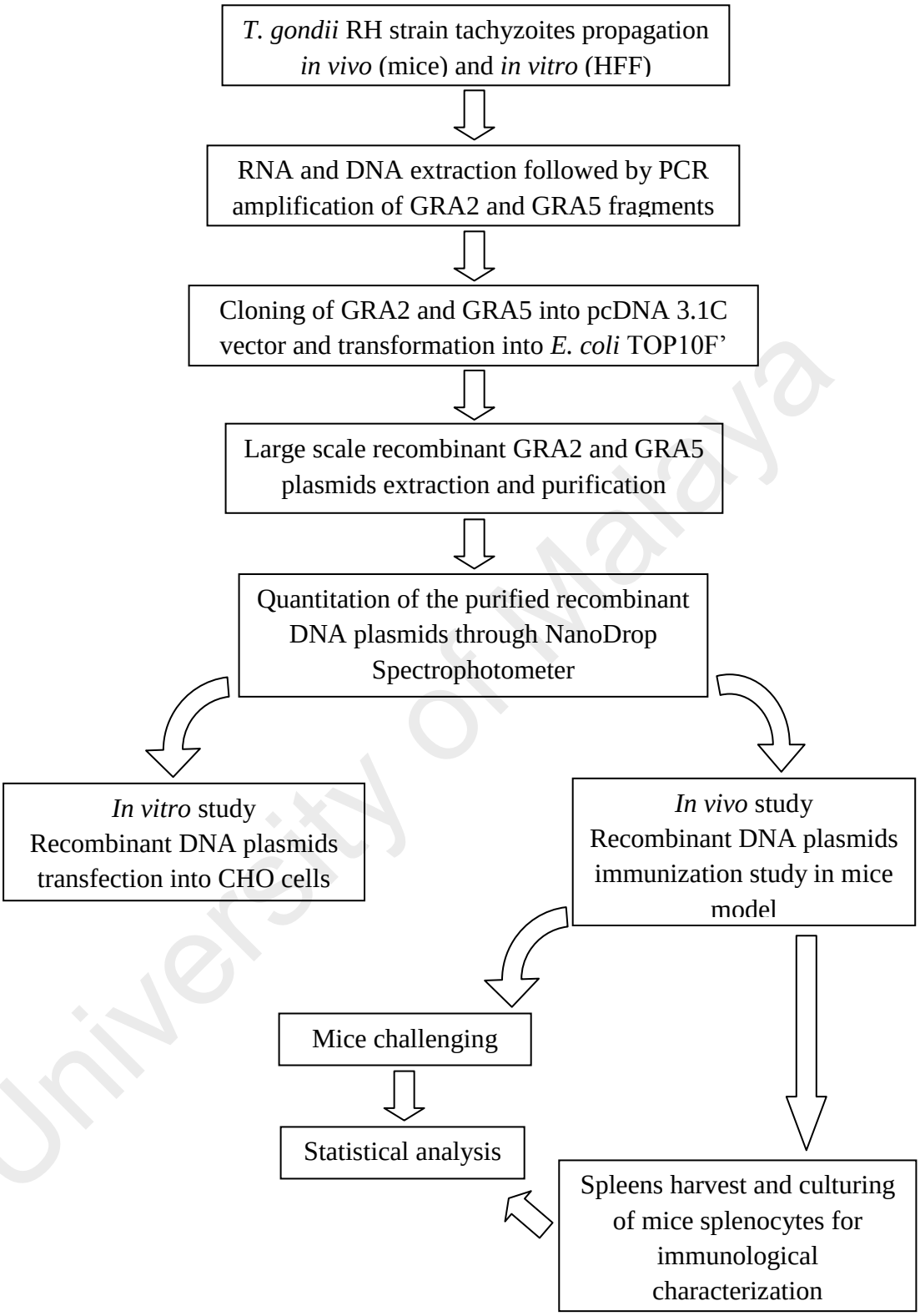

Figure 3.2: Overall approaches in recombinant plasmids immunization study

3.2 Reagents and chemicals

Culturing and cryopreservation of *T. gondii* tachyzoites required complete medium composed of Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, penicillin-streptomycin and fetal bovine serum (FBS) from Gibco, USA. Dimethyl sulfoxide (DMSO) and trypsin-EDTA were ordered from Sigma, USA.

Isolations of *T. gondii* tachyzoites RNA and DNA were performed with TRI reagent (MRC Medical Research Center, USA) and DNeasy® Blood and Tissue kit (Qiagen, Germany) respectively. PCR amplification was carried out with *i*-Taq™ DNA polymerase kit from iNtRON Biotechnology, Korea. Meanwhile, amplification of the target gene fragment from the extracted RNA was carried out with One-step Reverse Transcription Polymerase Chain Reaction (RT-PCR) kit from Qiagen, Germany. Primers involved in the amplifications were ordered from Bioneer, Korea. QIAquick® Gel Extraction kit and QIAprep® Spin Miniprep kit used in the purification of PCR products and isolation of plasmids respectively were purchased from Qiagen, Germany.

Agarose powder for the preparation of agarose gel was obtained from Amresco Inc., USA. Chemicals such as Tris base (Sigma, USA), glacial acetic acid (J.T.Baker, USA) and ethylenediaminetetraacetic acid (EDTA) (Amresco Inc., USA) were used for the preparation of Tris-acetate-EDTA (TAE) agarose gel electrophoresis buffer. GeneRuler™ Express DNA ladder including the 6X gel loading dye was purchased from Fermentas, USA whereas SYBR Safe DNA gel stain was ordered from Invitrogen, USA.

The pGEM®-T Vector System was purchased from Promega, USA for the cloning of PCR products. Culturing of bacteria, *E. coli* TOP10F' (Invitrogen, USA) required growth medium prepared from tryptone (Conda, Spain), yeast extract (Conda, Spain) and sodium chloride (NaCl) (J.T.Baker, USA). Antibiotics ampicillin and chloramphenicol were obtained from Bio Basic Inc., Canada. Several other chemicals

involved were glucose, glycerol and magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) from Sigma, USA, as well as polyethylene glycol (PEG) from Promega, USA.

T4 DNA ligase and restriction enzyme, *Eco*RI were purchased from Promega, USA and NEB, USA respectively. The reaction buffers were provided together with the respective enzymes.

Prokaryotic expression vector, pRSET B and host, *E. coli* BL21 pLysS (DE3) as well as Isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Invitrogen, USA. Phenylmethylsulfonyl fluoride (PMSF) was ordered from Merck, Germany. Chemicals involved in the recombinant protein purifications were guanidine hydrochloride, urea, sodium phosphate (monobasic and dibasic) and imidazole from Sigma, USA. Disposable polypropylene columns and nitrilotriacetic acid-nickel (Ni-NTA) resins were purchased from Qiagen, Germany. Purified recombinant protein concentration was determined by Bradford Assay Kit from Bio-Rad, USA.

On the other hand, chemicals such as acetonitrile (ACN), ammonium bicarbonate (NH_4HC0_3), DL-dithiothreitol (DL-DTT), formic acid and trypsin were bought from Sigma, USA; Iodoacetamide (IAA) was purchased from Amersham Pharmacia Biotech Inc., Sweden. Zip-Tip (Millipore) was from Merck, Germany.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) involved electrophoresis grade acrylamide/bis-acrylamide (29:1) solution, ammonium persulphate (APS), N,N,N',N'-tetramethylenediamine (TEMED), Tris-HCl (1.5mM, pH 8.8 & 0.5mM, pH 6.0), Coomassie brilliant blue (CBB) R-250, tris and glycine (from Bio-Rad, USA); SDS (Amresco Inc., USA), β-mercaptoethanol (Bio Basic Inc., Canada), glycerol (Sigma, USA), bromophenol blue (Fisher chemical, UK), methanol and glacial acetic acid (J. T. Baker, USA), as well as PageRuler™ Prestained Protein Ladder (Fermentas, USA). Polyvinylidene difluoride (PVDF) membrane, Whatman 3MM filter paper and blotting-grade blocker non-fat dry milk for western blot (WB) assay were

ordered from Bio-Rad, USA. Antibodies such as biotin-labeled goat anti-human lgG, biotin-labeled goat anti-human IgM, biotin-labeled goat anti-human IgG+IgM, biotin-labeled goat anti-mouse lgG, biotin-labeled goat anti-mouse IgM, biotin-labeled goat anti-mouse IgG+IgM, and streptavidin-AP (alkaline phosphatase conjugated) were purchased from KPL, USA. Xpress mouse monoclonal antibody was obtained from Invitrogen, USA. Tween-20 and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution were from Sigma, USA.

Eukaryotic expression vector, pcDNA 3.1C was bought from Invitrogen, USA. EndoFree® Plasmid Giga kit from Qiagen, Germany was used for the isolation of transfection-grade endotoxin-free DNA plasmid. Media required for the cell culture included Roswell Park Memorial Institute 1640 (RPMI 1640) medium, DMEM, penicillin-streptomycin, FBS, sodium pyruvate, and non-essential amino acid (NEAA) from Invitrogen, USA. TurboFect™ Transfection Reagent was purchased from Thermo Scientific, USA. Needles and syringes were purchased from Terumo, USA. Complete Freund's (CFA) and incomplete Freund's (IFA) adjuvants were both ordered from Sigma, USA.

Meanwhile, goat anti-mouse IgG-HRP, goat anti-mouse IgG1-HRP, goat anti-mouse IgG2a-HRP, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate were purchased from Abd Serotec, UK. Cell proliferation kit (MTT) was obtained from Roche Applied Science, Germany whereas mouse interferon-gamma (IFN-γ), mouse interleukin-4 (IL-4), mouse interleukin-2 (IL-2) and mouse interleukin-10 (IL-10) enzyme-linked immunosorbent assay (ELISA) kits were all ordered from Pierce, USA. Preparation of ACK lysis buffer required ammonium chloride (NH_4Cl) (Merck, Germany), sodium bicarbonate ($NaHCO_3$) (BDH, UK) and EDTA (Amresco Inc., USA). Concanavalin A (ConA) was purchased from Merck, Germany. Novalisa™ *Toxoplasma gondii* IgG and *Toxoplasma gondii* IgM ELISA kits were ordered from NovaTec, Germany.

3.3 Sterilization

3.3.1 Moist heat

Distilled and deionized water (ddH_2O), chemicals, medium, broth, micropipette tips, microcentrifuge tubes, centrifuge tubes (polycarbonate and polypropylene), flasks, measuring cylinders, Schott bottles with plastic caps, beakers, dissecting kits were sterilized by autoclaving at 15 p.s.i at 121˚C for 15 min.

3.3.2 Membrane filtration

Heat-labile media, solutions and antibiotics were sterilized through disposable syringe filters with pore size of 0.22 μm.

3.4 Stock solutions

3.4.1 Solutions and media for *E. coli*

3.4.1.1 Luria-Bertani (LB) medium

- Tryptone 1.0 g
- Yeast extract 0.5 g
- NaCl 1.0 g
- ddH_2O to 100.0 ml

Additional 1.5 g of bacteriological agar was added for the preparation of solid medium. The mixture was sterilized by autoclaving.

3.4.1.2 Ampicillin (100 mg/ml)

One gram of ampicillin sodium powder was dissolved in 10 ml of ddH_2O. The mixture was sterilized by membrane filtration before storing at -20˚C.

3.4.1.3 Chloramphenicol (34 mg/ml)

Zero point three four gram of chloramphenicol powder was dissolved in 10 ml of absolute ethanol and stored at -20˚C.

3.4.1.4 Medium A

- LB broth
- Glucose 0.2%
- $MgSO_4.7H_2O$ 10 mM

Glucose and $MgSO_4.7H_2O$ were prepared and sterilized through membrane filtration before adding into the sterile LB broth.

3.4.1.5 Medium B (Storage solution)

- LB broth
- Glycerin 36%
- PEG (MW7500) 12%
- $MgSO_4.7H_2O$ 12 mM

Glycerin, PEG and $MgSO_4.7H_2O$ were prepared and sterilized through membrane filtration before adding into the sterile LB broth.

3.4.2 Solutions for agarose gel electrophoresis (AGE)

3.4.2.1 50X Tris-acetate-EDTA (TAE) buffer stock solution

- Tris base (2 M) 242.0 g
- Glacial acetic acid (1 M) 57.1 ml
- 0.5 M EDTA (pH 8.0) 100.0 ml
- ddH_2O to 1000 ml

EDTA (0.5 M, pH 8.0) was prepared beforehand by dissolving 93.06 g of EDTA in 400 ml of ddH_2O and was adjusted to pH 8.0 with NaOH. The final volume was topped up to 500 ml with ddH_2O. The above compositions were mixed well before keeping at RT. The stock solution was diluted 50X with ddH_2O to achieve 1X working solution.

3.4.2.2 Preparation of 1% agarose gel

Zero point two five gram of electrophoresis-grade agarose powder was weighed into a small flask and topped up with 25 ml of TAE (1X) buffer. The mixture was heated in a microwave oven for 1 min to melt the agarose and was swirled to ensure even mixing. One µl of SYBR Safe DNA gel stain was added into the melted gel after being cooled to 55˚C under running tap water. Lastly, the gel was poured immediately onto a gel casting tray and gel comb was inserted.

3.4.3 Solution for protein expression in *E. coli* BL21 pLysS (DE3)

3.4.3.1 IPTG (100 mM)

Zero point two four gram of IPTG powder was dissolved in 10 ml of ddH_2O. The mixture was sterilized through membrane filtration before storing at -20˚C.

3.4.4 Solutions for protein purification

3.4.4.1 Buffer stock solutions (10X)

a) Stock solution A (10X)

- 200 mM Sodium phosphate, monobasic (NaH_2PO_4)
- 5 M NaCl

Twenty-seven point six gram of NaH_2PO_4 and 292.9 g of NaCl were dissolved in 800 ml ddH_2O. The solution was mixed well and the volume was adjusted to 1 L with ddH_2O.

b) Stock solution B (10X)

- 200 mM Sodium phosphate, dibasic (Na_2HPO_4)
- 5 M NaCl

Twenty-eight point four gram of Na_2HPO_4 and 292.9 g of NaCl were dissolved in 800 ml ddH_2O. The solution was mixed well and the volume was adjusted to 1 L with ddH_2O.

3.4.4.2 Native purification buffer (5X)

- 250 mM NaH_2PO_4, pH 8.0
- 2.5 M NaCl

Seven gram of NaH_2PO_4 and 29.2 g of NaCl were dissolved in 180 ml ddH_2O. The solution was mixed well and adjusted to pH 8.0 with NaOH. It was then topped up to a final volume of 200 ml with ddH_2O. For 1X working solution, this buffer was diluted 5X and was adjusted to pH 8.0 before used.

3.4.4.3 3M Imidazole, pH 6.0

- 3M Imidazole
- 500 mM NaCl
- 20 mM Sodium phosphate buffer, pH 6.0

Twenty point six gram of imidazole, 8.77 ml of stock solution A (10X) and 1.23 ml of stock solution B (10X) were dissolved in 90 ml ddH_2O. The solution was mixed

well and adjusted to pH 6.0 with HCl or NaOH. It was then topped up to a final volume of 100 ml with ddH_2O. The solution was heated if precipitates formed.

3.4.4.4 Native wash buffer (20 mM imidazole)

- 1X Native purification buffer 50 ml
- 3 M Imidazole, pH 6.0 335 µl

The compositions were mixed well and adjusted to pH 8.0 with NaOH or HCl.

3.4.4.5 Native elution buffer (250 mM imidazole)

- 1X Native purification buffer 13.75 ml
- 3 M Imidazole, pH 6.0 1.25 ml

The compositions were mixed well and adjusted to pH 8.0 with NaOH or HCl.

3.4.4.6 Guanidine lysis buffer

- 6 M Guanidine hydrochloride
- 20 mM Sodium phosphate, pH 7.8
- 500 mM NaCl

Fifty-seven point three gram of guanidine hydrochloride, 0.58 ml of stock solution A (10X) and 9.42 ml of stock solution B (10X) were dissolved in 90 ml ddH_2O. The solution was mixed well and was adjusted to pH 7.8 with HCl or NaOH. The volume was topped up to 100 ml with ddH_2O and filtered through membrane with pore size of 0.45 µm.

3.4.4.7 Denaturing binding buffer

- 8 M Urea
- 20 mM Sodium phosphate pH 7.8

- 500 mM NaCl

Forty-eight point one gram of urea, 0.58 ml of stock solution A (10X) and 9.42 ml of stock solution B (10X) were dissolved in 90 ml ddH_2O. The solution was stirred with gentle heating (50-60˚C) until dissolved completely and was adjusted to pH 7.8 with HCl or NaOH after cooled to RT. The volume was topped up to 100 ml with ddH_2O and filtered through membrane with pore size of 0.45 μm.

3.4.4.8 Denaturing wash buffer

- 8 M Urea
- 20 mM Sodium phosphate pH 6.0
- 500 mM NaCl

Forty-eight point one gram of urea, 7.38 ml of stock solution A (10X) and 2.62 ml of stock solution B (10X) were dissolved in 90 ml ddH_2O. The solution was stirred with gentle heating (50-60˚C) until dissolved completely and was adjusted to pH 6.0 with HCl or NaOH after cooled to RT. The volume was topped up to 100 ml with ddH_2O and filtered through membrane with pore size of 0.45 μm.

3.4.4.9 Denaturing elution buffer

- 8 M Urea
- 20 mM Sodium phosphate pH 4.0
- 500 mM NaCl

Forty-eight point one gram of urea and 10 ml of stock solution A (10X) were dissolved in 90 ml ddH_2O. The solution was stirred with gentle heating (50-60˚C) until dissolved completely and was adjusted to pH 4.0 with HCl or NaOH after cooled to RT. The volume was topped up to 100 ml with ddH_2O and filtered through membrane with pore size of 0.45 μm.

3.4.5 Solutions for SDS-PAGE

Solutions and gel preparation were based on the buffer systems of Laemmli (1970), and described in the Instruction Manual of Mini-Protean® II Electrophoresis Cell System, Bio-Rad, USA.

3.4.5.1 12% resolving gel solution (0.375 M Tris, pH 8.8)

- Acrylamide-bisacrylamide (30%) 4.00 ml
- 1.5 M Tris-HCl, pH 8.8 2.50 ml
- 10% SDS 100.0 µl
- 10% APS 100.0 µl
- ddH_2O 3.30 ml

The above compositions were mixed well and 4.0 µl of TEMED was added last.

3.4.5.2 5% stacking gel solution (0.125 M Tris, pH 6.8)

- Acrylamide-bisacrylamide (30%) 0.67 ml
- 0.5 M Tris-HCl, pH 6.8 1.00 ml
- 10% SDS 40.0 µl
- 10% APS 40.0 µl
- ddH_2O 2.20 ml

The above compositions were mixed well and 4.0 µl of TEMED was added last.

3.4.5.3 5X SDS running buffer, pH 8.3

- Tris base 15 g/l
- Glycine 72 g/l
- SDS 5 g/l

The SDS running buffer was diluted 5X with ddH_2O before used.

3.4.5.4 2X SDS gel loading buffer (sample buffer)

- Tris-HCl, pH 6.8 — 100 mM
- SDS — 4% (w/v)
- β-mercaptoethanol — 200 mM
- Glycerol — 20% (w/v)
- Bromophenol blue — 0.2% (w/v)

3.4.5.5 Coomassie staining solution

- CBB R-250 — 2.50 g
- Methanol — 500 ml
- Acetic acid — 100 ml
- ddH_2O — 400 ml

Two point five gram of CBB R-250 was dissolved in methanol, acetic acid and ddH_2O. The solution was filtered through Whatman filter paper to remove any undissolved powder and was stored at RT.

3.4.5.6 De-staining solution

- Methanol — 5%
- Acetic acid — 7%

Fifty milliliter of methanol was mixed with 70 ml of acetic acid and topped up with ddH_2O to a final volume of 1000 ml. The solution was stored at RT.

3.4.6 Solutions for WB

3.4.6.1 Semi-dry blotting / Transfer buffer, pH 8.3

- Tris base — 5.82 g
- Glycine — 2.93 g

- Methanol 200 ml
- ddH_2O to 1000 ml

3.4.6.2 5X Tris-borate-saline (TBS), pH 7.5

- Trizma base 12.11 g
- NaCl 48.85 g
- ddH_2O 800 ml

The pH of the solution was adjusted to pH 7.5 with HCI or NaOH. The final volume was topped up to 1000 ml. The buffer was diluted 5X to achieve 1X working solution with ddH_2O.

3.4.6.3 5% (w/v) blocking buffer

- Skim-milk powder 5.0 g
- 1X TBS 100 ml

3.4.6.4 2.5% (w/v) blocking buffer

- Skim-milk powder 2.5 g
- 1X TBS 100 ml

3.4.6.5 Washing buffer (0.002% TBS-T)

- Tween-20 2 ml
- 1X TBS 1000 ml

3.4.7 Solutions for Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF)

3.4.7.1 100 mM NH_4HCO_3

One point nine eight gram of NH_4HCO_3 was dissolved in 250 ml of ddH_2O and stored at RT.

3.4.7.2 10 mM L-DTT in 100 mM NH_4HCO_3

Zero point zero one five four gram of L-DTT was dissolved in 10 ml of 100 mM NH_4HCO_3 and stored at RT.

3.4.7.3 55 mM IAA in 100 mM NH_4HCO_3

Zero point one zero one eight gram of IAA was dissolved in 10 ml of 100 mM NH_4HCO_3 and stored at RT.

3.4.7.4 50% ACN in 50 mM NH_4HCO_3

Five milliliter of 100% ACN was mixed with 5 ml of 100 mM NH_4HCO_3 and stored at RT.

3.4.7.5 6 ng/µl trypsin in 50 mM NH_4HCO_3

The stock solution was prepared and diluted 10X with 50 mM NH_4HCO_3 before use. It was stored in -20˚C. E.g. 6 µl of stock solution + 54 µl of 50 mM NH_4HCO_3 = 60 µl of 6 ng/µl trypsin in 50 mM NH_4HCO_3.

3.4.7.6 50% ACN

Five hundred microliter of 100% ACN was mixed with 500 µl of miliQ water and stored at RT.

3.4.8 Medium for cell culture

3.4.8.1 Complete medium for Human Foreskin Fibroblast (HFF)

- DMEM
- FBS 10%
- Penicillin-streptomycin 1%
- L-glutamine 1%

The above compositions were mixed under sterile condition and stored at 4˚C. Parasite infection medium shared the same compositions but with lower FBS content, i.e. 2% instead of 10%.

3.4.8.2 Complete medium for Chinese Hamster Ovary (CHO)

- DMEM
- FBS 10%
- Penicillin-streptomycin 1%
- L-glutamine 1%
- Sodium Pyruvate 1%
- NEAA 1%

The above compositions were mixed under sterile condition and stored at 4˚C.

3.4.8.3 Freezing solution for cryopreservation

- DMEM complete medium 95%
- DMSO 5%

The above compositions were mixed under sterile condition and stored at -20˚C.

3.4.8.4 Complete medium for mice splenocytes

- RPMI 1640
- FBS 10%
- Penicillin-Streptomycin 1%

The above compositions were mixed under sterile condition and stored at 4˚C.

3.4.8.5 Ammonium-Chloride-Potassium (ACK) lysis buffer

- NH_4Cl 4.14 g
- $NaHCO_3$ 0.50 g
- EDTA 0.037 g
- ddH_2O to 500 ml

The above chemicals were dissolved in 500 ml ddH_2O. The buffer was then adjusted to pH 7.4 with NaOH or HCl and was sterilized by filtration through 0.22 μm filter.

3.4.8.6 ConA (1 mg/ml)

ConA (0.001 g) was dissolved in 1 ml of RPMI complete medium under sterile condition and kept at -20˚C.

3.4.9 Solutions for indirect ELISA

3.4.9.1 Coating buffer (0.05 M bicarbonate, pH 9.6)

- Sodium hydrogen carbonate 2.93 g
- Sodium carbonate 1.59 g
- Sodium azide 0.2 g
- ddH_2O 800 ml

The pH of the solution was adjusted to 9.6. The volume was made up to 1000 ml.

3.4.9.2 10X phosphate buffered saline (PBS), pH 7.2

- Sodium chloride 8.0 g/l
- Potassium chloride 0.2 g/l

- Di-sodium hydrogen phosphate (Na_2HPO_4) 1.15 g/l
- Potassium di-hydrogen phosphate 0.2 g/l

The stock solution was diluted ten times to achieve 1X working solution.

3.4.9.3 Washing buffer (0.05% PBS-T)

- Tween-20 5 ml
- PBS 100 ml

3.4.9.4 10% blocking buffer

- FBS 10 ml
- 1X PBS 100 ml

3.5 Mice

Six- to eight-week old female BALB/c mice were purchased from Monash University Sunway Campus. The mice were maintained in a pathogen free environment and were fed ad lib with commercial food pellets and water. Experiments were carried out in compliance with the animal ethics approved by Institutional Animal Care and Use Committee (IACUC) of the University of Malaya, Faculty of Medicine (2014-06-03/PARA/R/CXT) (APPENDIX A).

3.6 Monolayer cell culture

Human foreskin fibroblast (HFF) and Chinese hamster ovary (CHO) were purchased from American Type Culture Collection (ATCC, USA). Both cell lines were maintained in $75cm^3$ (T75) tissue culture flasks (TPP, Switzerland) and incubated in a sterile and humidified CO_2 incubator (Binder, Germany) at 37˚C under 5% of CO_2 supply.

3.6.1 Passaging of monolayer cell culture

The complete medium was aspirated from T75 flask when the adherent cells reached 90-95% confluence and was rinsed with 10 ml of sterile 1X PBS to remove all traces of serum containing trypsin inhibitor followed by incubation with 2 ml of trypsin-EDTA for 5 min at 37˚C. Five ml of new complete medium was dispensed into the flask when all cells were dislodged from the surface of the flask. The cells were pipetted up and down several times to ensure formation of single cell suspension. The single cell suspension was eventually divided and transferred into five new T75 flasks pre-filled with 10 ml of complete medium for a splitting ratio of 1:5 and incubated in the CO_2 incubator until confluent.

3.6.2 Cryopreservation of cell culture

Single cell suspension formed (section 3.6.1) was sedimented at 1,100 rpm for 10 min at 4˚C. Supernatant was discarded while the deposited cell pellet was resuspended in freezing solution and aliquoted into 2 ml cryovials. The cryovials were sealed in a Styrofoam box and was stored overnight in -80˚C to ensure gradual freezing of the cells. The frozen cells were then transferred to liquid nitrogen for a longer storage.

The frozen cells were revived by thawing at 37˚C waterbath directly and washed with complete medium when they had been thawed completely. The cells were sedimented, resuspended in complete medium and was seeded as described in 3.5.3.

3.6.3 Determination of cell density and viability

Density and viability of the cells was determined through trypan blue exclusion assay (Strober, 2001). The dye selectively stains only dead cells giving rise to its blue cytoplasm and appeared blue under the view of light microscope. Meanwhile, viable

cell which is resistant to the stain will have clear cytoplasm due to its intact cell membrane.

Ten µl of the single cell suspension formed (section 3.6.1) was mixed evenly with 10 µl of trypan blue dye (Sigma, USA) (1:1 ratio). Ten µl of the mixture was dispensed onto the hemocytometer (Marienfeld, Germany) under the glass cover slip. The hemocytometer was then observed under an inverted light microscope (Olympus, Japan) with 20X magnification. Viable cells were counted in the four main corner squares (0.1 mm^3) of the hemocytometer. Cell density was determined with the following formula:

$$\text{Cell density} = \frac{\text{Number of cells counted}}{4} \times \text{dilution factor} \times 10^4 \text{ cells/ml}$$

Meanwhile, viability of the cells was calculated using the formula below:

$$\text{Cell viability (\%)} = \frac{\text{Number of live cells}}{\text{Number of live cells} + \text{Number of dead cells}} \times 100$$

3.7 *T. gondii* parasite

T. gondii tachyzoites of the virulent wild-type RH strain were provided by the Department of Parasitology, University of Malaya, Kuala Lumpur, Malaysia. They were maintained by both *in vivo* and *in vitro* propagations with the starting inoculum of $1x10^6$ parasites.

3.7.1 *T. gondii* infection of mice

In vivo propagation involved serial intraperitoneal passage in BALB/c mice and were harvested from the peritoneal fluids 3 to 4 days post-infection. Tachyzoites-containing fluids were washed twice with sterile PBS. The cell suspension was centrifuged at 1,000 x g for 15 min between each wash and was resuspended in sterile PBS before filtering through 3 µm polycarbonate membrane (Merck, USA). The filtrate

containing only the parasites was sedimented and resuspended in either sterile PBS for further downstream works or in freezing solution for cryopreservation (section 3.6.2).

3.7.2 *T. gondii* infection of HFF cells

In vitro propagation involved tachyzoites infection of HFF cells. DMEM complete medium was replaced with parasite infection medium 12-16 h pre-infection when the growth of HFF cells reached 80-90% confluence. Freshly isolated or frozen parasites were washed with PBS and resuspended in parasite infection medium. The density and viability of parasites were determined (section 3.6.3) before infecting the HFF cells. After 24 h of incubation in the CO_2 incubator, the infected cells were replaced with new parasite infection medium in order to remove free floating tachyzoites. Incubation was continued until the lysis of the infected cells triggered by the actively-replicating tachyzoites thereby releasing them into the medium.

During lysis of the infected HFF cells, erupted tachyzoites and the infected cells were harvested with a cell scraper (TPP, USA). The entire cell suspension was transferred to a syringe attached to a 25 gauge needle which was placed into a 50 ml polypropylene tube beforehand. The cell suspension was forced to pass through the needle by the plunger to ensure the release of tachyzoites from the intact infected cells. The act was repeated twice followed by two times of washing with sterile PBS and was further processed as described in section 3.7.1.

3.8 Isolation of *T. gondii* RNA

Approximately 10^6-10^7 parasites (section 3.7.1, 3.7.2 and 3.6.3) were sedimented. The cell pellet acquired was lysed in 1.0 ml of TRI reagent by repetitive pipetting and incubated at RT for 5 min. The lysate obtained was added with 0.2 ml of chloroform before shaking vigorously for 15 s. The resulting mixture was incubated at RT for

another 2-15 min followed by centrifugation at 12,000 x g for 15 min at 4˚C which was able to separate the mixture into three main layers; lower organic phase (red-phenol chloroform), interphase and upper aqueous phase (colorless). The upper aqueous phase containing RNA was transferred to a new fresh tube and was incubated with 0.5 ml of isopropanol for 5-10 min at RT in order to precipitate the RNA. A gel-like or white pellet was formed on the side and bottom of the tube after centrifugation at 12,000 x g for 8 min at 4-25˚C. The supernatant was discarded while the RNA pellet was washed with at least 1 ml of 75% ethanol before subsequent centrifugation at 7,500 x g for 5 min at 4-25˚C. The RNA pellet was air-dried for 3-5 min after ethanol wash. The pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water by passing through pipette tip for few times and was incubated for 10-15 min at 55-60˚C. The dissolved RNA was ready to be used as a template for RT-PCR in the next step. All the consumables involved in the RNA isolation were treated with DEPC before usage. The concentration and purity of the isolated RNA was estimated using spectrophotometer.

3.9 Isolation of *T. gondii* DNA

Total genomic DNA of the parasites was extracted according to the manufacturer's protocol of the DNeasy® Blood and Tissue kit. Briefly, 1-5 x 10^6 parasites (section 3.7.1, 3.7.2 and 3.6.3) were sedimented. Cell pellet was resuspended in 200 µl of PBS followed by the addition of 20 µl Proteinase K and 200 µl Buffer AL. The suspension was vortexed to form a homogeneous solution before incubation at 56˚C for 10 min. Ten min later, 200 µl of absolute ethanol was added to the sample and was mixed thoroughly. The entire mixture was transferred into a DNeasy® Mini spin column assembled in a 2 ml collection tube and was centrifuged at 6,000 x g for 1 min. The flow-through was discarded and 500 µl Buffer AW1 was added into the same spin column and was centrifuged for another 1 min at the same speed. Flow-through was discarded and another 500 µl Buffer AW2 was

added before subjected to centrifugation at 20,000 x g for 3 min in order to dry the DNeasy® membrane. New collection tube was used after each washing step. The dried spin column was then placed in a clean 1.5 ml microcentrifuge tube before the addition of 100 µl sterile ddH_2O directly onto the DNeasy® membrane and was incubated for 1 min at RT. The column was spun at 6,000 x g for 1 min to elute the bound DNA. The concentration and purity of the eluted DNA was estimated using spectrophotometer.

3.10 Estimation of nucleic acid concentration and purity

Direct estimation was carried out with NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) which required only 1 µl of sample. Solution involved in solubilizing the isolated nucleic acid was employed as blank. Purity of the nucleic acid was determined according to the ratio of A_{260} to A_{280}, whereby a ratio between 1.8 and 2.0 indicated absence of protein contamination. The concentration was displayed as ng/µl by spectrophotometer.

3.11 Isolation of *T. gondii* total lysate antigen (TLA)

Parasites suspension prepared as described in section 3.7.1 and 3.7.2 was subjected to 5-10 cycles of freeze-thaw method which involved freezing in liquid nitrogen and thawing in water bath at 37˚C with 2-3 min each step. This was followed by centrifugation of the lysate at 3,000 x g for 15 min at 4˚C. The supernatant containing TLA was collected and stored at -80˚C until used.

3.12 Oligonucleotide primers

The stock concentration of all primers involved was 100 pmoles/µl and was diluted 10X to achieve 10 pmoles/µl working concentration.

3.12.1 GRA2 primers

The nucleotide sequence of the *T. gondii GRA2* (corresponding to nucleotides 497-1223) encoding GRA2 antigen was obtained from Genbank (Assession number: L01753.1). RNA extracted from the tachyzoites was used as a template for amplification of *GRA2* gene through One-step RT-PCR with sense primer (GRA2F- 5'-GAATTCGCCGAGTTTTCCGGAGTT-3') and antisense primer (GRA2R- 5'-GAATTCCTGCGAAAAGTCTGGGAC-3'). The primer set introduced *EcoR* I single restriction site (underlined) to facilitate cloning.

3.12.2 One-Step Reverse-Transcriptase Polymerase Chain Reaction

RT-PCR amplification of *GRA2* was carried out using the following reagents:

Reagents	Volume (µl)
5X OneStep RT-PCR buffer (2.5 mM Mg^{2+})	10.0
dNTP mix (400 µM each dNTP)	2.0
Forward primer (0.6 µM)	3.0
Reverse primer (0.6 µM)	3.0
OneStep RT-PCR Enzyme mix	2.0
RNase inhibitor (5-10 units/reaction)	0.3
Template* (1 pg-2 µg/reaction)	2.0
RNase-free water	27.7
Total volume	50.0

*RNase-free water was added into the negative control reaction; *T. gondii* RNA was added into the RT-PCR reaction tube.

RT-PCR amplification was initiated with reverse-transcription reaction at 50˚C for 32 min before the initial PCR activation step at 95˚C for 15 min. This was followed by 37 cycles of denaturation at 94˚C for 30 s, annealing at 55-60˚C for 1 min and

extension at 72˚C for 1 min. The cycles were eventually completed with a final extension at 72˚C for 10 min and a holding temperature at 16˚C.

3.12.3 GRA5 primers

T. gondii GRA5 gene sequence (corresponding to nucleotides 76–360), which encodes the GRA5 antigen, was obtained from Genbank (accession number: EU918733.1). DNA extracted was used as a template for PCR amplification of the *GRA5* gene with forward primer (5'-GCGGAATTCGGTTCAACGCGTGAC-3') and reverse primer (5'-GACGAATTCCTCTTCCTCGGCAACTTC-3'). The primer set introduced *EcoR* I restriction site (underlined) to facilitate cloning.

3.12.4 Polymerase Chain Reaction

PCR amplification of *GRA5* was carried out using the following reagents:

Reagents	Volume (µl)
10X PCR buffer (2.0 mM Mg^{2+})	2.0
dNTP mix (250 µM each dNTP)	2.0
Forward primer (0.5 µM)	1.0
Reverse primer (0.5 µM)	1.0
i-Taq^{TM} DNA polymerase (2.5 U)	0.5
Sterile ddH_2O	9.5
Template* (1 ng-1 µg)	4.0
Total volume	20.0

*sterile ddH_2O was added into the negative control reaction; *T. gondii* DNA was added into the PCR reaction tube.

PCR amplification was initiated at 95˚C for 10 min, followed by 35 cycles of denaturation at 94˚C for 45 s, annealing at 55-60˚C for 30 s and extension at 72˚C for 1

min. The cycles were eventually completed with a final extension at 72˚C for 10 min and a holding temperature at 16˚C.

3.13 Agarose gel electrophoresis

Preparation of 1% agarose gel was performed according to section 3.4.2.2. The gel comb was removed once the agarose gel had solidified and ready to be used. The gel was placed in the electrophoresis tank which was filled up with 1X TAE buffer until the gel was completely submerged. Samples were loaded into each well before running electrophoresis at 100 V for 30 min. DNA was visualized by Molecular Imager® Gel Doc™ XR+ System (Bio-rad, USA).

3.14 Gel purification of PCR product

Purification of the PCR product was performed according to the manufacturer's protocol of the QIAquick® Gel Extraction kit. Briefly, DNA fragment with expected size was excised from the gel (section 3.13) and transferred into a 1.5 ml microcentrifuge tube. Buffer QG (solubilization) with the volume equivalent to 3X the volume of the gel was added into the same tube. The mixture was incubated at 50˚C for 10 min with occasional vortexing in order to dissolve the gel. One gel volume of isopropanol was added to the dissolved sample and was mixed well. The entire volume of the mixture was transferred to a QIAquick® spin column assembled in a 2 ml collection tube and centrifuged at 17,900 x g for 1 min. Flow-through was discarded. The column was then washed with 750 µl Buffer PE before subjected to two rounds of centrifugations. Flow through was discarded after each centrifugation. The dried spin column was placed in a clean 1.5 ml micro-centrifuge tube before the addition of 30-50 µl sterile ddH_2O directly onto the membrane and was incubated for 1 min at RT. The column was spun at 17,900 x g for 1 min to elute the bound DNA.

3.15 Preparation of competent *E. coli* cells

TOP10F' and BL21 pLysS (DE3) competent cells were prepared according to the previous publication (Nishimura *et al.,* 1990). A single *E. coli* colony was picked and inoculated into 1 ml of LB broth for overnight incubation (approximately 16 h) in a shaking incubator at 37˚C. In the following day, 0.5 ml of the overnight culture was inoculated into 50 ml of Medium A and was incubated for another 3-4 h at 37˚C in a vigorously shaking incubator until the approximate OD_{600} of 0.5 was achieved. The cells were then chilled on ice for 10 min and harvested by centrifugation at 1,500 x g for 10 min at 4˚C. The supernatant was discarded whereas the cell pellet was resuspended gently and was mixed well in 0.5 ml of pre-cooled Medium A followed by 2.5 ml of Storage Medium B without vortexing. The competent cells were aliquoted with 100 µl in each sterile 1.5 ml micro-centrifuge tube and were kept in -80˚C until use.

3.16 Constructions of recombinant pGEM®-T-GRA2 and pGEM®-T-GRA5

3.16.1 Ligation of purified PCR product to pGEM®-T

Purified PCR product (fragment encoding GRA2 or GRA5 antigen) was ligated into the cloning vector, pGEM®-T through TA cloning as follows:

Reagents	Volume (µl)
Purified PCR product* (20-30 ng)	3.0
pGEM®-T vector (50 ng)	1.0
2X rapid ligation buffer	5.0
T4 ligase (3 U)	1.0
Total volume	10.0

*Control insert DNA (2 µl) provided in the kit was employed as a positive control and the total volume was topped up to 10 µl with sterile ddH_2O (1 µl). The ligation mixture was mixed well and incubated at 4˚C overnight.

3.16.2 Transformation into competent TOP10F' cells

Following the 4˚C overnight incubation, 10 µl of the ligation mixture was added into 100 µl of the pre-thawed competent cells. The cells were incubated on ice for 30 min followed by immediate heat-shock incubation in water bath at 42˚C for 60 s and were returned to ice for another 2 min. The cells were diluted 10-fold (1 ml) with LB broth before incubation in a shaking incubator at 37˚C for 1 h. Simultaneously, control insert DNA mixture was used to transform another 100 µl of competent cells as a positive control.

3.16.3 Selection of transformants

3.16.3.1 Cell plating

The transformed *E. coli* TOP10F' cells were sedimented at 5,000 x g for 5 min after the 1 h incubation. Supernatant was discarded while the cell pellet was resuspended in 200 µl LB broth. One hundred µl of the cell suspension was plated onto a LB agar containing 100 µg/ml of ampicillin with sterile glass beads. Positive control and negative control (competent cells without ligation mixture) were plated the same way. All plates were incubated overnight at 37˚C.

3.16.3.2 PCR colony of the transformants

Screening of the positive pGEM®-T-GRA2 or pGEM®-T-GRA5 clones were performed by picking 5-10 colonies from each ampicillin-incorporated LB plate incubated overnight. PCR amplification was carried out with gene specific forward and reverse primer set (GRA2F and GRA2R or GRA5F and GRA5 R) as described below:

Reagents	Volume (µl)
10X PCR buffer (2.0 mM Mg^{2+})	2.0
dNTP mix (250 µM each dNTP)	2.0

Forward primer (0.5 μM)	1.0
Reverse primer (0.5 μM)	1.0
i-Taq^{TM} DNA polymerase (2.5 U)	0.5
Sterile ddH_2O	12.5
Template*	1.0
Total volume	20.0

*sterile ddH_2O was added into the negative control reaction; picked colony was added into the PCR reaction tube.

PCR amplification was initiated at 95˚C for 10 min, followed by 35 cycles of denaturation at 94˚C for 45 s, annealing at 55-60˚C for 30 s and extension at 72˚C for 1 min. The cycles were eventually completed with a final extension at 72˚C for 10 min and a holding temperature at 16˚C.

3.16.3.3 Determination of positive clones

PCR products were analyzed and visualized in agarose gel (section 3.13). Selected positive clones with the expected size were inoculated into 5 ml of ampicillin (100 μg/ml) added LB broth for overnight incubation in shaking incubator at 37˚C.

3.16.4 Plasmid isolation and purification

DNA plasmids of the selected positive clones were isolated according to the manufacturer's protocol of the QIAprep® Spin Miniprep Kit. Briefly, the overnight culture was centrifuged where the cell pellet was resuspended in 250 μl Buffer P1. The cell suspension was transferred into a sterile 1.5 ml micro-centrifuge tube. This was followed by addition of 250 μl Buffer P2 then 350 μl Buffer N3 with gentle inversion between each addition. The mixture was spun at 17,900 x g for 10 min. The clear supernatant was transferred to the QIAprep® spin column and was centrifuged for 1 min

with same speed. Flow through was discarded and 500 µl Buffer PB was added to the column and was centrifuged again. The spin column was eventually washed with 750 µl Buffer PE before subjected to two rounds of centrifugations. Flow through was discarded after each centrifugation. The dried spin column was placed in a clean 1.5 ml micro-centrifuge tube followed by addition of 30-50 µl sterile ddH_2O directly onto the membrane and was incubated for 1 min at RT. The column was spun at 17,900 x g for 1 min to elute the bound DNA plasmid.

3.16.5 Verification of positive recombinant clones

The isolated DNA plasmids were sent to a commercial laboratory (Bioneer Corporation South Korea) for automated sequencing with universal primer set for pGEM®-T vector; M13F and M13R.

3.16.6 Analysis of the sequencing results

The sequencing results obtained were analyzed and compared with the published sequences in the GenBank database with accession number of L01753.1 and EU918733.1 for *GRA2* and *GRA5* genes respectively. The nucleotides sequences were translated and analyzed using software available online at the Expert Protein Analysis System of the Swiss Institute of Bioinformatics (http://www.expasy.org). The deduced amino acids of GRA2 or GRA5 coded proteins were determined.

3.16.7 Storage and maintenance of positive recombinant clones

Glycerol stocks of the verified positive clones were prepared by mixing 1 volume of sterile 50% glycerol with 1 volume of overnight culture (1:1) and were stored at -80˚C. The clones were re-streaked onto fresh antibiotic-incorporated plates and new glycerol stocks were prepared every 6 months.

3.17 Constructions of recombinant pRSET B-GRA2 and pRSET B-GRA5

Positive recombinant DNA plasmids of pGEM®-T-GRA2 and pGEM®-T-GRA5 as well as the empty vector, pRSET B were isolated (section 3.16.4), excised, gel purified (section 3.14) and finally ligated at the corresponding *Eco*RI site.

3.17.1 *Eco*RI single digestion

Reagents	Volume (µl)
DNA plasmid (1 µg)	10.0
10X RE buffer	5.0
*Eco*RI enzyme (10 U)	0.5
ddH_2O	34.5
Total volume	50.0

*DNA plasmid can be pGEM®-T-GRA2, pGEM®-T-GRA5 or pRSET B.

The above compositions were mixed and incubated at 37˚C for 3 h.

3.17.2 Dephosphorylation

Linearized pRSET B vector was dephosphorylated with alkaline phosphatase, calf intestinal (CIP) to prevent self-ligation.

Reagents	Volume (µl)
Linearized vector DNA (1 µg)	20.0
10X reaction buffer	2.5
CIP (1U/µl)	0.5
ddH_2O	2.0
Total volume	25.0

The above compositions were mixed well and incubated at 37˚C for another 30 min. The enzymatic reaction was halted by heating at 65˚C for 10 min.

3.17.3 Ligation of purified inserts to pRSET B

Following the enzymatic reaction of restriction digestion and dephosphorylation, the linearized products were analyzed with 1% agarose gel (section 3.13) and gel purified (section 3.14) before performing ligation.

Reagents	Volume (µl)
Purified insert (20-30 ng)	6.0
Purified vector (pRSET B) (50 ng)	2.0
10X ligation buffer	2.0
T4 ligase (400 U)	1.0
ddH_2O	9.0
Total volume	20.0

*purified insert can be *GRA2* or *GRA5* gene

The above compositions were mixed well and incubated at 4˚C overnight.

The ligation products were transformed into competent TOP10F' cells the next day. The entire steps depicted under section 3.16 were repeated for positive clones of pRSET B-GRA2 and pRSET B-GRA5. However, different primer set was involved in both PCR colony and automated sequencing. Universal forward primer (T7F) and gene specific reverse primer (GRA2R or GRA5R) were used instead in order to ascertain the correct orientation of the cloned fragment. The isolated DNA plasmids were sent to a commercial laboratory (Bioneer Corporation South Korea) for automated sequencing with T7F primer. The resulting recombinant pRSET B constructs permitted expressions of polyhistidine (His)-tagged at the N-terminal.

3.17.4 Transformation into competent BL21 pLysS (DE3) cells

Recombinant pRSET B constructs and the empty vector propagated in TOP10F' cells were extracted and transformed into competent BL21 (DE3) pLysS cells before performing protein expression. Positive clones were selected against two antibiotics; ampicillin and chloramphenicol with final concentration of 100 μg/ml and 34 μg/ml respectively.

3.18 Optimization of heterologous proteins expressions in BL21 pLysS (DE3)

Optimal conditions for recombinant proteins expressions (rGRA2 and rGRA5) in *E. coli* were determined prior to scaling up the proteins productions protocol for further study. Two main parameters that are critical to be optimized are the a) time point and b) concentration of inducer, IPTG (Table 3.1). A single GRA2-pRSET B or GRA5-pRSET B-containing colony was picked and inoculated into 5 ml of LB broth supplemented with ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). The culture was grown overnight at 37˚C (200 rpm) and then diluted to a final volume of 10 mL with LB broth to yield an optical density of 0.1 at 600 nm (OD_{600}). The culture was then grown at 37˚C (~250 rpm) until reaching an OD_{600} of 0.4 - 0.5, at which point protein expression was induced by addition of 1 mM IPTG for various incubation periods (0, 2, 3, 4 and 5 h). After time point determination, the same set of protocols was repeated for both recombinant proteins with different IPTG concentrations (0.1, 0.5, and 1.0 mM) but with the optimum time point determined earlier. The cells were harvested by centrifugation at 5,000 x g for 10 min at 4˚C before assessing protein expression through SDS-PAGE.

Table 3.1: Parameters for optimization of heterologous protein expression

Parameter	Range
IPTG concentration (mM)	0.1, 0.5, 1.0
Time Point (hour)	0, 2, 3, 4, 5

3.19 Scaled-up protein expressions and determination of the solubility

Scaled-up protein productions were achieved according to the parameters tested earlier (section 3.18) but with an increased culture volume of 20 fold. It is crucial to determine the solubility of the overexpressed target proteins before choosing the appropriate purification method. About 2-5 ml of the *E. coli* culture was sedimented and the pellet was resuspended in 500 µl PBS. The cell suspension was sonicated on ice for 60 s, with 10 s pulses. The total cell lysate was centrifuged at 12,000 x g for 10 min at 4˚C and was separated into soluble (supernatant) and insoluble (pellet) fractions. The insoluble cell pellet was further resuspended in 500 µl 6M urea in 1X PBS before being subjected to sonication again with the same condition. The lysate was centrifuged and the supernatant containing insoluble protein was collected. Total cell lysate, soluble and insoluble fractions of the target protein were analyzed with SDS-PAGE.

3.20 Purifications of the recombinant proteins

The Probond^{TM} Purification System (Invitrogen, USA) and Ni-NTA resins were used to purify the recombinant proteins according to the manufacturers' instructions. Recombinant GRA2 protein was purified under Native condition. Cell lysate was prepared under Native condition prior to purification steps. Eight ml of Native Binding Buffer was added to resuspend the cell pellet (section 3.19) and 8 mg of lysozyme was added into the cell suspension with a 30 min incubation period on ice, followed by sonication on ice with six 10 s pulses at high intensity. Following sonication, the cell lysate was separated from the cellular debris by centrifugation at 3,000 x g for 15 min and poured into a column with resin for 60 min to allow binding. After 60 min of binding, the supernatant was aspirated. The column was washed with 8 ml of Native Wash Buffer four times. The supernatant was aspirated after each washing step. After

the last wash, the rGRA2 protein was eluted from the nickel resin with 5 ml of Native Elution Buffer.

On the other hand, recombinant GRA5 protein was purified under Denature condition. Briefly, cell lysate of the recombinant protein was prepared under denaturing condition prior to the purification steps. The cell pellet (section 3.19) obtained was resuspended in 8 ml of Guanidine Lysis Buffer and rocked slowly for 5 to 10 min at RT to ensure thorough cell lysis, followed by sonication on ice with three 5-second pulses (high intensity). After sonication, the lysate was separated from cellular debris by centrifugation at 3,000 x g for 15 min, added to a column with resin, and allowed to bind for 30 min. The supernatant was aspirated after 30 min of binding. The column was washed with three types of buffer (4 ml each wash) continuously, which were Denaturing Binding Buffer for two times, Denaturing Wash Buffer with pH 6.0 and pH 5.3 for two times respectively. The supernatant was aspirated after each washing step. After the last wash, recombinant GRA5 protein was eluted from the nickel resin with 5 ml of Denaturing Elution Buffer before being subjected to dialysis against PBS overnight at 4˚C.

BL21 (DE3) pLysS carrying the empty pRSET B vector was used as a negative control for both expression and purification. The concentrations of purified recombinant proteins were measured with Bradford Assay Kit (section 3.22) while their identities were further confirmed by MALDI-TOF mass spectrometry (MS) (section 3.24-3.25) as well as WB against human serum and monoclonal antibody (section 3.26, 3.26.2.1 and 3.26.2.2).

3.21 Dialysis

Purified rGRA2 and rGRA5 proteins were dialysed using Slide-A-Lyzer® G2 Dialysis Cassettes (Pierce, USA) with membrane molecular weight cutoff value of 10

kDa and 7 kDa respectively, against PBS to remove unnecessary salts. The dialysis cassette was hydrated by immersing the membrane in PBS for 2 min before the protein sample was loaded in with the aid of a syringe connected to a needle. Dialysis cassette together with the protein sample was immersed in PBS with constant stirring at 4˚C. Two hours later, the dialysis buffer was replaced with fresh buffer for two times after every 2 h and was continued overnight. Dialyzed protein was collected from the cassette the next day with a new syringe with needle. The collected protein sample was then analysed by SDS-PAGE and quantitated before used.

3.22 Protein Quantitation

Protein concentration was estimated using Quick Start™ Bradford Protein Assay Kit. Pre-diluted Bovine Serum Albumin (BSA) with 7 concentrations (2.0, 1.5, 1.0, 0.75, 0.5, 0.25 and 0.125 mg/ml) provided in the kit was used as the protein standard. Ten µl of each standard/sample was mixed well with 500 µl of pre-warmed Quick Start™ Bradford 1X dye reagent in 1.5 ml micro-centrifuge tube and was incubated at RT for 5 min. The mixture was aliquoted into 96-well flat bottom microplate (TPP, Switzerland) in duplicate with 250 µl in each well. The absorbance was read at 595 nm with Infinite® M200 PRO NanoQuant Microplate Reader (Tecan, Switzerland). PBS was employed as blank sample. Protein concentration was calculated based on the standard curve plotted (APPENDIX B).

3.23 SDS-PAGE

SDS-PAGE was conducted based on the discontinuous method of Laemmli (1970). Protein sample was denatured when heated with sample buffer containing SDS and β-mercaptoethanol.

3.23.1 Preparation of SDS-PAGE gel

A rectangular 8.3 cm x 10.2 cm glass plate and a 7.3 cm x 10.2 cm notched plate separated by 0.75 mm spacers were assembled and clamped to the gel caster. Resolving gel solution was pipetted into the assembled glass plates and a few drops of isopropanol were layered onto the gel in order to achieve an even surface. Polymerization occurred within 30-40 min. Stacking gel solution was layered on top of the polymerized resolving gel and a comb was inserted immediately. The gel was ready to use upon polymerization of the stacking gel or could be stored in 4˚C up to 14 days.

3.23.2 Preparation of protein sample and running of SDS-PAGE gel

Protein sample was mixed well with sample buffer in 1:1 ratio and was boiled for 5-10 min. Polymerized gel (section 3.23.1) was placed into the running chamber of the electrophoresis apparatus, as described in the instruction manual of the Mini-Protean II (Bio-Rad, USA). The running chamber was filled up with 1X SDS running buffer followed by loading of boiled sample into the gels. Electrophoresis was carried out at constant voltage of 120 V with PowerPac Basic Power Supply (Bio-Rad, USA) and was halted when the bromophenol blue marker reached 1 cm from the bottom (45-60 min) of the glass plates.

3.23.3 Staining and de-staining of the SDS gel

Following electrophoresis, the SDS gel was stained with fresh CBB R-250 for 10-15 min or re-used stain for 1-2 h. The stained gel was rinsed in ddH_2O to remove excess stain before soaking in the de-staining solution until clear bands were visible on the gel. All steps were carried out at RT with gentle shaking.

3.24 In-gel tryptic digestion of the purified proteins

Affinity purified recombinant protein band of interest was excised from the Coomassie-stained gel (based on size) (section 3.23) and further de-stained with 50 µl of 50% ACN in 50 mM NH_4HCO_3. This step was repeated several times (15–20 min washes, discarding the de-staining solution after each wash) until the excised gel was completely de-stained. The protein-containing gel plug was then incubated with 150 µl of 10 mM DTT in 100 mM NH_4HCO_3 for 30 min at 60˚C. The gel was subsequently cooled to RT, the DTT solution was discarded, and the band was incubated with 150 µl of 55 mM IAA in 100 mM NH_4HCO_3 in the dark for 20 min. The gel plug was then washed four times with 50% ACN in 50 mM NH_4HCO_3 (500 µl washes, 20 min each), dehydrated via incubation with 50 µl of 100% ACN for 15 min, and subjected to speed vacuum for 15 min at ambient temperature to remove the ACN. The gel plug was then incubated with 25 µl of trypsin (6 ng/µl) in 50 mM NH_4HCO_3 at 37˚C. Following overnight digestion, 50 µl of 50% ACN was added to the gel plug, and it was incubated for 15 min in order to disintegrate the trypsin enzyme and extract protein from the gel plug. The resulting liquid (containing the digested protein) was transferred into a new tube. The gel plug was further incubated with another 50 µl of 100% ACN for 15 min and the liquid was added to the liquid in the new tube. The protein-containing solution was then dried completely via speed vacuum. Prior to MALDI-TOF MS analysis, the protein sample was reconstituted in 10 µl of 0.1% formic acid and desalted using a Zip-Tip. For this, the Zip-Tip membrane was wetted and equilibrated with 50% ACN and 0.1% formic acid respectively. The protein sample was bound onto the Zip-Tip membrane which was washed with 0.1% formic acid. Finally, the protein was eluted with 0.1% formic acid in 50% ACN and analyzed by MALDI-TOF MS.

3.25 MALDI-TOF MS analysis

The Zip-Tip–eluted protein sample was mixed with matrix solution provided by UMCPR staff at a 1:1 ratio before spotting onto the MALDI plate. The analysis was carried out by University Malaya Center for Proteomics Research (UMCPR).

3.26 WB

3.26.1 Electrophoretic trans-blotting of proteins

WB was based on the method of Towbin *et al.* (1979), adopting electrical current for eluting proteins from polyacrylamide gel and transferred onto WB membrane such as nitrocellulose or PVDF membrane. Semi-dry blotting which was first reported by Kyhse-Andersen in 1984 was used in this study through Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, USA) where the blotting was performed with plate electrodes in a horizontal configuration. The unit was assembled and operated according to the instruction manual provided by the manufacturer.

Following electrophoresis, the SDS gel (section 3.23), methanol-activated PVDF membrane and Whatman 3MM filter papers were soaked in semi-dry transfer buffer for 15 min. PVDF membrane and SDS gel were then sandwiched between filter papers placed on the platinum anode as depicted in (Figure 3.3). Excessive buffer and air-bubbles were removed with a roller. The cathode and safety cover unit were placed onto the stack before carrying out the transfer for 30-40 min at a constant voltage of 15 V.

3.26.2 Detection of the trans-blotted proteins

3.26.2.1 Detection with human serum

Protein blotted-PVDF membrane was soaked in 5% blocking buffer for 2 h and was subsequently probed with primary antibody; diluted human serum sample (1:200)

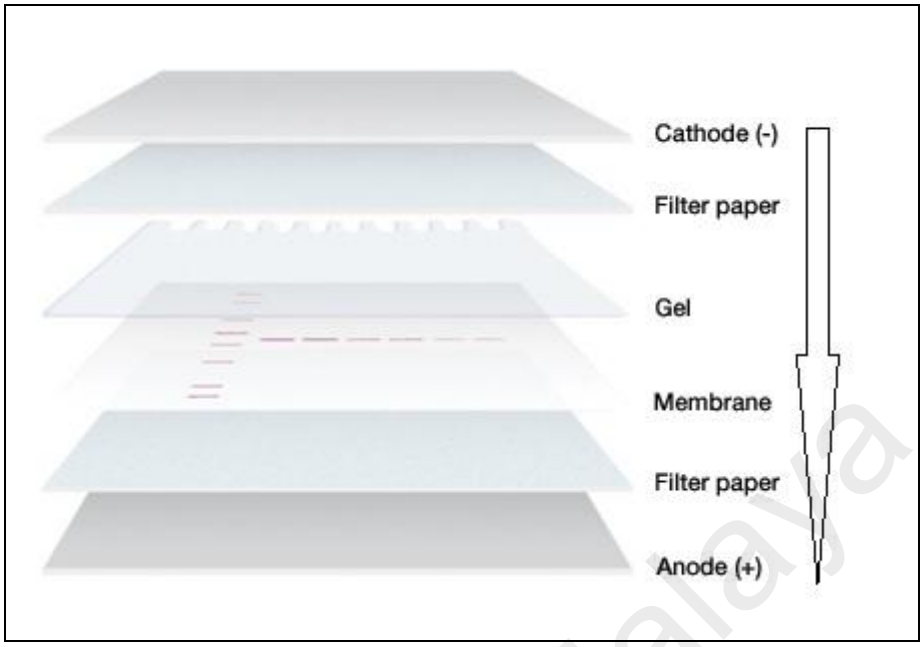

Figure 3.3: Arrangement of western blot components (adapted from http://www.bio-rad.com/en-my/applications-technologies/protein-blotting-methods#3).

for 2-3 h. The membrane was then incubated for 1 h with secondary antibody; biotinylated goat anti-human IgG (1:2500) and then with streptavidin-alkaline phosphatase (1:2,500) for another 1 h. Antibody was diluted in 2.5% blocking buffer. The membrane was washed with 0.002% TBS-T after each incubation step for 30 min (six times with 5 min each). The membrane was eventually developed with NBT/BCIP as the chromogenic substrate. The entire incubation steps were performed at RT with constant gentle shaking. The developed membrane was rinsed with ddH_2O for 2-5 min to stop the enzymatic reaction once protein bands were visible in order to prevent non-specific background coloration. The membrane was air-dried before keeping.

3.26.2.2 Detection with monoclonal antibody

Section 3.26.2.1 was repeated with different antibody. Primary and secondary antibodies involved in this section were Xpress mouse monoclonal antibody (Anti-Xpress™) and biotin-labeled goat anti-mouse IgG respectively. The Anti-Xpress™ antibody allowed detection of the expression of N-terminal Xpress™ fusion proteins from bacterial, insect, and mammalian cells, recognizing the Xpress™ epitope sequence Asp-Leu-Tyr-Asp-Asp-Asp-Asp-Lys.

3.26.2.3 Detection with mouse serum

Primary and secondary antibodies involved in this section were mouse serum collected from non-immunized or immunized mouse through tail-bleeding and biotin-labeled goat anti-mouse IgG respectively.

3.27 Evaluation of sensitivity and specificity of the recombinant proteins

Diagnostic sensitivity and specificity of the recombinant proteins were evaluated by WB analysis (section 3.26) using sera from both toxoplasmosis-diagnosed patients

and toxoplasmosis-negative individuals. Toxoplasmosis cases were divided into three groups: (1) patients with early acute toxoplasmosis (IgM positive, IgG negative); (2) patients with acute toxoplasmosis (IgM positive, IgG positive); and (3) patients with chronic toxoplasmosis (IgM negative, IgG positive). A fourth group was comprised of toxoplasmosis-negative healthy serum samples (IgM negative, IgG negative). These human serum samples were grouped based on results obtained from Novalisa™ *Toxoplasma gondii* IgG and *Toxoplasma gondii* IgM ELISA kits. In addition, specificity of the recombinant proteins were determined using serum samples from patients diagnosed with amoebiasis (3 samples), cysticercosis (3 samples), filariasis (3 samples), and toxocariasis (3 samples). Diagnosis of these infections was carried out using serological tests for the respective infections. All serum samples were obtained from the Diagnostic Laboratory at the Department of Parasitology, University of Malaya. Sensitivity and specificity of the screening were calculated and tabulated, with the following formulae:

$$\text{Sensitivity } (\%) = \frac{\text{Number of true positive}}{\text{Number of true positive} + \text{Number of false negative}} \times 100$$

$$\text{Specificity } (\%) = \frac{\text{Number of true negative}}{\text{Number of true negative} + \text{Number of false positive}} \times 100$$

3.28 Classification of the serological status of human sera

Serological status of human serum samples was categorized through Novalisa™ *Toxoplasma gondii* IgM μ-capture and *Toxoplasma gondii* IgG ELISA kits.

3.28.1 Novalisa™ *Toxoplasma gondii* IgM μ-capture

This kit allows qualitative determination of IgM against *T. gondii*. The assay is a Sandwich ELISA (Figure 3.4) and was performed according to manufacturer's manual.

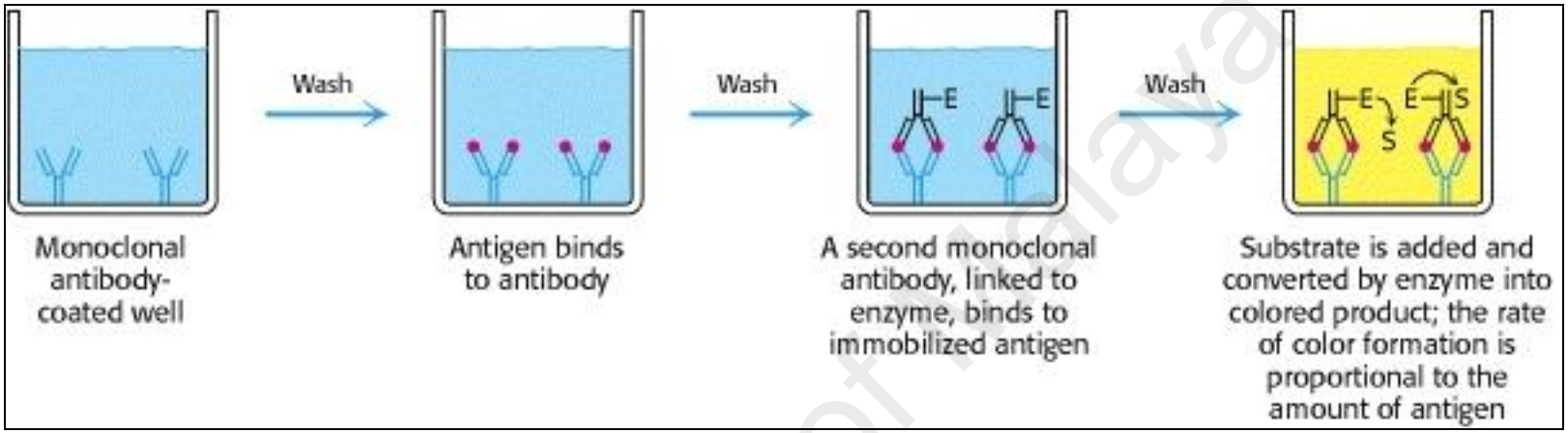

Figure 3.4: Sandwich ELISA (adapted from https://exploreable.files.wordpress.com/2011/05/ch4f35.jpg).

The microtiter wells are pre-coated with anti-human IgM antibody. A substrate blank, negative control, positive control and cut-off control were run with each assay. Briefly, samples were diluted 100X with Sample Diluent. One hundred µl of the controls and diluted serum samples were dispensed into respective wells (in duplicate) and incubated at 37˚C for 1 h. An hour later, the contents were aspirated and the wells were washed three times with 300 µl washing buffer. One hundred µl *T. gondii* Conjugate was dispensed into all wells except the blank well before incubation at 37˚C for another hour. The wells were washed thrice again followed by incubation with 100 µl TMB Substrate Solution for exactly 30 min at RT in dark. The enzymatic reaction was eventually halted by addition of 100 µl Stop Solution. Absorbance was measured at 450 nm with Infinite® M200 PRO NanoQuant Microplate Reader (Tecan, Switzerland).

3.28.2 Novalisa™ *Toxoplasma gondii* IgG

This kit allows quantitative determination of IgG against *T. gondii*. The microtiter wells provided are pre-coated with inactivated *T. gondii* antigens. The assay is an Indirect ELISA (Figure 3.5) and was performed according to the manufacturer's manual. A substrate blank, standards A, B, C and D were run with each assay. Briefly, samples were diluted 100X with Sample Diluent. One hundred µl of the each standard and diluted serum samples were dispensed into respective wells (in duplicate) and incubated at 37˚C for 1 h. An hour later, the contents were aspirated and the wells were washed three times with 300 µl washing buffer. One hundred µl *Toxoplasma* anti-IgG Conjugate was dispensed into all wells except the blank well before incubation at RT for 30 min. The wells were washed thrice again followed by incubation with 100 µl TMB Substrate Solution for exactly 15 min at RT in dark. The enzymatic reaction was

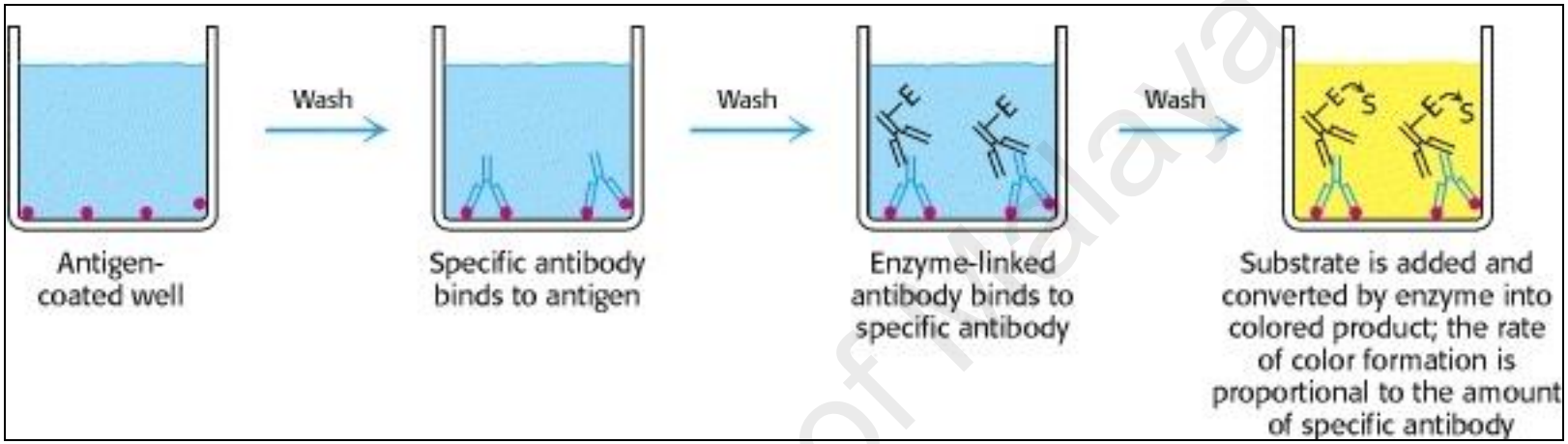

Figure 3.5: Indirect ELISA (adapted from https://exploreable.files.wordpress.com/2011/05/ch4f35.jpg).

eventually halted by addition of 100 µl Stop Solution. Absorbance was measured at 450 nm.

3.28.3 Novalisa™ *Toxoplasma gondii* IgG avidity

IgG avidity refers to the binding affinity between IgG in serum samples and the pre-coated *T. gondii* antigens. Low avidity reflects the early stage of infection while high avidity correlates past infection. Generally, the entire steps in section 3.28.2 were repeated except for the conjugate incubation step whereby one well was incubated with 100 µl Avidity reagent and the other with 100 µl of diluted washing solution instead of *Toxoplasma* anti-IgG Conjugate for 5 min at RT.

3.29 Immunization of mice with recombinant proteins

Six- to eight-week old female inbred BALB/c mice were divided into 4 immunization groups with 13 mice in each group. First two groups of mice were given subcutaneous injection with two negative controls; PBS and vector protein while the other two groups with 10 µg purified recombinant proteins; rGRA2 and rGRA5. Following prime injection at the mice tail base, another two boosters were administered with the same protein dose at both sides of the mice at two weeks intervals. Adjuvants (CFA for prime injection whereas IFA for remaining two boosts) were emulsified with the injection samples at 1:1 ratio through vortexing before immunizing the mice. Blood (50-100 µl) were collected from the immunized mice through tail-bleeding on day 0, 14, 28 and 42.

3.30 Evaluation of humoral responses

Blood samples collected from mice were allowed to coagulate overnight at 4˚C followed by sedimentation at 4,000 rpm for 20 min at same temperature to harvest the

serum samples. Mice serum samples harvested were then analysed by WB assay and ELISA against the respective purified recombinant proteins or *T. gondii* TLA in order to evaluate the antigen-specific humoral immune response. Presence of antigen-specific IgG antibodies and the titers were determined by WB (section 3.26 and 3.26.2.3) and in-house ELISA respectively.

3.30.1 IgG titer and subclass determination

The 96-well flat bottom microplate was coated overnight at 4˚C with 10 µg/ml TLA, rGRA2 or rGRA5 diluted in 100 µl coating buffer. The antigen solutions were aspirated and the wells were washed three times with 0.05% PBS-T after overnight incubation. The subsequent incubation steps were all carried out at 37˚C. Non-specific binding sites of the wells were blocked by incubation with 200 µl of 10% blocking buffer for 1 h. The blocking buffer was then aspirated and the plate was washed thrice followed by incubation with 100 µl of serially diluted mice sera (control and vaccinated) for another 1 h to determine the optimal working dilution. The plate was washed again in the same way. Bound antigen-specific IgG was detected through incubation with 100 µl of diluted HRP-conjugated goat anti-mouse IgG (1:2000) for 1 h. The plate was washed five times and the enzymatic reaction was developed by addition of 100 µl 3,3',5,5'-Tetramethylbenzidine (TMB), a chromogenic substrate and was incubated for 10-15 min at RT. The reaction was eventually stopped with 2 M of sulphuric acid and the absorbance was measured at 450 nm with microplate reader. Primary and secondary antibodies were diluted in 10% blocking buffer. All samples were run in triplicates. Vaccinated mice sera were considered positive if the mean optical density (OD) of triplicate determinations was greater than the cut-off limit of the negative control groups; cut-off = mean OD + 2(standard deviation). The entire steps were repeated for IgG

subclass determination assay whereby different secondary antibodies involved were HRP-conjugated goat anti-mouse IgG1 and IgG2a.

3.30.2 *In vitro* splenocyte proliferation assay

Three mice per group were euthanized with CO_2 and spleens were harvested aseptically two weeks after final immunization (section 3.29). Single cell suspension was prepared by mashing the spleen over a 70 μm cell strainer on a petri dish with 10 ml RPMI complete medium and a syringe plunger. The cell suspension was transferred and passed through the cell strainer fitted to the 50 ml polypropylene tube. The petri dish was rinsed with another 5 ml complete medium which was then passed through the same cell strainer. The collected cell suspension was centrifuged to remove the supernatant while the pellet was resuspended and incubated with ACK lysis buffer for 5 min at RT in order to lyse the red blood cells. Five min later the cell suspension was topped up with 20 ml complete medium and mixed well before centrifuged. The supernatant was discarded while the pellet was resuspended in 10 ml complete medium and was centrifuged again. Finally, the pellet obtained was resuspended in another 10 ml complete medium. All centrifugation steps were performed at 1,500 rpm for 10 min.

Splenocytes were cultured in 96-well flat bottom microplate with cell density of 2×10^5 cells/well (section 3.6.3) in triplicates. The cells were induced with culture medium alone (negative control) or with 10 μg/ml TLA, rGRA2 or rGRA5 or with 5 μg/ml conA (positive control) and were incubated at 37˚C in a 5% CO_2 incubator for 24, 72 and 96 h. ConA is potent mitogen therefore personal protective equipment (PPE) such as protective gloves and goggles should be worn whenever handling the chemical in order to prevent direct contact with skin and eyes. Besides, prolonged exposures and breathing dust of conA should be avoided.

Splenocytes proliferation was analysed with MTT Cell Proliferation Kit. After 72 h of incubation, MTT labeling reagent was added into each well of cells and incubated for an additional of 4 h followed by 100 µl Solubilization solution for further overnight incubation. The plate was read at 570 nm with microplate reader the next day.

$$\text{Stimulation index (SI)} = \frac{\text{mean } OD_{570} \text{ values of stimulated cells}}{\text{mean } OD_{570} \text{ values of unstimulated cells}}$$

3.30.3 Cytokine assays

Splenocytes cultured and incubated at different time point (24, 72 and 96 h) (section 3.30.2) were subjected to centrifugation at 2,000 x g for 20 min. Culture supernatants were collected for various cytokine assays such as IFN-γ, IL-2, IL-4 and IL-10 assays. These assays were based on Sandwich ELISA (Figure 3.4) and were performed in accordance with the instruction's manuals.

3.30.3.1 IFN-γ assay

Desired numbers of anti-mouse IFN-γ precoated strips were placed in a microwell frame. The assay was performed at RT. Serially diluted standards or samples (50 µl) were added into each well in duplicate for 2 h incubation followed by addition of 50 µl Biotinylated Antibody Reagent and were incubated for another 1 h. The plate content was discarded and was washed three times with 400 µl Wash Buffer before incubation with 100 µl of diluted Streptavidin-HRP Solution for 30 min. The plate was washed thrice the same way and 100 µl of TMB substrate was added into each well. Enzymatic reaction was developed in dark for 30 min before terminated by 100 µl of Stop Solution. The absorbance was read at 450 nm with microplate reader. Concentration of IFN-γ for each test samples were calculated based on the standard curve generated (APPENDIX C). Sensitivity limit for this assay was 10 pg/ml.

3.30.3.2 IL-2 assay

Desired numbers of anti-mouse IL-2 precoated strips were placed in a microwell frame. Plate Reagent (50 µl) was added into each well followed by another 50 µl of serially diluted standards or samples in duplicate for 2 h incubation in a 37˚C humidified incubator. Two hours later, the plate content was discarded and was washed five times with 400 µl of Wash Buffer. After washing, 100 µl of diluted Conjugate Reagent was added and was incubated at 37˚C for 1 h. The plate was washed five times the same way before addition of 100 µl TMB substrate into each well. Enzymatic reaction was developed in dark for 30 min at RT before terminated by 100 µl Stop Solution. The absorbance was read at 450 nm with microplate reader. Concentration of IL-2 for each test samples were calculated based on the standard curve generated (APPENDIX D). Sensitivity limit for this assay was 3 pg/ml.

3.30.3.3 IL-4 assay

Entire steps in section 3.30.3.2 were repeated for IL-4 assay. Anti-mouse IL-4 precoated strips and the respective antibody solutions as provided were used. Concentration of IL-4 for each test samples were calculated based on the standard curve generated (APPENDIX E). Sensitivity limit for this assay was 5 pg/ml.

3.30.3.4 IL-10 assay

Desired numbers of anti-mouse IL-10 precoated strips were placed in a microwell frame. This assay was performed at RT. Assay Buffer (50 µl) was initially added into each well followed by addition of serially diluted standards or samples (50 µl) in duplicate for 3 h incubation. Three hours later, the plate content was emptied and washed three times with 400 µl Wash Buffer. Premixed Biotinylated Antibody Reagent (50 µl) was then added and the plate was incubated for 1 h. The plated was washed the

same way followed by 30 min incubation with 100 µl of diluted Streptavidin-HRP Solution. The plate was washed again before adding 100 µl of TMB substrate into each well. Enzymatic reaction was developed in dark for 30 min before terminated by 100 µl Stop Solution. The absorbance was read at 450 nm with microplate reader. Concentration of IL-10 for each test samples were calculated based on the standard curve generated (APPENDIX F). Sensitivity limit for this assay was 12 pg/ml.

3.31 Mice challenge

The remaining vaccinated and control mice were subjected to lethal parasitic challenge study through intraperitoneal injection of 1×10^3 live tachyzoites of *T. gondii* virulent RH strain in 100 µl PBS (section 3.7 and 3.6.3). Mortality rate of the mice were monitored and recorded daily. Dose of tachyzoites used was based on previous studies.

3.31.1 Statistical analysis

Significance levels of the differences between groups of mice were analysed through Student's t-test or analysis of variance (ANOVA). $P < 0.05$ indicates statistical significance. The survival rate was calculated based on χ^2 (chi-square) test while the survival graph was drawn based on Kaplan-Meier method (Kaplan & Meier, 1958).

3.32 Constructions of recombinant pcDNA 3.1C-GRA2 and pcDNA 3.1C-GRA5

The entire steps depicted under section 3.16 were repeated for cloning these two same target genes into pcDNA 3.1C and propagated in TOP10F' cells. The selected positive recombinant clones of pcDNA 3.1C-GRA2 and pcDNA 3.1C-GRA5 were used for *in vitro* (transfection into CHO cells) and *in vivo* (immunization of mice) studies. Empty vector, pcDNA 3.1C was employed as negative control.

3.33 Endotoxin-free DNA plasmid purification

DNA plasmids of the selected positive clones of pcDNA 3.1C-GRA2, pcDNA 3.1C-GRA5 and the empty vector were isolated according to the manufacturer's protocol of EndoFree® Plasmid Giga kit. Briefly, single colony was inoculated into 3-5 ml LB broth containing 100 µg/ml of ampicillin and was incubated for 8 h with constant shaking (300 rpm) at 37˚C. The starter culture was then diluted 1000X with the same growing medium for an overnight incubation.

Overnight culture was sedimented at 6,000 x g for 15 min at 4˚C the next day. Bacterial pellet obtained was resuspended in 125 ml Buffer P1 before mixing with 125 ml Buffer P2 and was incubated at RT for 5 min to ensure complete cell lysis. The lysate obtained was neutralized with 125 ml chilled Buffer P3 through vigorous inversion. The lysate was then decanted into a QIAfilter Cartridge which was fitted to a glass bottle and connected to a vacuum system. The lysate was allowed to incubate in the cartridge for 10 min before being filtered through the cartridge by switching on the vacuum. Another 50 ml Buffer FWB2 was added and filtered through the same cartridge. The clear filtrate was then mixed with 30 ml Buffer ER by 10X inversions before incubated on ice for 30 min. At the same time, QIAGEN-tip 10000 column was equilibrated with 75 ml Buffer QBT. The column was emptied by gravity flow. After 30 min incubation on ice, the filtrate was poured into the equilibrated column. The emptied column was washed with 600 ml Buffer QC followed by elution of bound DNA from the resin with 100 ml Buffer QN. Eluted DNA was precipitated with 70 ml isopropanol and was centrifuged at 16,000 x g for 30 min at 4˚C. Sedimented DNA pellet was washed with endotoxin-free 70% ethanol followed by centrifugation for 10 min. Ethanol was discarded while DNA pellet obtained was air-dried for 10-20 min before dissolving in PBS. DNA plasmid purity and concentration were determined (section 3.10) before used.

3.34 DNA plasmid transfection

Six-well flat bottom microplate was seeded with of 0.8-2.4 × 10^5 CHO cells (section 3.6.3) in 4 ml of DMEM complete medium 24 h before transfection. When the cells reached 70-90% confluence, 4 µg of the isolated endotoxin-free DNA plasmid (section 3.33) was diluted in 400 µl of serum-free DMEM. Diluted DNA plasmid was then mixed immediately with 6 µl Turbofect™ Protein Transfection reagent through vortexing. The Turbofect™/DNA mixture was incubated for 20 min at RT before adding 400 µl of the mixture into each well containing CHO cells. The cells were then incubated in CO_2 incubator at 37˚C for 24-48 h before harvested for the analysis of recombinant protein expressions by WB (section 3.26 and 3.26.2.2).

3.35 Immunization of mice with DNA plasmids

Section 3.29-3.31 was repeated but with several changes. In DNA vaccination study, four different groups of BALB/c mice were given intramuscular injection at tibialis anterior muscle of both leg with 100 µl (50 µl in each leg) of PBS (negative control), empty vector (negative control), 100 µg of pcDNA 3.1C-GRA2 and 100 µg of pcDNA 3.1C-GRA5. A total of three injections were carried out at three weeks interval. Blood samples (50-100 µl) were collected from the injected mice through tail-bleeding on day 0, 21, 42 and 63.

CHAPTER 4: RESULTS

4.1 *T. gondii* propagation

The tachyzoites of *T. gondii* RH strain were used as the starting materials for obtaining genomic DNA, RNA, total lysate antigen or live parasites throughout the study. Laboratory maintenance of the parasites has been routinely carried out by *in vitro* propagation in HFF cells (Figure 4.1). The parasites successfully invaded and multiplied within the host cells 24 h post-infection. Rupturing of the infected cell occurred when ~64-128 tachyzoites (Radke & White, 1998) were accumulated in each infected cell, thereby freeing more tachyzoites to infect neighboring host cells. It took about 72-96 h for the whole flask of HFF cells to be infected and erupted in order to release up to 10^7 parasites. Meanwhile, *T. gondii* tachyzoites were also propagated in mice in order to maintain the parasites' viability. The harvested intraperitoneal fluid from the infected mice usually contains up to 10^7 parasites about 3 to 4 days post-infection.

4.2 PCR amplifications of *GRA2* and *GRA5* gene fragments

RT-PCR amplification of *GRA2* (RNA as template) and PCR amplification of *GRA5* (DNA as template) generated PCR products with expected sizes of 486 bp and 285 bp, respectively upon analysis on 1% agarose gel (Figure 4.2). The primers used were designed to include *Eco*RI restriction sites in both 5' and 3' end of GRA2 and GRA5 gene fragments in order to facilitate cloning into the expression vectors. The forward primers did not include start codon at the 5' end of both gene sequences, allowing genes translations to initiate from the expression vectors incorporating the N-terminal polyhistidine tag and Xpress™ epitope sequences.

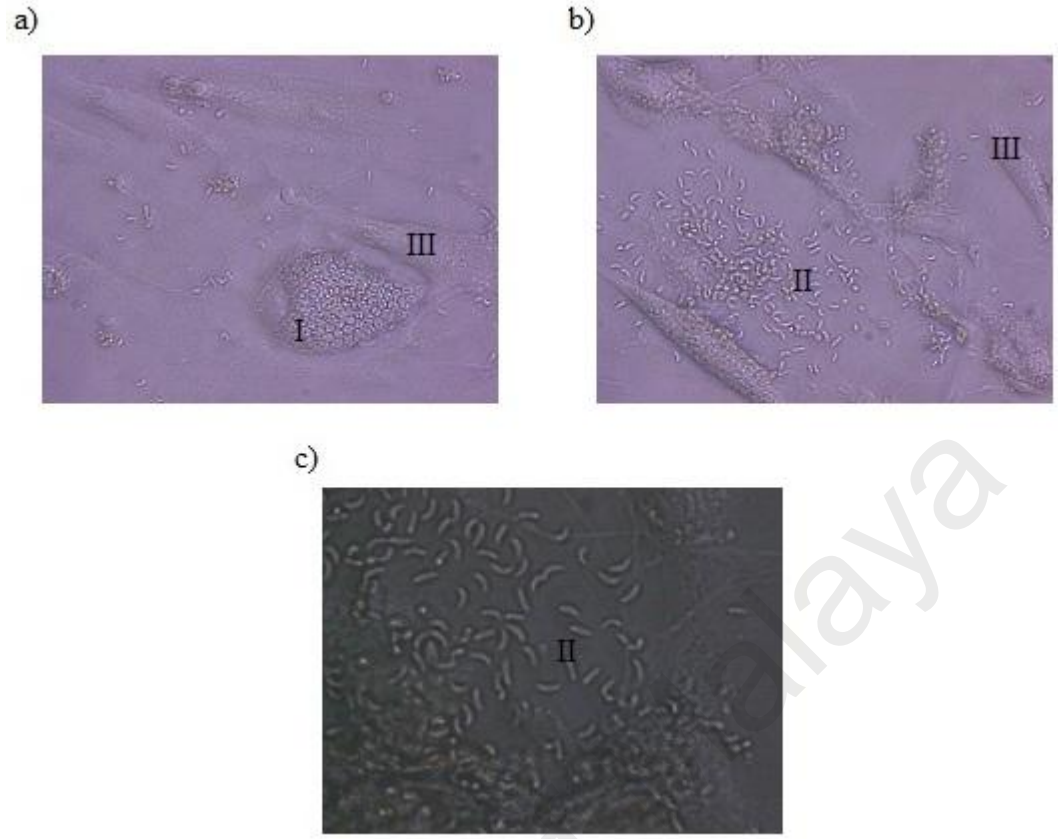

Figure 4.1: ***T. gondii*** **propagation in HFF cells.** Tachyzoites-infected HFF cells imaged by inverted microscope at magnification of 10x (a and b) and 40x (c). I) Multiplication of tachyzoites within HFF cell; II) Rupture of HFF cell leading to the release of tachyzoites; III) uninfected HFF cell.

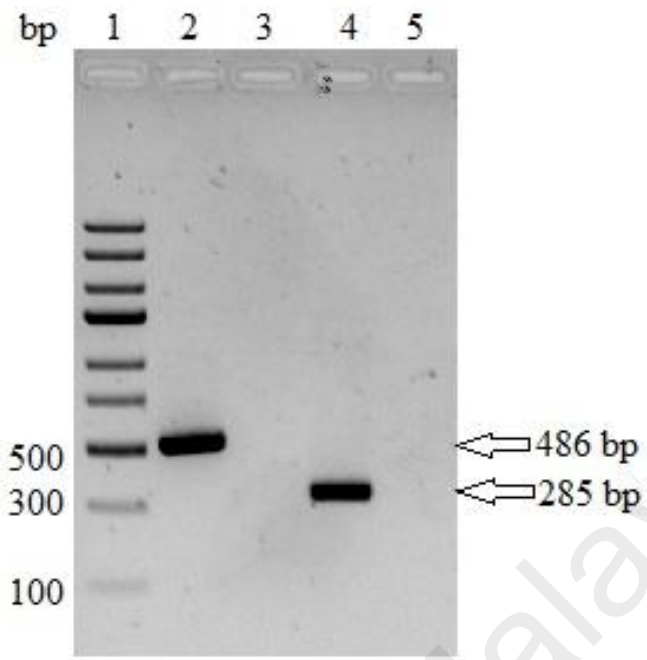

Figure 4.2: PCR amplifications of *GRA2* and *GRA5*. Agarose gel electrophoresis analysis of RT-PCR amplified GRA2 and PCR amplified GRA5 using *T. gondii* RNA and genomic DNA as template respectively. Lane 1 contained GeneRuler Express DNA Ladder. Lane 2 and 4 contained amplified GRA2 and GRA5 products with expected sizes of 486 bp and 285 bp respectively (arrow). Lane 3 and 5 contained negative controls with double distilled water as template.

4.3 Constructions of recombinant pGEM®-T-GRA2 and pGEM®-T-GRA5

Amplified GRA2 and GRA5 fragments were cloned into pGEM®-T cloning vector and propagated in TOP10F' cells before introducing the fragments into expression vectors. Deoxyadenosine (A) residues added at the 3' ends of GRA2 and GRA5 gene sequences by *Taq* DNA polymerase were used to ligate into the pGEM®-T cloning vector having 3'-thymidine (T) overhang residues through the A-T complementary pairing. Transformed TOP10F' colonies with the ligated products were plated on ampicillin-containing LB agar plate. Single isolated TOP10F' white colonies were picked and plated onto another fresh ampicillin-containing LB agar plate for further characterization. Selected TOP10F' colonies were characterized through colony PCR, *Eco*RI restriction digestion as well as automated DNA sequencing with M13 primers set. Colony PCR amplification on TOP10F' colonies involved gene-specific primers set and produced PCR products with expected sizes of 486 bp and 285 bp for target gene of *GRA2* and *GRA5* respectively as illustrated in Figure 4.3. The selected positive clones that were digested with *Eco*RI generated both GRA2 or GRA5 fragment and linearized pGEM®-T vector as shown in Figure 4.4. Meanwhile, sequencing analysis of the recombinant pGEM®-T clones harboring GRA2 and GRA5 inserts revealed that the cloned genes shared 100% identity with the published GRA2 (Accession number: L01753.1) and GRA5 (Accession number: EU918733.1) sequences respectively (APPENDIX G). The deduced amino acids of GRA2 and GRA5 translated proteins were determined (APPENDIX H). M13 primers used are the universal primers of pGEM®-T vector.

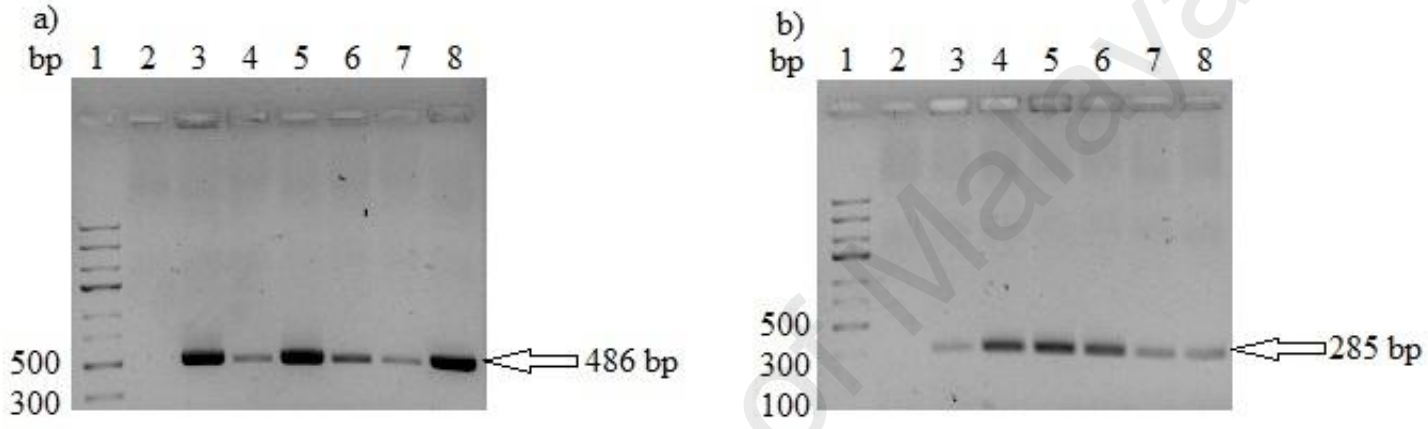

Figure 4.3: Colony PCR amplification of recombinant pGEM®-T clones in TOP10F'. Agarose gel electrophoresis analysis of colony PCR amplified a) pGEM®-T-GRA2 and b) pGEM®-T-GRA5 using gene-specific primers. Lane 1 (panel a - b) contained GeneRuler Express DNA Ladder. Lane 2 (panel a - b) contained negative control with double distilled water as the template. Lane 3 (a) to 8 (a) contained positive colonies 1 to 6 with expected size of 486 bp (arrow). Lane 3 (b) to 8 (b) contained positive colonies 1 to 6 with expected size of 285 bp (arrow).

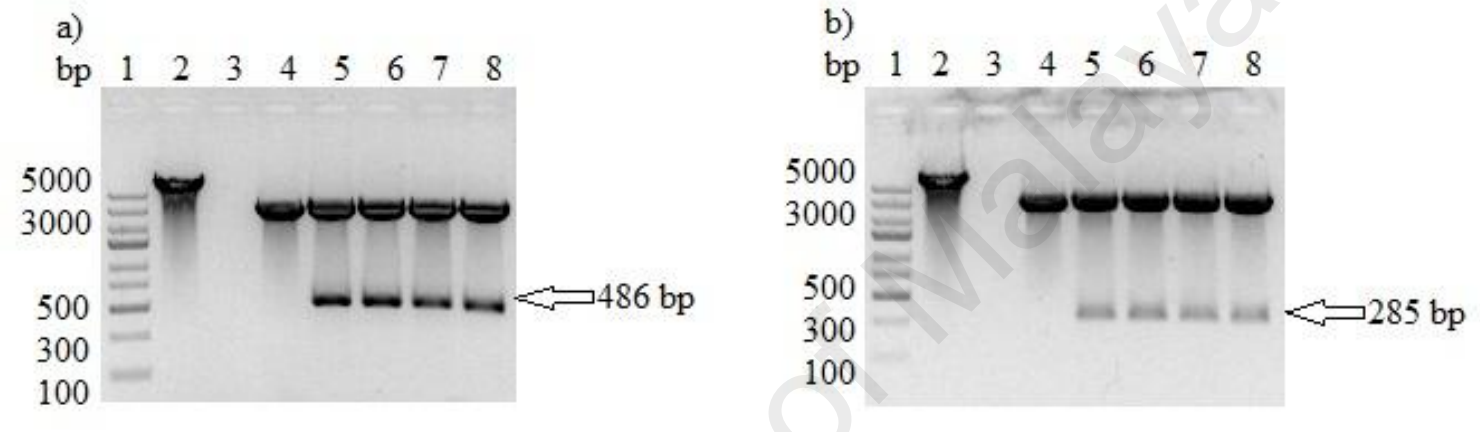

Figure 4.4: Restriction digestion analysis of recombinant pGEM®-T clones. Agarose gel electrophoresis analysis of *Eco*RI digested a) pGEM®-T-GRA2 and b) pGEM®-T-GRA5. *Eco*RI digestion was also performed on two expression vectors; pRSET B and pcDNA 3.1C. Lane 1 (panel a - b) contained GeneRuler Express DNA Ladder. Lane 3 (panel a - b) contained empty lane. Lane 2 (panel a - b) contained digested pcDNA 3.1C vector (5500 bp). Lane 4 (panel a - b) contained digested pRSET B vector (2900 bp). Lane 5 to 8 (panel a - b) contained digested pGEM®-T-GRA2 and pGEM®-T-GRA5 yielding 486 bp and 285 bp inserts (arrow) respectively as well as linearized pGEM®-T vector (3000 bp).

4.4 Constructions of recombinant pRSET B-GRA2 and pRSET B-GRA5

The pRSET vectors are pUC-derived expression vectors designed for high-level protein expression and purification from cloned genes in *E. coli*. The presence of the strong bacteriophage T7 promoter allows high-level protein expression. pRSET vectors are available in three form, namely pRSET A, B and C which have the same sequences encoding the N-terminal fusion peptide but different reading frame respective to the multiple cloning sites to simplify in-frame cloning of the genes of interest. The N-terminal fusion peptide consisted of initiation ATG, six-histidine tag and the Xpress™ epitope. Among the three, pRSET B vector (APPENDIX I) was chosen as both the *GRA2* and *GRA5* inserts were in-frame with the downstream multiple cloning sites and the stop TGA codon. The N-terminal six-histidine tag plays an important role as a metal binding domain for rapid and simple protein purification. Both N-terminal six-histidine tag and Xpress™ epitope are involved in the detection of the expressed protein in western blot analysis by monoclonal Anti-His antibody and monoclonal Anti-Express™ antibody respectively.

Overnight *Eco*RI digestion performed on the positive clones of pGEM®-T-GRA2, pGEM®-T-GRA5 as well as the prokaryotic expression vector, pRSET B produced linearized fragments of GRA2, GRA5 and pRSET B (dephosphorylated) with same sticky ends to enable ligation to occur (Figure 4.4). Successfully transformed TOP10F' colonies with the respective ligated products were selected and grown on ampicillin-containing LB agar plate. Single isolated TOP10F' white colonies were picked and plated onto another fresh ampicillin-containing LB agar plate for further characterization through colony PCR amplification, *Eco*RI restriction digestion analysis and verification by automated DNA sequencing with T7 primers set.

Positive clones with the correct sense orientation were selected through colony PCR amplification of the TOP10F' colonies which involved T7 forward primer and

gene-specific reverse primer. T7 forward primer has a fixed position in the vector sequence, therefore amplification of the target gene depends mainly on its ligation orientation which means that amplification will only occur if the target gene has been ligated in the correct direction. Figure 4.5 showed that such PCR amplification yielded fragments with expected sizes of 704 bp and 503 bp for GRA2 and GRA5 clones respectively. An increase in fragment size indicated inclusion of the upstream region of the expression vector (218 bp) covered by T7 forward primer.

Positive clones were further confirmed through *Eco*RI digestion, thereby releasing a 2900 bp linearized pRSET B vector and a 486 bp of *GRA2* insert or a 285 bp of *GRA5* insert as depicted in Figure 4.6. Meanwhile, sequencing analysis of positive recombinant pRSET B clones harboring *GRA2* and *GRA5* inserts confirmed that the cloned genes shared 100% identity with the published GRA2 (Accession number: L01753.1) and GRA5 (Accession number: EU918733.1) sequences respectively (APPENDIX G). The deduced amino acids of GRA2 and GRA5 translated proteins were determined (APPENDIX H). T7 primer is a universal primer originated from pRSET B vector sequence. The sequence analysis confirmed both gene fragments are in the sense orientation and are in frame with both the *Eco*RI restriction site and stop codon of the vector sequence (Figure 4.7). It also showed that the resulting recombinant pRSET B-GRA2 and pRSET B-GRA5 constructs retained the open reading frame encoding amino acid residues 24-185 and 26-120 of GRA2 and GRA5 proteins respectively, excluding the putative hydrophobic N-terminal signal sequences (APPENDIX G and H).

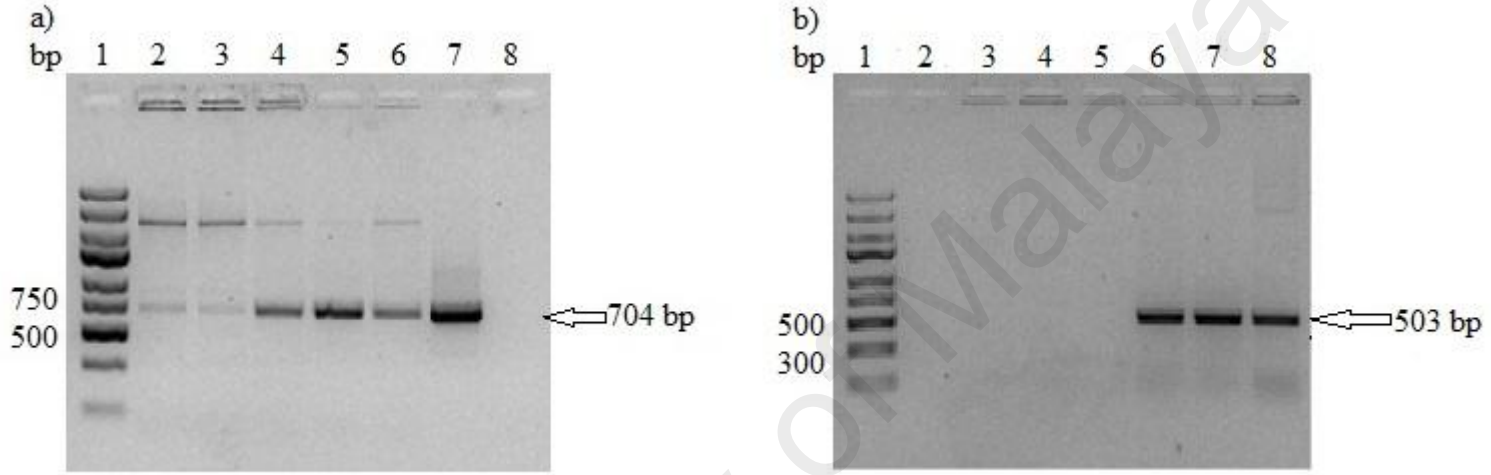

Figure 4.5: Colony PCR amplification of recombinant pRSET B clones in TOP10F'. Agarose gel electrophoresis analysis of directional colony PCR amplified a) pRSET B-GRA2 and b) pRSET B-GRA5 with T7 forward and gene-specific reverse primers. Lane 1 (panel a - b) contained GeneRuler Express DNA Ladder. Lane 8 (a) and 2 (b) contained negative control with double distilled water as template. Lane 2 (a) to 7 (a) contained colony 1 to 6; colony 4 and 6 were selected as positive colonies of 704 bp (arrow). Lane 3 (b) to 8 (b) contained colony 1 to 6; colony 4 and 5 were selected as positive colonies of 503 bp (arrow).

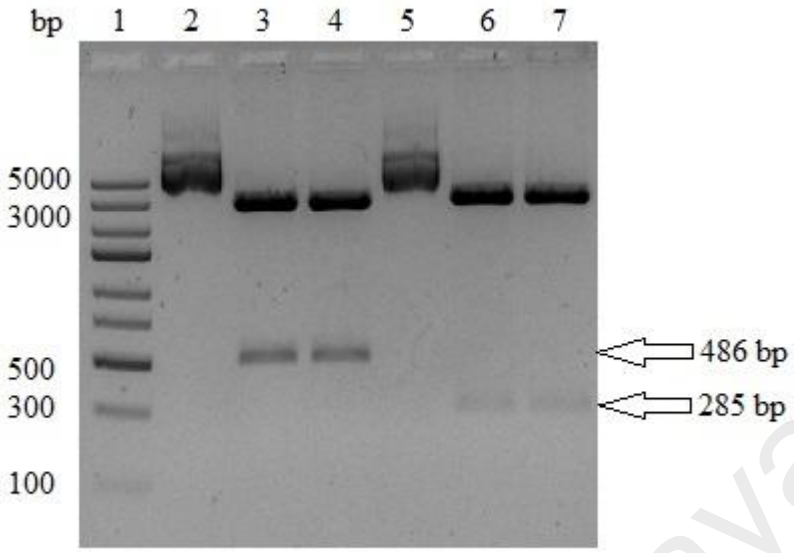

Figure 4.6: Restriction digestion analysis of recombinant pRSET B clones. Agarose gel electrophoresis analysis of *Eco*RI digested pRSET B-GRA2 and pRSET B-GRA5. Lane 1 contained GeneRuler Express DNA Ladder. Lane 2 contained undigested recombinant pRSET B-GRA2 plasmid. Lane 3 and 4 contained digested pRSET B-GRA2 producing a 486 bp insert (arrow). Lane 5 contained undigested recombinant pRSET B-GRA5 plasmid. Lane 6 and 7 contained digested pRSET B-GRA5 yielding a 285 bp insert (arrow). A linearized pRSET B fragment of 2900 bp was produced as well upon *Eco*RI digestion of the positive recombinant clones as shown in lane 3, 4, 6 and 7.

a) pRSET B-GRA2

ATG CGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAT CCG AGC TCG AGA TCT GCA GCT GGT ACC ATG **GAA TTC** GCC GAG TTT TCC GGA GTT GTT AAC CAG GGA CCA GTC GAC GTG CCT TTC AGC GGT AAA CCT CTT GAT GAG AGA GCA GTT GGA GGA AAA GGT GAA CAT ACA CCA CCA CTC CCA GAC GAG AGG CAA CAA GAG CCA GAA GAA CCG GTT TCC CAA CGT GCA TCC AGA GTG GCA GAA CAA CTG TTT CGC AAG TTC TTG AAG TTC GCT GAA AAC GTC GGA CAT CAC AGT GAG AAG GCC TTC AAA AAA GCA AAG GTG GTG GCA GAA AAA GGC TTC ACC GCG GCA AAA ACG CAC ACG GTT AGG GGT TTC AAG GTG GCC AAA GAA GCA GCT GGA AGG GGC ATG GTG ACC GTT GGC AAG AAA CTC GCG AAT GTG GAG AGT GAC AGA AGC ACT ACG ACA ACG CAG GCC CCC GAC AGC CCT AAT GGC CTG GCA GAA ACC GAG GTT CCA GTG GAG CCC CAA CAG CGG GCC GCA CAC GTG CCC GTC CCA GAC TTT TCG CAG **GAA TTC** GAA GCT **TGA**

Figure 4.7: Sequencing analysis of recombinant pRSET B clones. Sequence analysis of the positive recombinant a) pRSET B-GRA2 and b) pRSET B-GRA5 plasmids shows that both cloned genes (underlined; excluding signal sequences) are in the correct sense orientation and are in frame with the *Eco*RI restriction digestion site (**GAATTC**) and **TGA** stop codon. As mentioned earlier, genes translations will initiate from the **ATG** start codon of the expression vector.

b) pRSET B-GRA5

ATG CGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAT CCG AGC TCG AGA TCT GCA GCT GGT ACC ATG **GAA TTC** GGT TCA ACG CGT GAC GTA GGG TCA GGC GGG GAT GAC TCC GAA GGT GCT AGG GGG CGT GAA CAA CAA CAG GTA CAA CAA CAC GAA CAA AAT GAA GAC CGA TCG TTA TTC GAA AGG GGA AGA GCA GCG GTG ACT GGA CAT CCA GTG AGG ACT GCA GTG GGA CTT GCT GCA GCT GTG GTG GCC GTT GTG TCA CTA CTG CGA TTG TTG AAA AGG AGG AGA AGA CGC GCG ATT CAA GAA GAG AGC AAG GAG TCT GCA ACC GCG GAA GAG GAA GAA GTT GCC GAG GAA GAG **GAA TTC** GAA GCT **TGA**

Figure 4.7 (cont.)

4.5 Transformation into competent BL21 (DE3) pLysS cells

Successfully transformed BL21 (DE3) pLysS cells with verified positive recombinant pRSET B plasmids and its empty vector were seen growing on ampicillin-chloramphenicol-containing LB agar pate as single isolated white colonies. Few selected white colonies were PCR amplified with gene-specific primers which generated the respective expected sizes as illustrated in Figure 4.8. This was followed by *E. coli* expression of the recombinant GRA2 and GRA5 proteins.

4.6 *E. coli* expression of recombinant proteins

A pilot expression is necessary to determine the optimal condition for expressing protein of interest since every recombinant protein has different characteristic and thus different requirement for its optimal expression. Production of the recombinant proteins were optimized by altering various parameters, and their expressions levels were analyzed by SDS-PAGE as shown in Figure 4.9 and 4.10. Upon induction of rGRA2 expression from GRA2-pRSET B-containing *E. coli*, we observed a 30-kDa band of increasing intensity, which was absent in the negative control (empty pRSET B). Expression of this protein increased up to five hours post-induction. Three different IPTG concentrations were tested, and 1.0 mM was found to result in maximum expression. On the other hand, a 20-kDa band was observed in rGRA5 expression with maximum intensity at second hour and remained constant from third to fifth hour after induction with 1.0 mM IPTG. Taken together, these data suggested that optimal rGRA2 and rGRA5 expressions were achieved following induction with 1.0 mM IPTG for 5 hours and 1.0 mM IPTG for 2 hours respectively. These same conditions were applied to scaled-up productions as illustrated in Figure 4.11. Meanwhile, protein solubility determination performed showed that rGRA2 is a soluble protein as the target protein size was only seen in the soluble fraction but absent in the insoluble fraction whereas

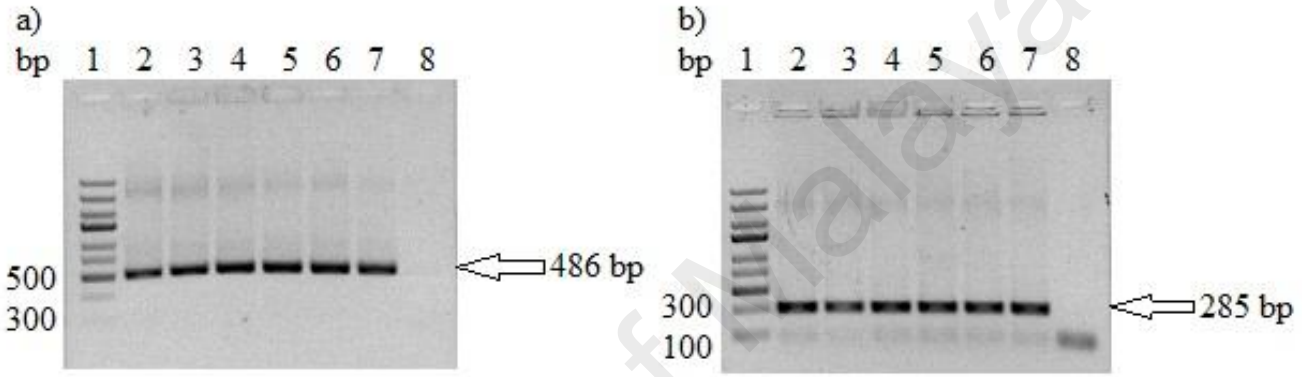

Figure 4.8: Colony PCR amplification of recombinant pRSET B clones in BL21 (DE3) pLysS. Agarose gel electrophoresis analysis of colony PCR amplified a) pRSET B-GRA2 and b) pRSET B-GRA5 with gene-specific primers set. Lane 1 (panel a - b) contained GeneRuler Express DNA Ladder. Lane 8 (panel a - b) contained negative control with double distilled water as template. Lane 2 (a) to 7 (a) contained positive colonies 1 to 6 with expected size of 486 bp (arrow). Lane 2 (b) to 7 (b) contained positive colonies 1 to 6 with expected size of 285 bp (arrow).

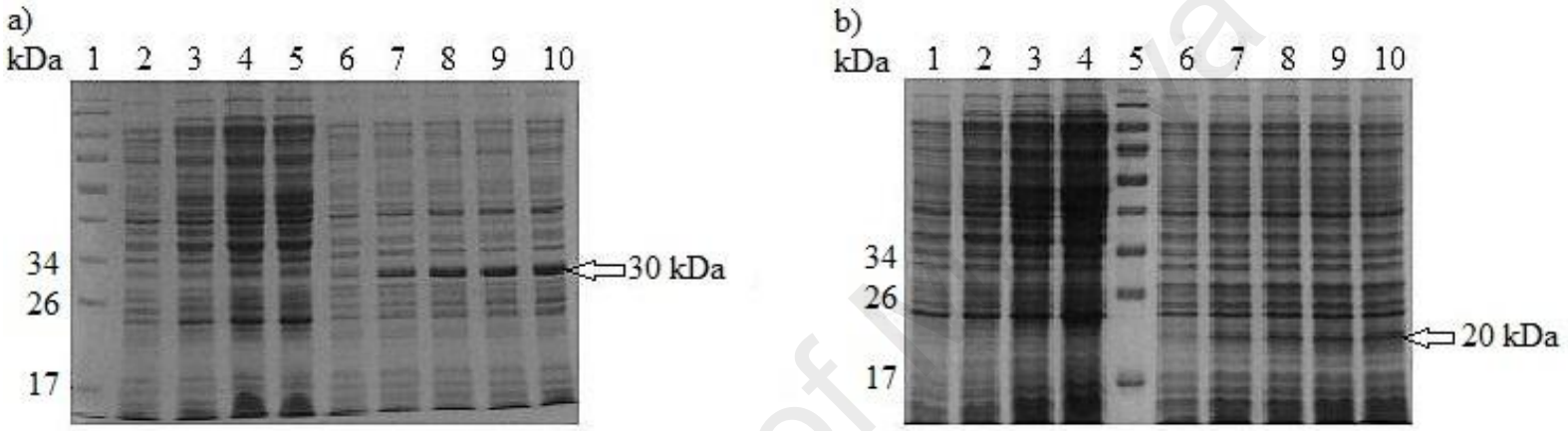

Figure 4.9: Time point analysis of *E. coli* expression. SDS-PAGE of time point analysis for a) rGRA2 and b) rGRA5 proteins in BL21 pLysS (DE3) with constant IPTG concentration (1.0 mM) stained with coomassie blue. (a) Lane 1 contained PageRuler™ Prestained Protein Ladder. Lane 2 to 5 contained cell pellet fractions of pRSET B plasmid as negative control (0, 3, 4, 5 h). Lane 6 to 10 contained cell pellet fractions of GRA2 clone (0, 2, 3, 4, 5 h). The GRA2 protein band of interest was observed at molecular weight of 30 kDa (arrow) compared to the negative control. The band intensity increased from 0 to 5 h post-induction. (b) Lane 5 contained PageRuler™ Prestained Protein Ladder. Lane 1 to 4 contained cell pellet fractions of pRSET B plasmid as negative control (0, 3, 4, 5 h). Lane 6 to 10 contained cell pellet fractions of GRA5 clone (0, 2, 3, 4, 5 h). The GRA5 protein band of interest was observed at molecular weight of 20 kDa (arrow) compared to the negative control. The band intensity increased from 0 to 2 h post-induction and remained constant at the 3 h.

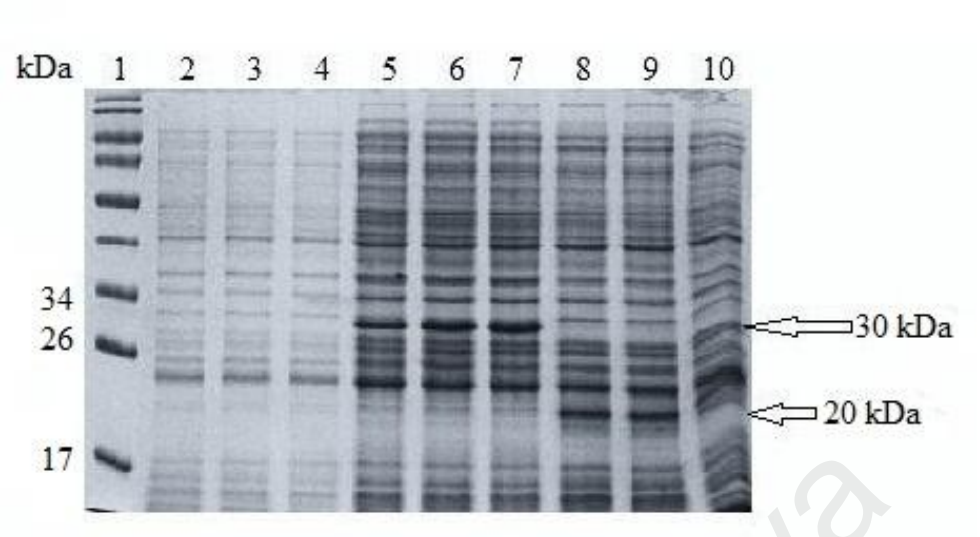

Figure 4.10: IPTG concentration optimization study of *E. coli* expression. SDS-PAGE analysis of IPTG (inducer) concentration optimization for expressions of rGRA2 and rGRA5 proteins in BL21 pLysS (DE3) with pre-determined time point (5 h and 2 h respectively) stained with coomassie blue. Lane 1 contained PageRuler™ Prestained Protein Ladder. Lane 2 to 4 contained cell pellet fractions of pRSET B plasmid as negative control (0.1, 0.5, 1.0 mM). Lane 5 to 7 contained cell pellet fractions of GRA2 clone (0.1, 0.5, 1.0 mM). Lane 8 to 10 contained cell pellet fractions of GRA5 clone (1.0, 0.5, 0.1 mM). The optimal IPTG concentration for both rGRA2 and rGRA5 proteins is 1.0 mM.

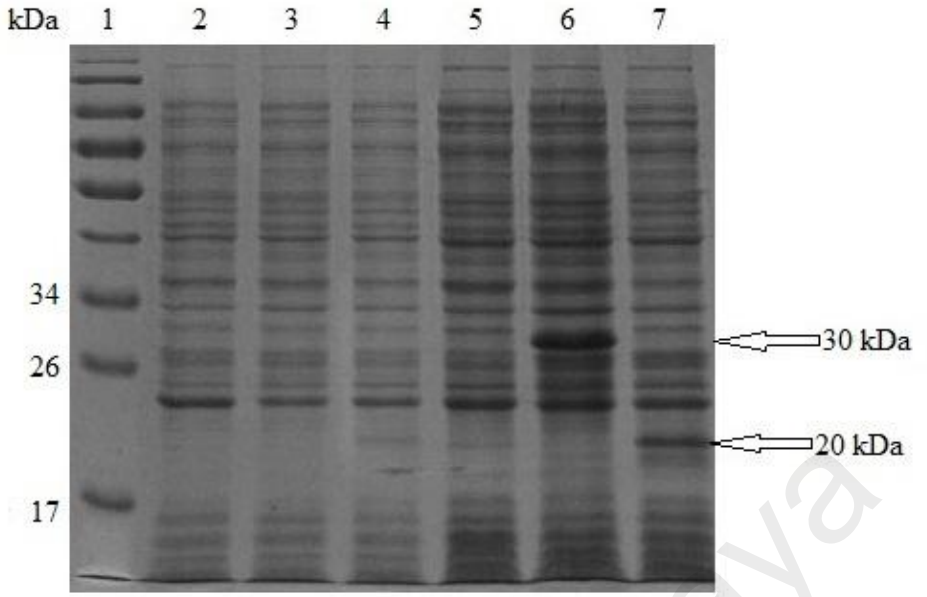

Figure 4.11: Scaled-up *E. coli* expression of recombinant proteins. SDS-PAGE analysis of the optimized bacterial expressions of rGRA2 and rGRA5 proteins in BL21 pLysS (DE3) induced with 1.0 mM IPTG stained with coomassie blue. Lane 1 contained PageRuler™ Prestained Protein Ladder. Lane 2 and 5 contained cell pellet fractions of pRSET B plasmid as negative control (0 and 5 h). Lane 3 and 6 contained cell pellet fractions of GRA2 clone (0 and 5 h). Lane 4 and 7 contained cell pellet fractions of GRA5 clone (0 and 2 h). The GRA2 and GRA5 proteins bands of interest were observed at molecular weights of 30 kDa and 20 kDa (arrow) respectively compared to the negative control.

rGRA5 is an insoluble protein due to its presence in the insoluble fraction (Figure 4.12).

4.7 Purifications of recombinant proteins

It is crucial to determine the solubility of each recombinant protein expressed before choosing the appropriate protein purification technique to ensure purification efficacy. The solubility results showed that rGRA2 and rGRA5 proteins could be purified through native and denaturing purifications protocols respectively, using nickel resin columns. Dialyzed fractions of the purified proteins were quantitated to be in the range of 0.2-0.4 mg/ml and were visualized on SDS-PAGE which showed single major band of their respective expected sizes as well as several minor faint bands (Figure 4.13 and 4.14). These purified proteins could be detected by western blot analysis using serum from a *Toxoplasma*-infected patient as illustrated in Figure 4.13 and 4.14.

4.8 Identities confirmation of recombinant proteins

Purified rGRA2 and rGRA5 proteins could be detected by western blot analysis against anti-Xpress™ monoclonal antibody showing their respective expected sizes as depicted in Figure 4.15 which serves as primary confirmation of the proteins' identities. Furthermore, analysis by MALDI-TOF MS of the tryptic peptides from 20 and 30 kDa bands revealed by the Mascot search results for having highest protein scores ($p<0.05$) which means that the digested peptide masses of these two targets possessed highest match with the amino acid sequences within protein GRA5 and GRA2 respectively (APPENDIX J), thus double confirming that the isolated proteins were *T. gondii* GRA5 and GRA2.

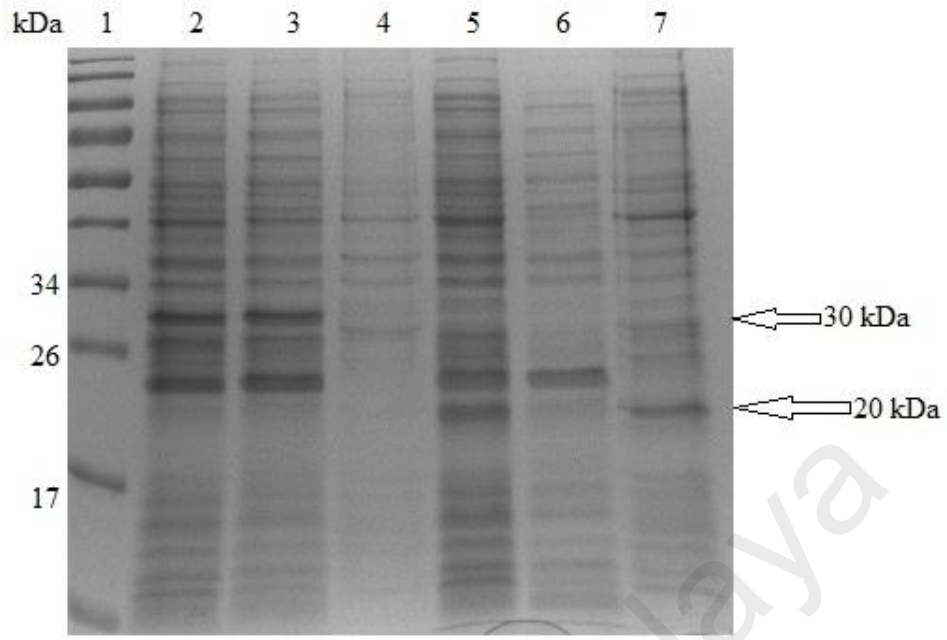

Figure 4.12: Recombinant proteins solubility determination. SDS-PAGE analysis of the solubility of rGRA2 and rGRA5 proteins expressed. Coomassie blue stained. Lane 1 contained PageRuler™ Prestained Protein Ladder. Lane 2 to 4 contained cell pellet fractions of GRA2 clone with different fractions (total cell lysate, soluble and insoluble). Lane 5 to 7 contained cell pellet fractions of GRA5 clone with different fractions (total cell lysate, soluble and insoluble. The GRA2 and GRA5 proteins bands of interest were observed in the soluble (Lane 3) and insoluble (Lane 7) fractions respectively.

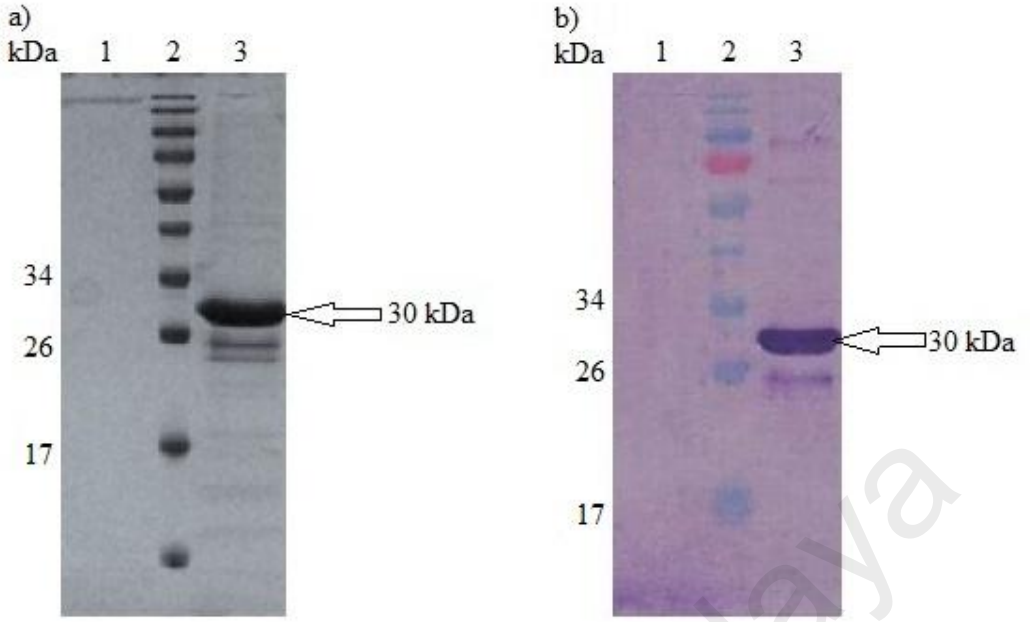

Figure 4.13: Purification of recombinant GRA2 protein. SDS-PAGE and western blot analysis of purified rGRA2 protein. (a) Coomassie blue stained. Lane 1 contained purified pRSET B. Lane 3 contained purified rGRA2. (b) Western blot probed with toxoplasmosis-infected patient's serum. Lane 1 contained purified pRSET B. Lane 3 contained purified rGRA2. Lane 2 (a) and (b) contained PageRuler™ Prestained Protein Ladder. The 30 kDa purified rGRA2 was detected (arrow).

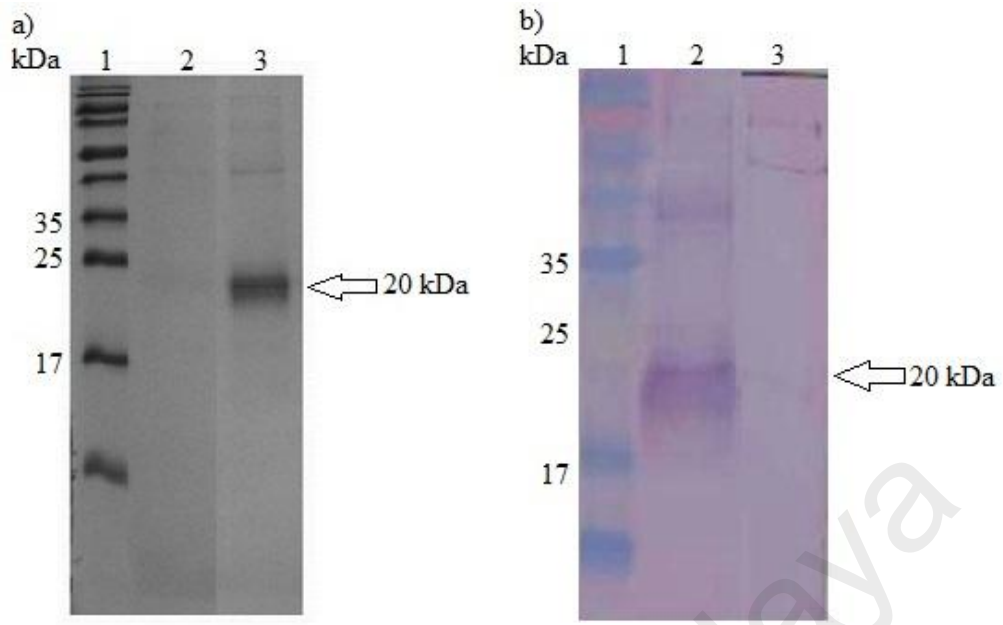

Figure 4.14: Purification of recombinant GRA5 protein. SDS-PAGE and western blot analysis of purified rGRA5 protein. (a) Coomassie blue stained. Lane 2 contained purified pRSET B. Lane 3 contained purified rGRA5. (b) Western blot probed with toxoplasmosis-infected patient's serum. Lane 2 contained purified rGRA5. Lane 3 contained purified pRSET B. Lane 1 (a) and (b) contained Pre-stained Broad Range Protein Marker. The 20 kDa purified rGRA5 was detected (arrow).

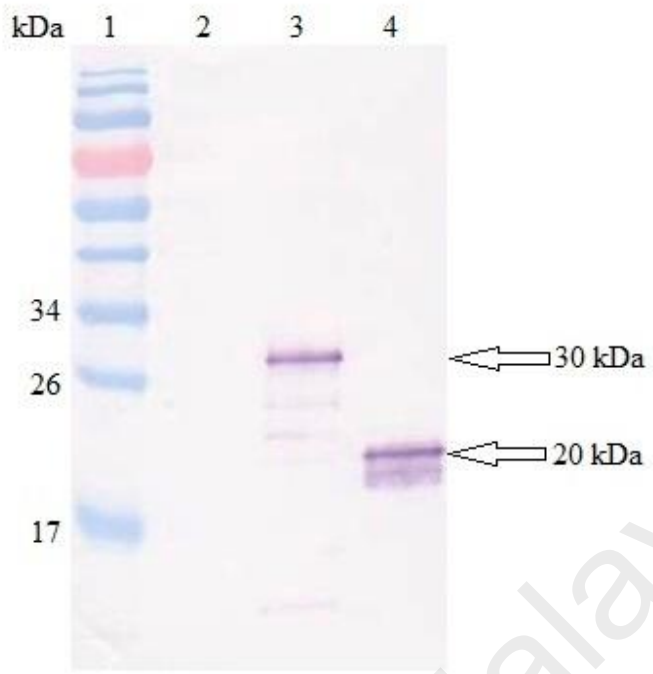

Figure 4.15: Identities confirmation of recombinant GRA2 and GRA5 proteins. Western blot analysis of purified rGRA2 and rGRA5 proteins, probed with anti-Xpress™ monoclonal antibody. Lane 1 contained PageRuler™ Prestained Protein Ladder. Lane 2 contained purified pRSET B. Lane 3 contained purified rGRA2 (30 kDa; arrow). Lane 4 contained purified rGRA5 (20 kDa; arrow).

4.9 Western blot analysis of rGRA2 protein with human serum samples

The purified rGRA2 protein was further tested for its sensitivity and specificity through western blot analysis with serum samples from toxoplasmosis-positive (Group 1, 2 and 3) and toxoplasmosis-negative (Group 4) patients as well as patients infected with other infections such as amoebiasis, cysticercosis, filariasis and toxocariasis. Results tabulated in Table 4.1 showed that rGRA2 protein has a sensitivity of 100.0% (13 out of 13 sera), 100.0% (19 out of 19 sera) and 61.5% (16 out of 26 sera) for early acute, acute and chronic infection respectively (five positive results for each group were shown in Figure 4.16). On the other hand, 3 out of 30 sera from toxoplasmosis-negative control patients (five negative results were shown in Figure 4.16) and 2 out of 12 sera from patients infected with other diseases (toxocariasis) reacted with the rGRA2 protein giving rise to a specificity of 90.0% and 83.3% respectively.

4.10 Western blot analysis of rGRA5 protein with human serum samples

The purified rGRA5 protein was tested for its diagnostic sensitivity and specificity through western blot analysis with serum samples from toxoplasmosis-positive (Group 1, 2, and 3) and toxoplasmosis-negative (Group 4) patients. In addition, specificity was tested using sera from patients infected with other parasites, including amoebiasis, cysticercosis, filariasis, and toxocariasis. We observed that the rGRA5 protein had sensitivities of 0% (0 out of 44 sera), 46.8% (22 out of 47 sera), and 61.2% (52 out of 85 sera) for early acute, acute, and chronic infections, respectively (Table 4.2). In contrast, 0 out of 24 control sera from the toxoplasmosis-negative patients reacted with rGRA5 (100% specificity). In Figure 4.17, five example results are shown for each group (positive results for Groups 2 and 3; negative results for Group 4). Also, only 1 (toxocariasis) out of the 12 sera from patients infected with other parasites reacted with the rGRA5 protein (91.7% specificity).

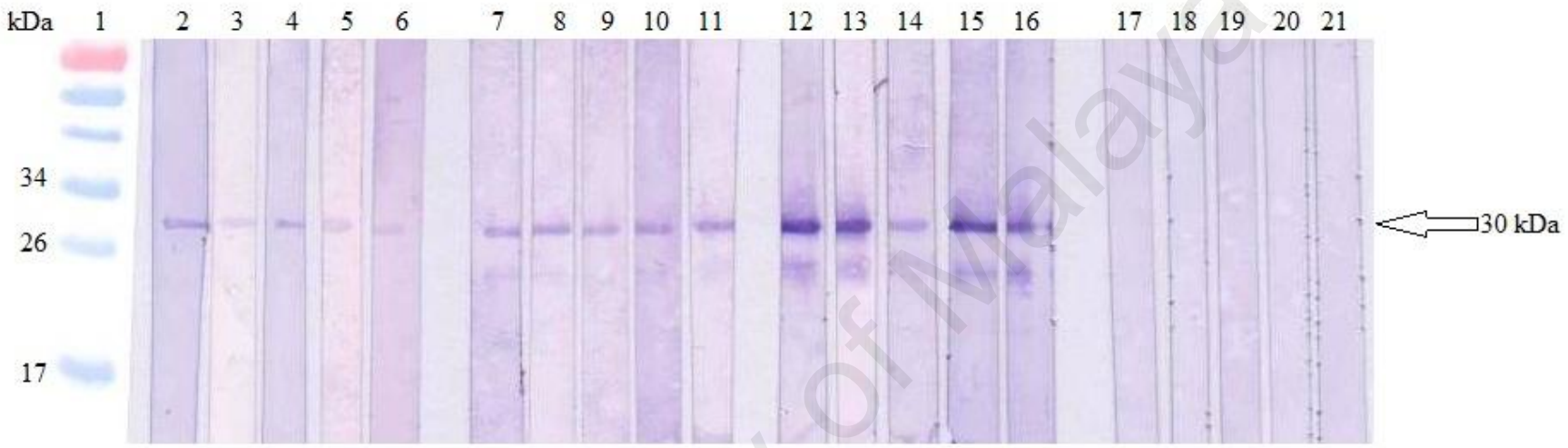

Figure 4.16: Evaluation of sensitivity and specificity of purified rGRA2 protein. Western blots of purified rGRA2 protein (100 ng) with sera of toxoplasmosis and toxoplasmosis-negative patients. Lane 1 contained PageRuler™ Prestained Protein Ladder. Lane 2 to 6 contained results of 5 sera from early acute-profile patients (Group 1: IgG-ve, IgM+ve), lane 7 to 11 contained results of 5 sera from acute-profile patients (Group 2: IgG+ve, IgM+ve), lane 12 to 16 contained results of 5 sera from chronic-profile patients (Group 3: IgG+ve, IgM-ve) and lane 17 to 21 contained results of 5 sera from toxoplasmosis-negative patients (Group 4: IgG-ve, IgM-ve). The 30 kDa purified rGRA2 was detected by toxoplasmosis-positive sera (arrow).

Table 4.1: Immunoreactivities (sensitivity and specificity) of the rGRA2 antigen to serum samples from toxoplasmosis-positive and toxoplasmosis-negative patients

Serum samples group	Number of human serum samples	Immunoreactivities			
		Positive		Negative	
		No.	%	No.	%
1 (early acute: IgG-ve, IgM+ve)	13	13	100	0	0
2 (acute: IgG+ve, IgM+ve)	19	19	100	0	0
3 (chronic: IgG+ve, IgM-ve)	26	16	61.5	10	38.5
4 (toxoplasmosis-negative: IgG-ve, IgM-ve)	30	3	10.0	27	90.0
Other infection	12	2	16.7	10	83.3
Amoebiasis	3	0	0	3	100
Cysticercosis	3	0	0	3	100
Filariasis	3	0	0	3	100
Toxocariasis	3	2*	66.7	1	33.3

* Two out of three toxocariasis-positive sera samples reacted with the rGRA2 antigen. These two toxocariasis-positive serum samples were shown to be IgG positive for toxoplasmosis based on the commercial kits.

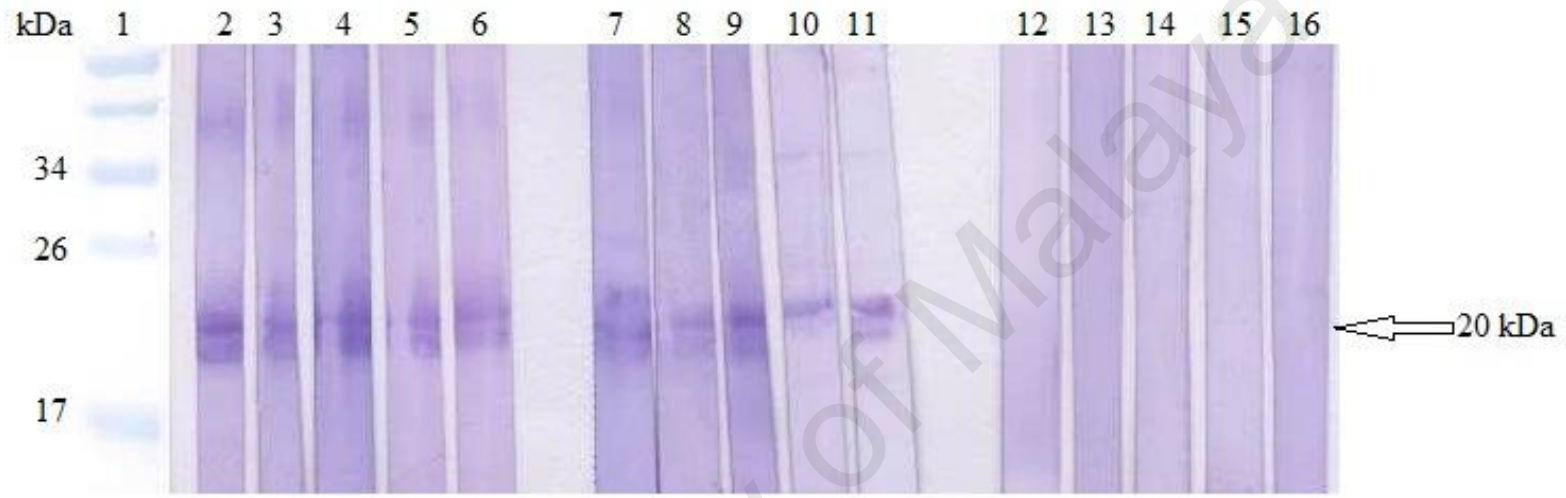

Figure 4.17: Evaluation of sensitivity and specificity of purified rGRA5 protein. Western blots of purified rGRA5 protein (200 ng) with sera of toxoplasmosis and toxoplasmosis-negative patients. Lane 1 contained PageRuler™ Prestained Protein Ladder. Lane 2 to 6 contained results of 5 sera from chronic-profile patients (Group 3: IgG+ve, IgM-ve), lane 7 to 11 contained results of 5 sera from acute-profile patients (Group 2: IgG+ve, IgM+ve), lane 12 to 16 contained results of 5 sera from toxoplasmosis-negative patients (Group 4: IgG-ve, IgM-ve). The 20 kDa purified rGRA5 was detected by toxoplasmosis-positive sera (arrow).

Table 4.2: Immunoreactivities (sensitivity and specificity) of the rGRA5 antigen to serum samples from toxoplasmosis-positive and toxoplasmosis-negative patients

Serum samples group	Number of human serum samples	Immunoreactivities			
		Positive		Negative	
		No.	%	No.	%
1 (early acute:IgG-ve, IgM+ve)	44	0	0	44	100
2 (acute:IgG+ve, IgM+ve)	47	22	46.8	25	53.2
3 (chronic:IgG+ve, IgM-ve)	85	52	61.2	33	38.8
4 (toxoplasmosis-negative: IgG-ve, IgM-ve)	24	0	0	24	100
Other infection	12	1	8.3	11	91.7
Amoebiasis	3	0	0	3	100
Cysticercosis	3	0	0	3	100
Filariasis	3	0	0	3	100
Toxocariasis	3	1*	33.3	2	66.7

* One out of three toxocariasis-positive sera sample reacted with the rGRA5 antigen. This particular toxocariasis-positive serum sample was shown to be IgG positive for toxoplasmosis based on the commercial kits.

4.11 Immunological characterization of recombinant GRA2 and GRA5 proteins

Immunological responses of both humoral and cellular-mediated induced by recombinant GRA2 and GRA5 proteins emulsified with complete-incomplete Freund's adjuvant were characterized in the respective immunized mice.

4.11.1 Induction of humoral immunity

Induction of humoral immune response was evaluated through determination of specific anti-GRAs IgG antibody level, antibody titers as well as IgG subclass.

4.11.1.1 IgG antibody detection

Total specific anti-GRA2 and anti-GRA5 IgG antibodies were detected in the sera collected from the mice immunized with rGRA2 and rGRA5 respectively through western blot (Figure 4.18) and ELISA (Figure 4.19 and Table 4.3) against purified recombinant proteins. Faint protein bands with target size of 30 kDa and 20 kDa were observed at week 2 after prime injection of mice with rGRA2 and rGRA5 respectively as shown in Figure 4.18. The intensity increased at week 4 and 6 following first and second booster injections. Meanwhile, Figure 4.19 also showed the same phenomena whereby significantly higher levels of IgG antibodies were observed in the recombinant protein-vaccinated groups compared to two control groups ($p<0.001$) and the level gradually elevated with successive immunizations. There was no statistical difference between the two control groups at week 2 and 4 ($p>0.05$). However, IgG level in pRSET B-injected group was found significantly higher than that of PBS-injected group ($p<0.05$) at week 6 post-prime injection. The level of IgG antibodies in mice sera collected from rGRA5-vaccinated group was significantly higher than that of rGRA2-vaccinated group ($p<0.001$) at week 4 but both groups reached highest level two weeks after the last injection (week 6) without statistical difference ($p>0.05$). These results

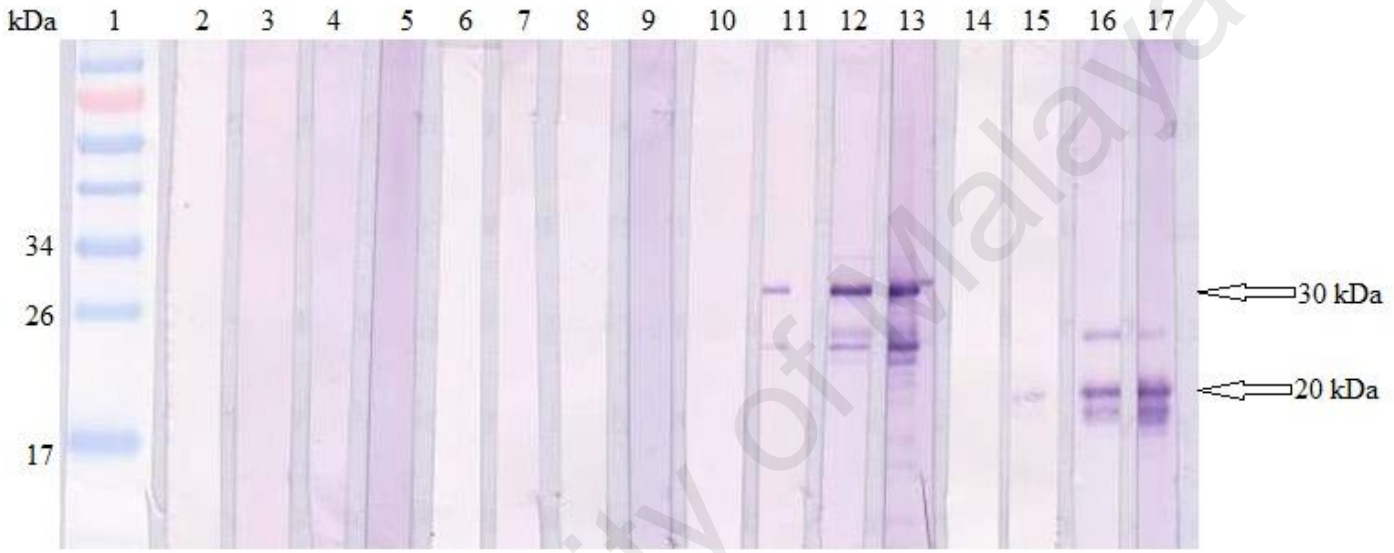

Figure 4.18: Qualitative detections of total specific anti-GRAs IgG antibodies in mice sera. Western blots of purified recombinant proteins (rGRA2 or/and rGRA5) with sera of the immunized mice. Lane 1 contained PageRuler™ Prestained Protein Ladder. Lane 2 to 5 contained results of 4 sera from PBS-injected mice, lane 6 to 9 contained results of 4 sera from pRSET B-injected mice, lane 10 to 13 contained results of 4 sera from rGRA2-immunized mice and lane 14 to 17 contained results of 4 sera from rGRA5-immunized mice. The 4 sera represented sera collected at week 0, 2, 4 and 6 post-prime injections. The 30 kDa purified rGRA2 and 20 kDa purified rGRA5 were first detected at week 2 (lane 11 and 15 respectively) followed by an increase in the band intensity at week 4 and 6. No bands were observed in the mice sera of the two control groups.

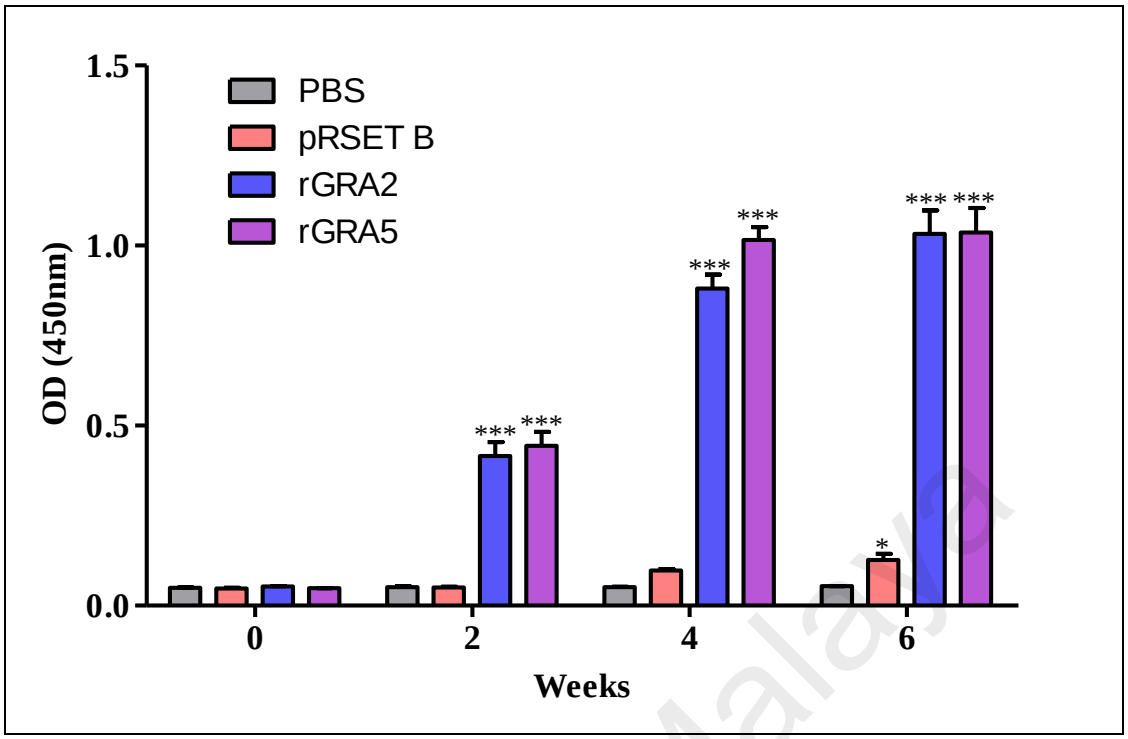

Figure 4.19: Quantitative detections of total specific anti-GRAs IgG antibodies in mice sera. Total anti-GRAs IgG antibodies in mice sera were detected and evaluated by ELISA. Sera were collected from each mice group one day before each immunization. Data are expressed as mean OD_{450} ±SD (n=3). Statistical differences are represented by * (significant; $p<0.05$) and *** (highly significant; $p<0.001$) in comparison with the control groups (PBS or pRSET B).

Table 4.3: Specific anti-GRAs IgG antibody profile in sera collected from the immunized BALB/c mice two weeks after last injection

Group (n=3)	OD_{450}		
	IgG	IgG1	IgG2a
rGRA2	1.0330 ±0.0645***	2.9760 ±0.2025***	2.5850 ±0.0303***
rGRA5	1.0370 ±0.0679***	3.0770 ±0.0528***	2.6820 ±0.0768***
pRSET B	0.1267 ±0.0170*	0.0801 ±0.0020	0.0686 ±0.0033
PBS	0.0540 ±0.0007	0.1256 ±0.0060	0.0717 ±0.0078

Data are expressed as mean ±SD. Statistical differences are represented by * (significant; $p<0.05$) and *** (highly significant; $p<0.001$) in comparison with the control groups (PBS or pRSET B). Each group consisted of three mice.

indicated that both recombinant GRA2 and GRA5 proteins are immunogenic and capable of stimulating significantly strong humoral immune response in the respective vaccinated mice compared to the negative control groups.

4.11.1.2 IgG titer determination

Antibody titer is a quantitative measurement of the amount of antibody capable of recognizing the respective epitope. It is usually expressed as reciprocal of the highest dilution with an OD450 greater than the positive cut-off value of IgG (mean+2SD) relative to the control mice sera at the same dilution. Antibody titer of both the anti-GRA2 and anti-GRA5 IgG was determined to range from 1:409,600 to 1:819,200 by ELISA.

4.11.1.3 IgG antibody isotypes determination

Polyclonal antibody isotypes (IgG1 and IgG2a) in the immunized BALB/c mice sera were further assessed by ELISA in order to identify type of immunity being triggered. The levels of specific anti-GRAs IgG1 and IgG2a being produced are depicted in Figure 4.20 and tabulated in Table 4.3. Generally, the level of IgG isotypes present in the sera of the two vaccinated mice groups were highly significantly greater than that of the two control groups ($p<0.001$). No statistical difference was observed between the two control groups ($p>0.05$) and also between the two vaccinated groups ($p>0.05$). On top of that, it was shown that high levels of two IgG isotypes were detected in all the rGRA2- and rGRA5-immunized mice sera, with slightly higher level of IgG1 compared to IgG2a, giving rise to an IgG2a/IgG1 ratio of <1 (≈ 0.87). The result obtained indicated that both Th1 and Th2 immune responses were driven in all the vaccinated mice.

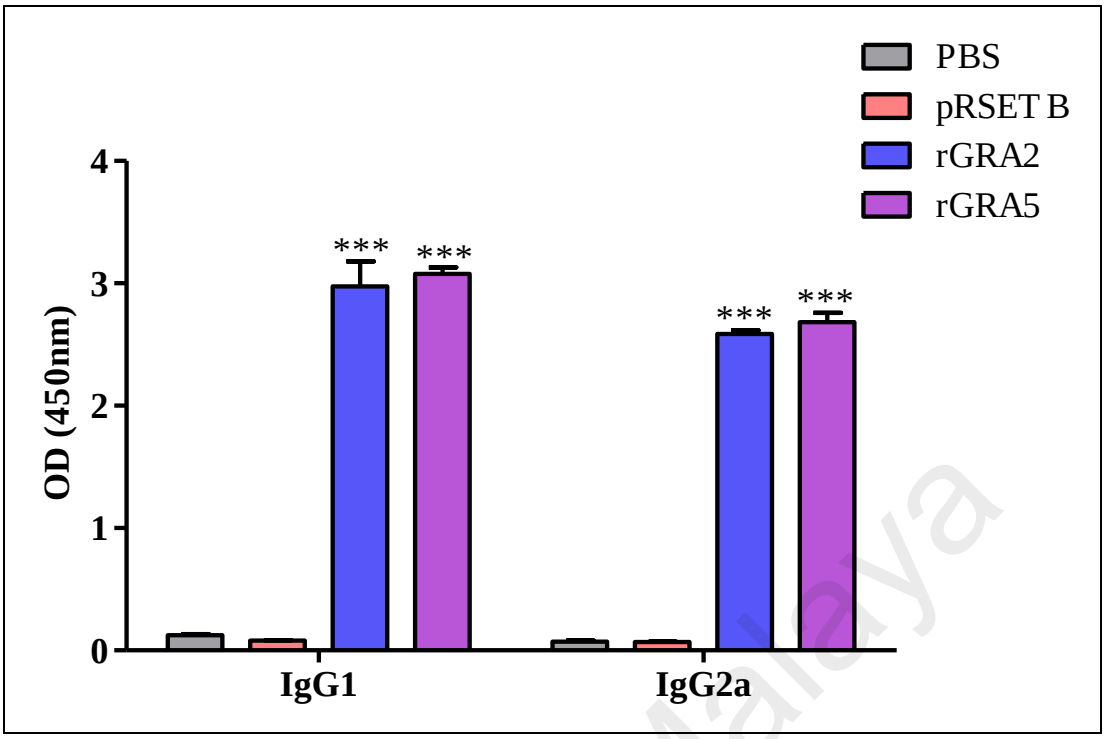

Figure 4.20: IgG isotypes determination in the immunized BALB/c mice sera. Polyclonal antibody isotypes (IgG1 and IgG2a) in the immunized mice sera were determined by ELISA. Sera from each mice group were collected two weeks after the last injection. Data are expressed as mean OD_{450} ±SD (n=3). Statistical difference is represented by *** (highly significant; $p<0.001$) in comparison with the control groups (PBS or pRSET B).

4.11.2 Induction of cellular-mediated immunity

Induction of cellular-mediated immune response was evaluated through antigen-specific splenocytes proliferation assay and cytokine production assay.

4.11.2.1 *In vitro* splenocytes proliferation assay

Antigen-specific proliferative response of splenocytes from each mice group to rGRA2 or/and rGRA5 stimulus was determined using MTT assay and represented by the SI value as illustrated in Figure 4.21 and Table 4.4. Generally, significantly higher SI value was observed in the recombinant protein-vaccinated groups compared to the control groups ($p<0.05$). On top of that, splenocytes from rGRA5-vaccinated mice had significantly stronger proliferation compared to that of rGRA2-vaccinated mice in response to their respective stimulus ($p<0.05$). Nevertheless, there was no statistical difference between the two control groups ($p>0.05$). Meanwhile, SI value for all mice groups had comparable levels in response to the mitogen conA. These results indicated that T lymphocytes of the vaccinated mice were successfully stimulated.

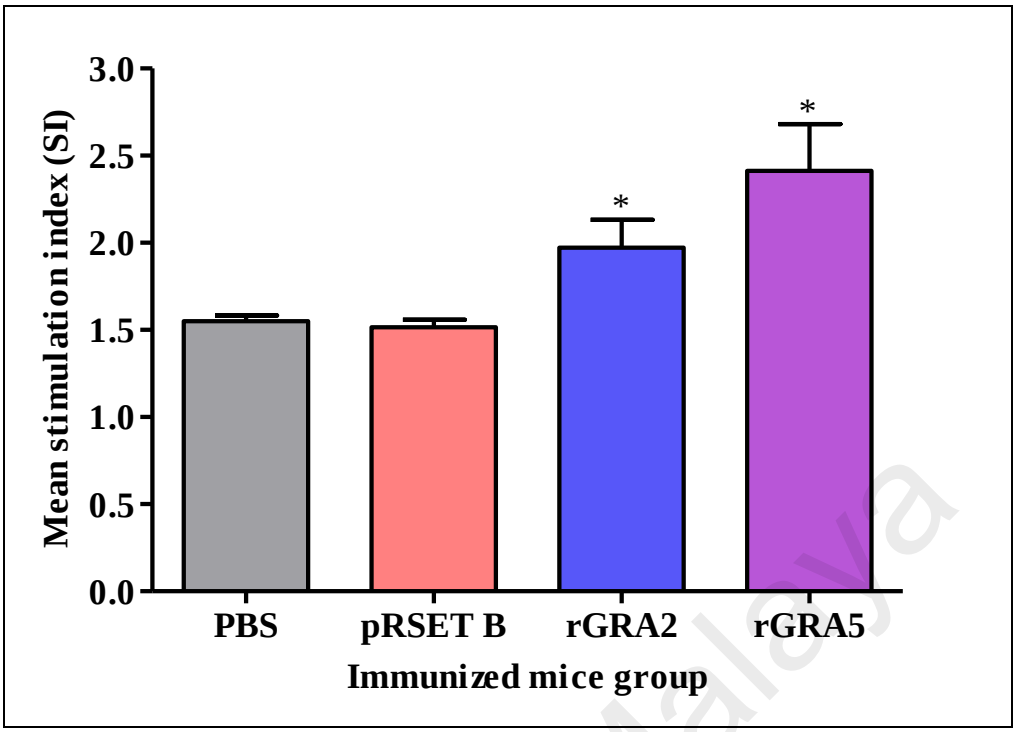

Figure 4.21: *In vitro* splenocytes proliferation response in mice. Spleen lymphocytes were harvested from mice immunized with rGRA2, rGRA5, pRSET B and PBS two weeks after last injection. The splenocytes were cultured and stimulated with the respective recombinant proteins. Proliferative response was measured by MTT assay. Data are expressed as mean stimulation index (SI) ±SD (n=3). Statistical difference is represented by * ($p<0.05$) in comparison with the control groups (PBS or pRSET B).

Table 4.4: Characterization of cellular-mediated immunity in the vaccinated mice

Group (n=3)	Proliferation (SI)	Cytokine level (pg/ml)			
		IFN-γ	IL-2	IL-4	IL-10
rGRA2	1.973 ±0.1589*	4645 ±1032*	1527 ±247*	9.989 ±2.231	121.6 ±43.94*
rGRA5	2.414 ±0.2674*	4724 ±372.5*	1232 ±95.07*	25.46 ±11.98*	178.3 ±27.71*
pRSET B	1.514 ±0.0447	197.4 ±61.45	146 ±42.01	Undetectable	61.55 ±16.18
PBS	1.549 ±0.0345	142.7 ±77.15	123 ±13.67	Undetectable	26.98 ±8.667

SI stands for stimulation index.

IFN-γ activity was assayed at 96 h, IL-2 and IL-4 activities were assayed at 24 h, and IL-10 activity was assayed at 72 h.

Undetectable IL-4 level was observed in the stimulated splenocytes culture supernatant of the negative control mice groups.

Data are expressed as mean ±SD (n=3). Statistical difference is represented by * ($p<0.05$) in comparison with the control groups (PBS or pRSET B).

4.11.2.2 Cytokine production assay

Stimulated spleen T lymphocytes of the vaccinated mice were evaluated for their cytokine production level. Cytokines (IFN-γ, IL-2, IL-4 and IL-10) secreted and released into the supernatant of the culture of antigen-stimulated splenocytes were collected (96 h, 24 h, 24 h and 72 h, respectively) and assayed by ELISA. The assay was performed in order to determine the type of immunity being polarized, either Th1- or Th2-type cellular immune response.

Results obtained showed that splenocytes of the vaccinated mice produced significantly higher levels of IFN-γ and IL-2 compared to the control groups ($p<0.05$) as demonstrated in Figure 4.22 and Table 4.4. No statistical difference was observed between two vaccinated groups ($p>0.05$) and between two control groups ($p>0.05$). In contrast, relatively low levels of IL-4 and IL-10 were released by the stimulated splenocytes of the mice immunized with rGRA2 and rGRA5 (Figure 4.23 and Table 4.4). Undetectable level of IL-4 was observed in the control groups (Table 4.4). However, IL-10 level produced by splenocytes from the recombinant protein-vaccinated mice was significantly higher compared to PBS and pRSET B-injected mice ($p>0.05$). Production of huge amount of IFN-γ and IL-2 indicated that Th1 immune response was favored in the vaccinated mice.

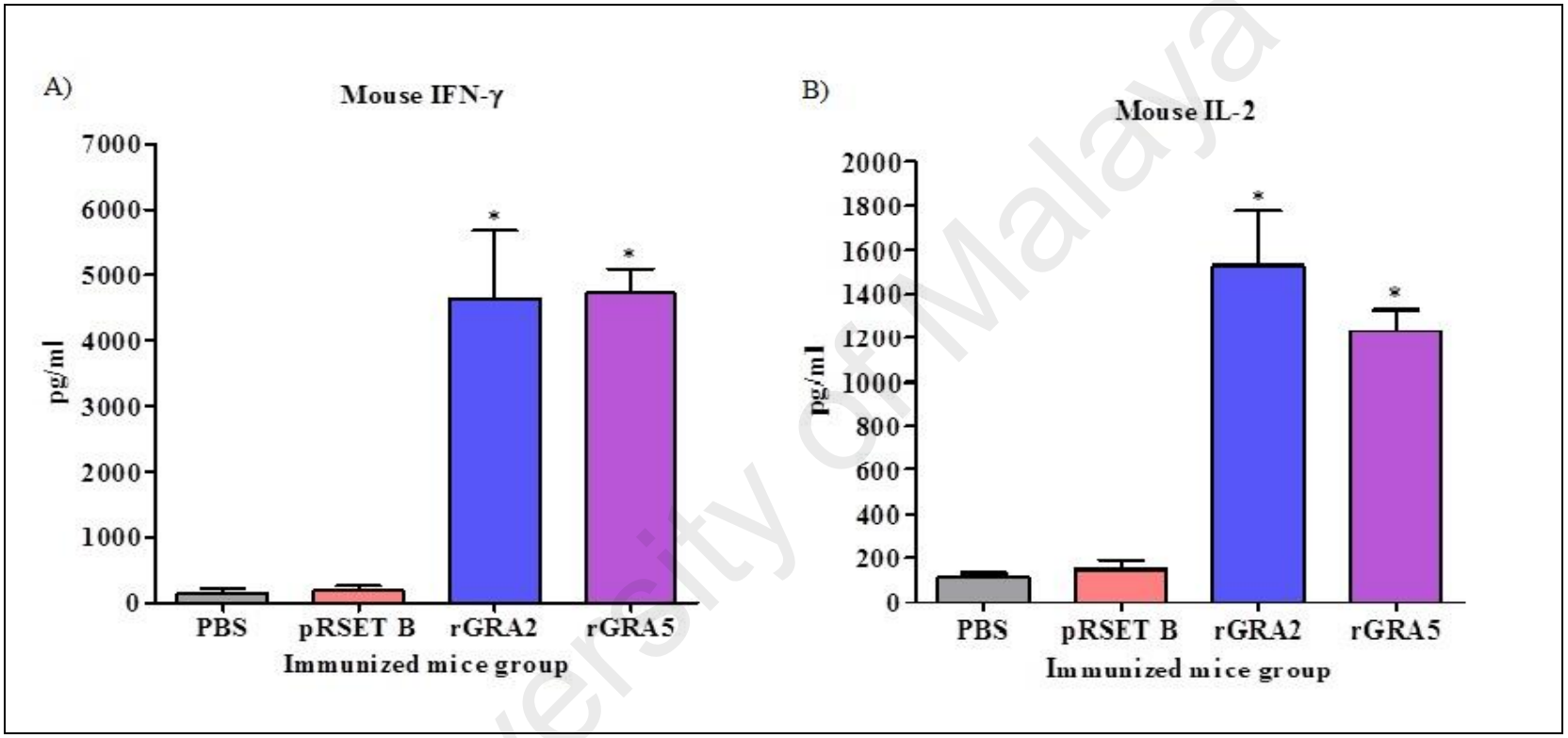

Figure 4.22: IFN-γ and IL-2 production by the stimulated splenocytes of the immunized mice. Culture supernatants from the antigen-stimulated immunized mice splenocytes were harvested at 96 h and 24 h post-incubation for the evaluation of A) IFN-γ (96 h) and B) IL-2 (24 h) production respectively via ELISA. Data are expressed as mean ±SD (n=3). Statistical difference is represented by * ($p<0.05$) in comparison with the control groups (PBS or pRSET B).

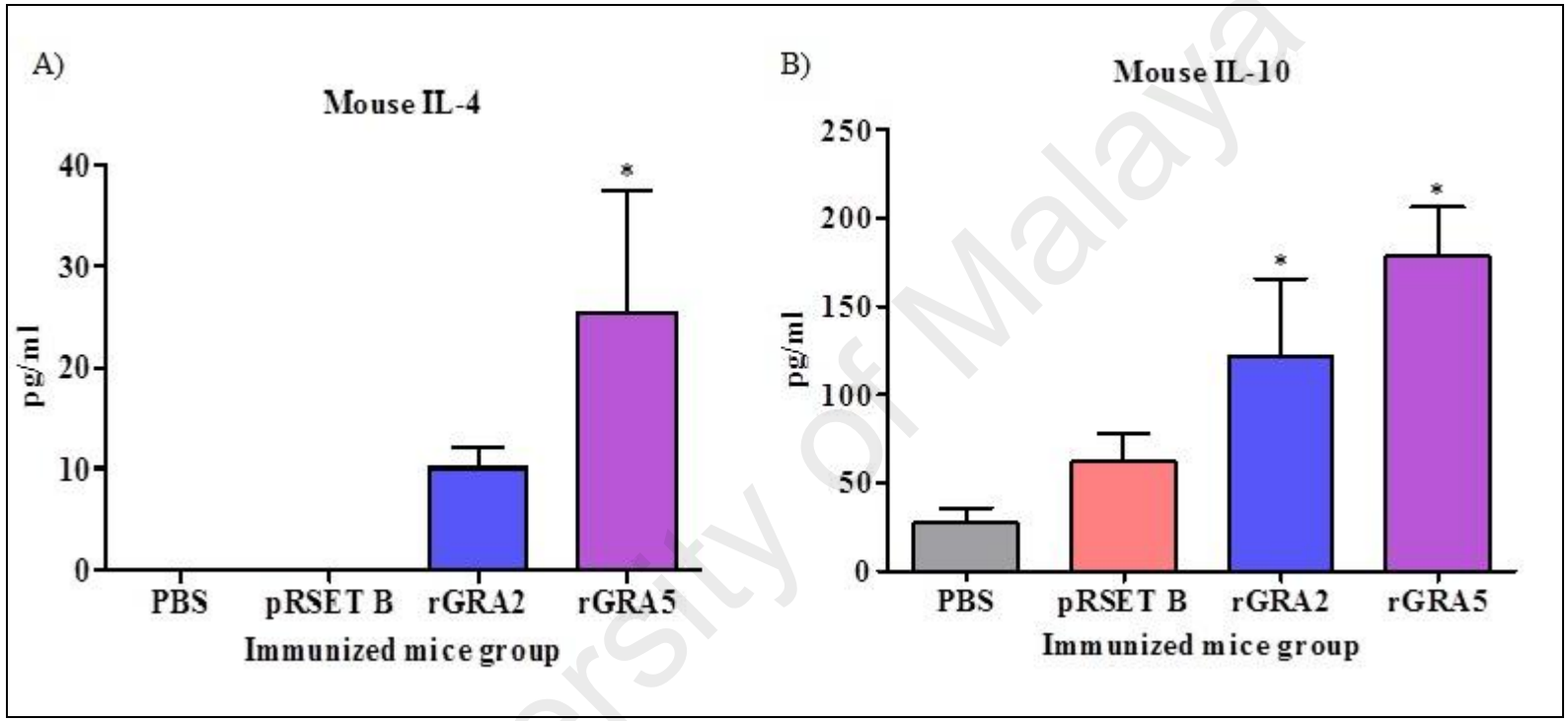

Figure 4.23: IL-4 and IL-10 production by the stimulated splenocytes of the immunized mice. Culture supernatants from the antigen-stimulated immunized mice splenocytes were harvested at 24 h and 72 h post-incubation for the evaluation of A) IL-4 (24 h) and B) IL-10 (72 h) production respectively via ELISA. Data are expressed as mean ±SD (n=3). Statistical difference is represented by * ($p<0.05$) in comparison with the control groups (PBS or pRSET B).

4.11.3 Protective efficacy of recombinant protein vaccination in BALB/c mice

Protective efficacy of recombinant GRA2 and GRA5 proteins in the immunized BALB/c mice were evaluated against lethal challenge with *T. gondii*. The survival rates of the four challenged mice groups were illustrated in Figure 4.24. It was shown that the two vaccinated mice groups had significantly prolonged survival rates as compared to the two control mice groups (PBS and pRSET B) ($p<0.05$). All PBS- and pRSET B-injected mice succumbed to the parasite infection on day 6 (median survival of 6 days) and day 9 (median survival of 8 days) respectively. Meanwhile, rGRA2- and rGRA5-immunized mice died within 8-18 days post-infection with the median survival of 16.5 and 16 days respectively. Although all the immunized mice died on day 18, but these two subunit vaccines were successfully demonstrated to increase the survival rates of the vaccinated BALB/c mice against *T. gondii* infection.

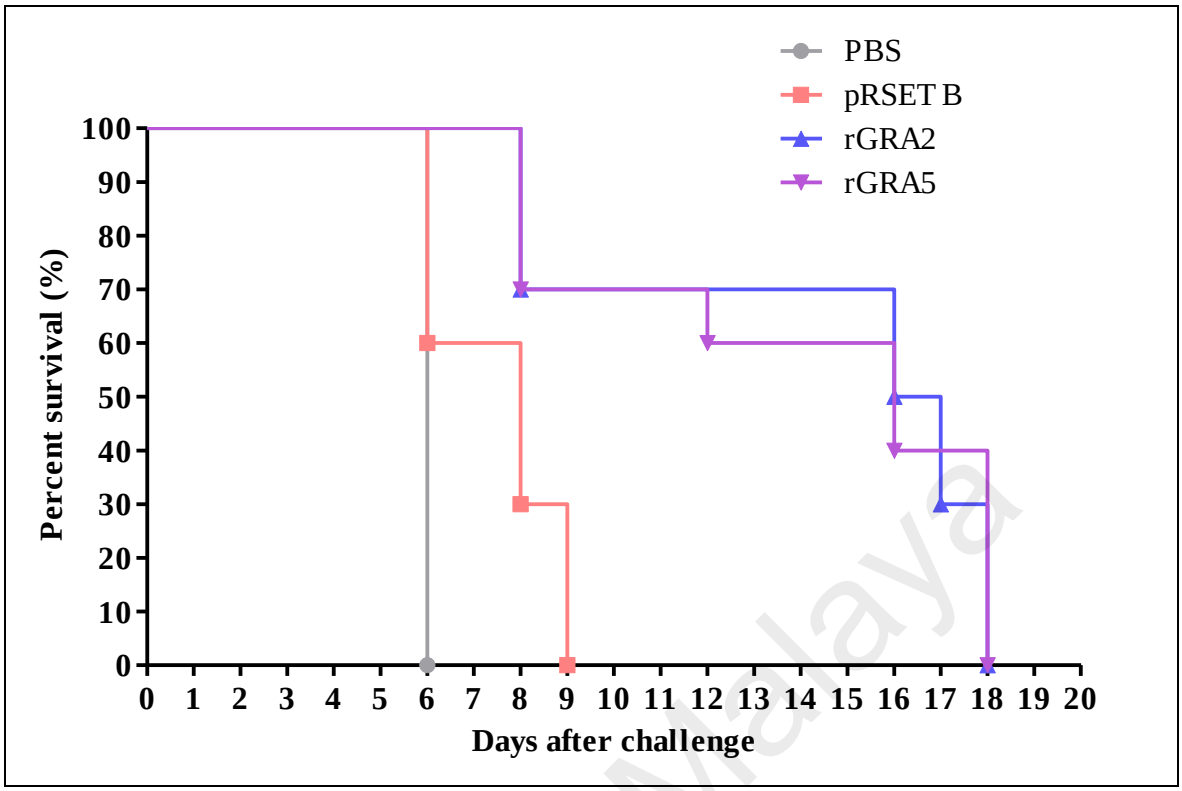

Figure 4.24: Survival rate of the immunized mice. All four groups of the immunized mice (PBS, pRSET B, rGRA2, rGRA5) were subjected to lethal challenge with 1000 live tachyzoites of *T. gondii* virulent RH strain 2 weeks after the last immunization. Mice immunized with rGRA2 and rGRA5 exhibited a significant increase in the survival days (median survival of 16.5 and 16 days respectively) in comparison to the control mice injected with PBS and pRSET B (median survival of 6 and 8 days respectively). Each group consisted of 10 mice.

4.12 Constructions of recombinant pcDNA 3.1C-GRA2 (pcGRA2) and pcDNA 3.1C-GRA5 (pcGRA5)

The pcDNA 3.1 vectors are pcDNA 3.1-derived expression vectors containing the human cytomegalovirus (CMV) immediate-early promoter designed for high-level expression in a wide range of mammalian cells, such as CHO cells. They are available in three forms, namely pcDNA 3.1A, B and C which have the same sequences encoding the N-terminal fusion peptide but different reading frame respective to the multiple cloning sites to simplify in-frame cloning of the genes of interest. The N-terminal fusion peptide consisted of initiation ATG, six-histidine tag and the Xpress™ epitope. Among the three, pcDNA 3.1C vector (APPENDIX K) was chosen as both the *GRA2* and *GRA5* inserts were in-frame with the downstream multiple cloning sites and the vector stop codon. High-level stable and non-replicative transient expression can be carried out in most mammalian cells. Meanwhile, pcDNA 3.1/His/*lac*Z, is actually the pcDNA 3.1B vector with a 3.2 kb fragment containing the β-galactosidase gene cloned in frame with the N-terminal peptide and acts as a positive control for transfection, expression, and purification in the experiment.

Overnight *Eco*RI digestion performed on positive clones of pGEM®-T-GRA2, pGEM®-T-GRA5 as well as the eukaryotic expression vector, pcDNA 3.1C produced digested fragments of GRA2, GRA5 and pcDNA 3.1C (dephosphorylated) with the same sticky ends to enable ligation to occur (Figure 4.4). Successfully transformed TOP10F' colonies with the respective ligated products were selected and grown on ampicillin-containing LB agar plate. Single isolated TOP10F' white colonies were picked and plated onto another fresh ampicillin-containing LB agar plate for further characterization through colony PCR amplification, *Eco*RI restriction digestion analysis and verification by automated DNA sequencing with T7 forward and BGH reverse primers.

Colony PCR amplification performed on the selected TOP10F' colonies involved T7 forward primer and gene-specific reverse primer to ensure correct sense orientation of the cloned gene. T7 forward primer has a fixed position in the vector sequence, therefore amplification of the target gene depends mainly on its ligation orientation which means that amplification will only occurs if the target gene has been ligated correctly and vice versa. Figure 4.25 showed that such PCR amplification yielded fragments with expected sizes of 669 bp and 468 bp for GRA2 and GRA5 clones respectively. An increase in fragment size indicated inclusive of the upstream region of the expression vector (183 bp) covered by T7 forward primer.

Positive clones were further confirmed through *Eco*RI digestion, thereby releasing a 5500 bp linearized pcDNA 3.1C vector and a 486 bp of *GRA2* insert or a 285 bp of *GRA5* insert as depicted in Figure 4.26. Meanwhile, sequencing analysis of positive recombinant pcDNA 3.1C clones harboring *GRA2* and *GRA5* inserts confirmed that the cloned genes shared 100% identity with the published GRA2 (Accession number: L01753.1) and GRA5 (Accession number: EU918733.1) sequences respectively (APPENDIX G). The deduced amino acids of GRA2 and GRA5 translated proteins were determined (APPENDIX H). T7 forward and BGH reverse primers involved are the primers originated from pcDNA 3.1C vector sequence. The sequence analysis confirmed both gene fragments are in the sense orientation and are in frame with the *Eco*RI restriction site and the stop codon of the vector sequence (Figure 4.27). It also showed that the resulting recombinant pcGRA2 and pcGRA5 constructs retained the open reading frame encoding amino acid residues 24-185 and 26-120 of GRA2 and GRA5 proteins respectively, both excluding the putative hydrophobic N-terminal signal sequences (APPENDIX G and H).

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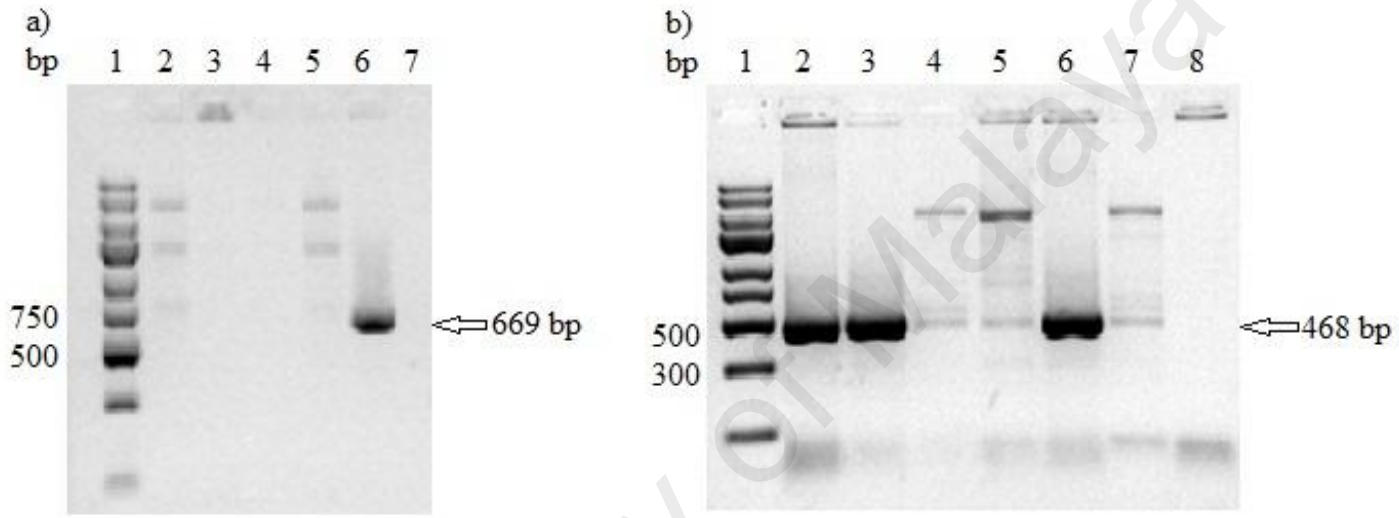

Figure 4.25: Colony PCR amplification of recombinant pcDNA 3.1C clones in TOP10F'. Agarose gel electrophoresis analysis of directional colony PCR amplified a) pcGRA2 and b) pcGRA5 using T7 forward and gene-specific reverse primers. Lane 1 (panel a – b) contained GeneRuler Express DNA Ladder. Lane 7 (a) and 8 (b) contained negative control with double distilled water as template. Lane 2 (a) to 6 (a) contained colony 1 to 5 respectively; colony 5 was selected as positive colony with 669 bp (arrow). Lane 2 (b) to 7 (b) contained colony 1 to 6; colony 1, 2 and 5 were selected as positive colonies with 468 bp (arrow).

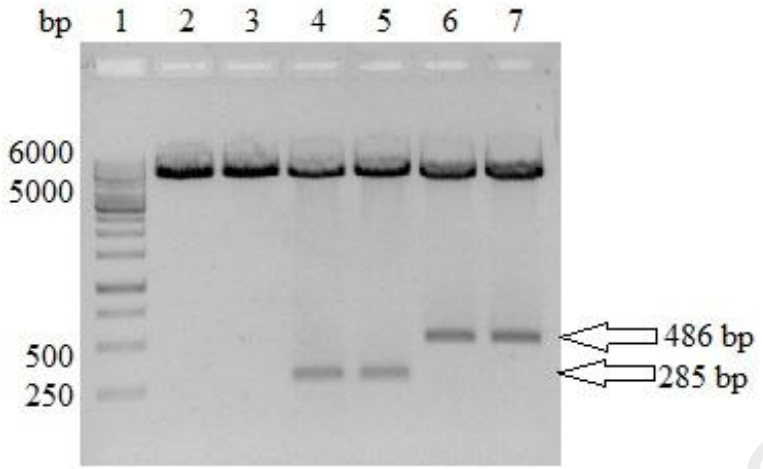

Figure 4.26: Restriction digestion analysis of recombinant pcDNA 3.1C clones. Agarose gel electrophoresis analysis of *Eco*RI digested GRA2 and GRA5 clones in pcDNA 3.1C vector. Lane 1 contained O'GeneRuler 1 kb DNA Ladder. Lane 2 and 3 contained linearized pcDNA 3.1C vector of 5500 bp. Lane 4 and 5 contained digested pcGRA5 producing a 285 bp insert (arrow). Lane 6 and 7 contained digested pcGRA2 generating a 486 bp insert (arrow).

a) pcGRA2

ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GTA CCA GGA TCC AGT GTG GTG **GAA TTC** GCC GAG TTT TCC GGA GTT GTT AAC CAG GGA CCA GTC GAC GTG CCT TTC AGC GGT AAA CCT CTT GAT GAG AGA GCA GTT GGA GGA AAA GGT GAA CAT ACA CCA CCA CTC CCA GAC GAG AGG CAA CAA GAG CCA GAA GAA CCG GTT TCC CAA CGT GCA TCC AGA GTG GCA GAA CAA CTG TTT CGC AAG TTC TTG AAG TTC GCT GAA AAC GTC GGA CAT CAC AGT GAG AAG GCC TTC AAA AAA GCA AAG GTG GTG GCA GAA AAA GGC TTC ACC GCG GCA AAA ACG CAC ACG GTT AGG GGT TTC AAG GTG GCC AAA GAA GCA GCT GGA AGG GGC ATG GTG ACC GTT GGC AAG AAA CTC GCG AAT GTG GAG AGT GAC AGA AGC ACT ACG ACA ACG CAG GCC CCC GAC AGC CCT AAT GGC CTG GCA GAA ACC GAG GTT CCA GTG GAG CCC CAA CAG CGG GCC GCA CAC GTG CCC GTC CCA GAC TTT TCG CAG **GAA TTC** TGC AGA TAT CCA GCA CAG TGG CGG CCG CTC GAG TCT AGA GGG CCC GTT **TAA**

Figure 4.27: Sequencing analysis of recombinant pcDNA 3.1C clones. Sequencing analysis of the positive recombinant a) pcGRA2 and b) pcGRA5 plasmids shows that both cloned genes (underlined; excluding signal sequences) are in the correct sense orientation and are in frame with the *Eco*RI restriction digestion site (**GAATTC**) and the **TAA** stop codon. Gene translations will initiate from the **ATG** start codon of the expression vector.

b) pcGRA5

ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GTA CCA GGA TCC AGT GTG GTG **GAA TTC** GGT TCA ACG CGT GAC GTA GGG TCA GGC GGG GAT GAC TCC GAA GGT GCT AGG GGG CGT GAA CAA CAA CAG GTA CAA CAA CAC GAA CAA AAT GAA GAC CGA TCG TTA TTC GAA AGG GGA AGA GCA GCG GTG ACT GGA CAT CCA GTG AGG ACT GCA GTG GGA CTT GCT GCA GCT GTG GTG GCC GTT GTG TCA CTA CTG CGA TTG TTG AAA AGG AGG AGA AGA CGC GCG ATT CAA GAA GAG AGC AAG GAG TCT GCA ACC GCG GAA GAG GAA GAA GTT GCC GAG GAA GAG **GAA TTC** TGC AGA TAT CCA GCA CAG TGG CGG CCG CTC GAG TCT AGA GGG CCC GTT **TAA**

Figure 4.27 (cont.)

4.12.1 Mammalian cell expression of pcDNA 3.1C constructs

Recombinant constructs of pcDNA 3.1C were transfected into mammalian cells to ensure that the target genes are functional and able to direct protein expression in mammalian cells. The ability to direct protein expression is important for eliciting immune response when the constructs are being injected into the mice model. Transfection study involved two negative controls; pcDNA 3.1C empty vector and non-transfected cells, one positive control; pcDNA 3.1/His/*lacZ*, and two target genes; pcGRA2 and pcGRA5. Protein expressions of the constructs were analysed through western blot assay using Xpress mouse monoclonal antibody as depicted in Figure 4.28. Results obtained showed that pcGRA2 and pcGRA5-transfected CHO cells produced antigenic proteins with their respective expected protein sizes. Positive control-transfected CHO cells produced β-galactosidase protein with approximate protein size of 120 kDa. Meanwhile, no protein bands were detected in both the negative control-transfected and non-transfected CHO cells.

4.13 Immunological characterization of recombinant GRA2 and GRA5 DNA plasmids

Immunological responses of both humoral and cellular-mediated induced by recombinant GRA2 and GRA5 DNA plasmids were characterized in the respective immunized mice.

4.13.1 Induction of humoral immunity

Induction of humoral immune response was evaluated through determination of specific anti-TLA IgG antibody level, antibody titers as well as IgG isotypes.

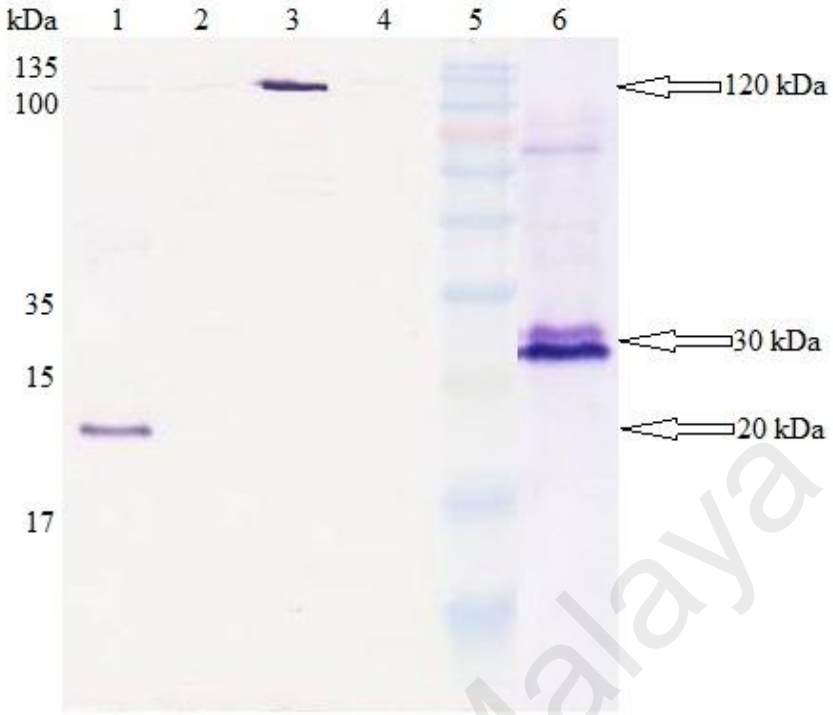

Figure 4.28: CHO cells expression of recombinant proteins. Western blot analysis of mammalian cell expressions of pcDNA 3.1C constructs using Xpress mouse monoclonal antibody. Lane 5 contained Pre-stained Broad Range Protein Marker. Lane 1 contained cell pellet fraction transfected with pcGRA5. Lane 2 contained cell pellet fraction transfected with negative control, pcDNA 3.1C empty vector. Lane 3 contained cell pellet fraction transfected with positive control, pcDNA 3.1/His/*lacZ*. Lane 4 contained non-transfected cell pellet fraction. Lane 6 contained cell pellet fraction transfected with pcGRA2. The GRA2 and GRA5 proteins bands of interest were observed at molecular weights of 30 kDa and 20 kDa (arrow) respectively compared to the negative control. The positive control expressed β-galactosidase protein with expected size of 120 kDa (arrow).

4.13.1.1 IgG antibody detection

Specific anti-TLA IgG antibody was at an undetectable level in the sera collected from all the injected mice at 0, 3, 6 and 9 weeks after first injections through western blot (Figure 4.29) and ELISA (Figure 4.30) assays. The cut-off (mean+2SD) OD450 value of the IgG level in the sera of pcGRA2- and pcGRA5-immunized mice groups was approximately the same as that of both PBS- and pcDNA 3.1C-injected mice groups. On the other hand, antibody titers and IgG isotypes were unable to be determined as well.

4.13.2 Induction of cellular-mediated immunity

Induction of cellular-mediated immune response was evaluated through TLA-specific splenocytes proliferation assay and cytokine production assay.

4.13.2.1 *In vitro* splenocytes proliferation assay

Proliferative response of splenocytes harvested from each mice group to TLA as stimulus was determined using MTT assay and represented by the SI value as illustrated in Figure 4.31 and Table 4.5. Generally, significantly higher SI value was observed in the recombinant DNA plasmid-vaccinated groups compared to the control groups ($p<0.05$). There was no statistical difference found between two vaccinated groups ($p>0.05$) and also between two control groups ($p>0.05$). Meanwhile, SI value for all mice groups had comparable levels in response to the mitogen conA. These results indicated that T lymphocytes of the vaccinated mice were successfully stimulated.

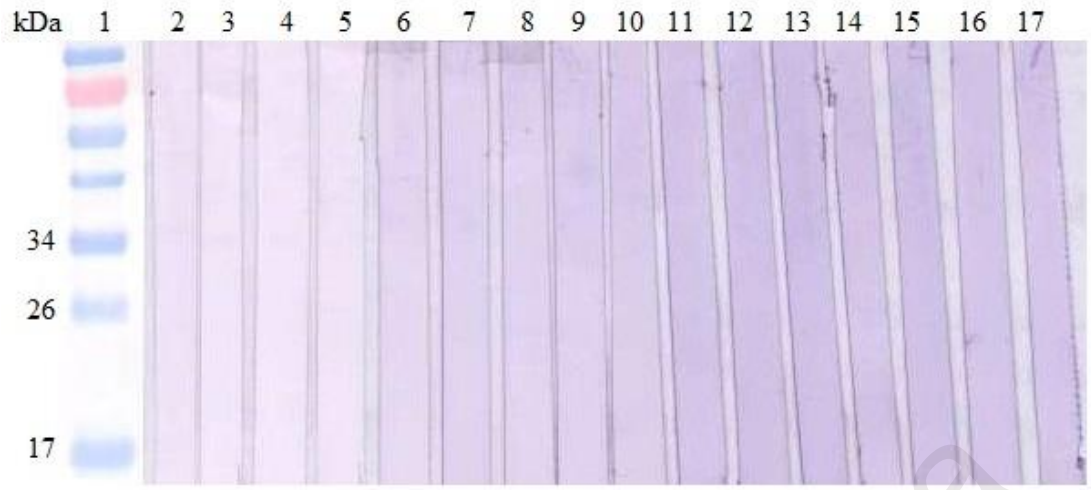

Figure 4.29: Qualitative detections of total specific anti-TLA IgG antibodies in mice sera. Western blots of *T. gondii* TLA with sera of the immunized mice. Lane 1 contained PageRuler™ Prestained Protein Ladder. Lane 2 to 5 contained results of 4 sera from PBS-injected mice, lane 6 to 9 contained results of 4 sera from pcDNA 3.1C-injected mice, lane 10 to 13 contained results of 4 sera from pcGRA2-injected mice and lane 14 to 17 contained results of 4 sera from pcGRA5-injected mice. The 4 sera represented sera collected at week 0, 3, 6 and 9 post-prime injections. No bands of interest were observed in the sera of all the injected mice at all the time point.

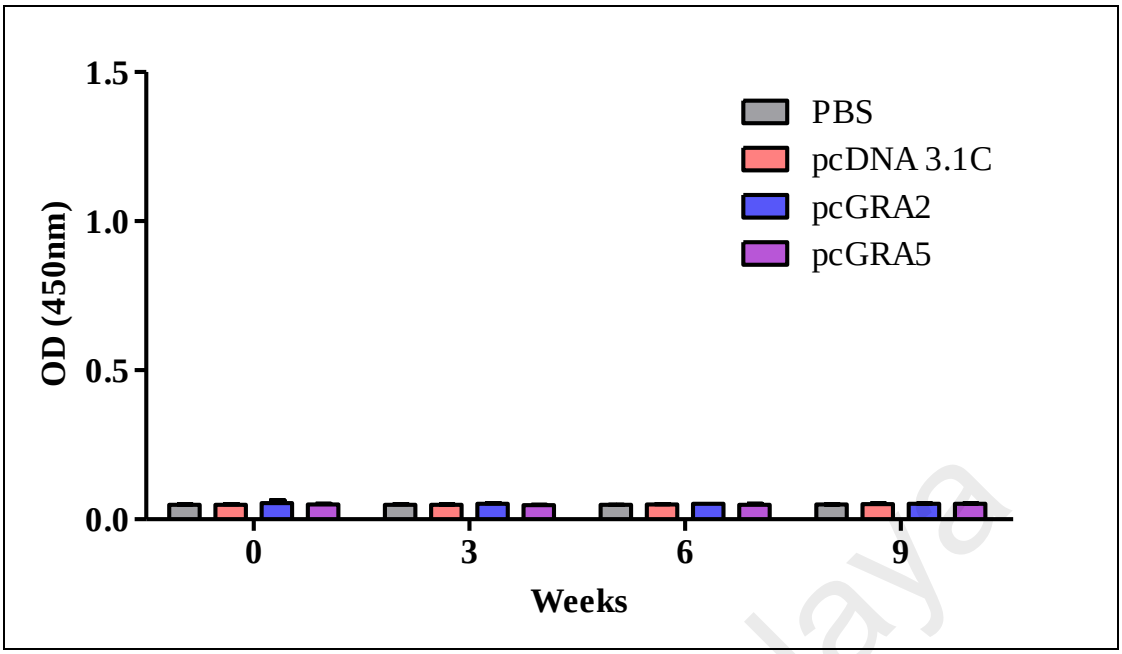

Figure 4.30: Quantitative detections of total specific anti-TLA IgG antibodies in mice sera. Total anti-TLA IgG antibodies in the immunized mice sera were evaluated by ELISA. Sera were collected from each mice group one day before each immunization. Data are expressed as mean OD_{450} ±SD (n=3). Undetectable level of anti-TLA IgG antibodies is observed in the sera of all the injected mice at all the time point.

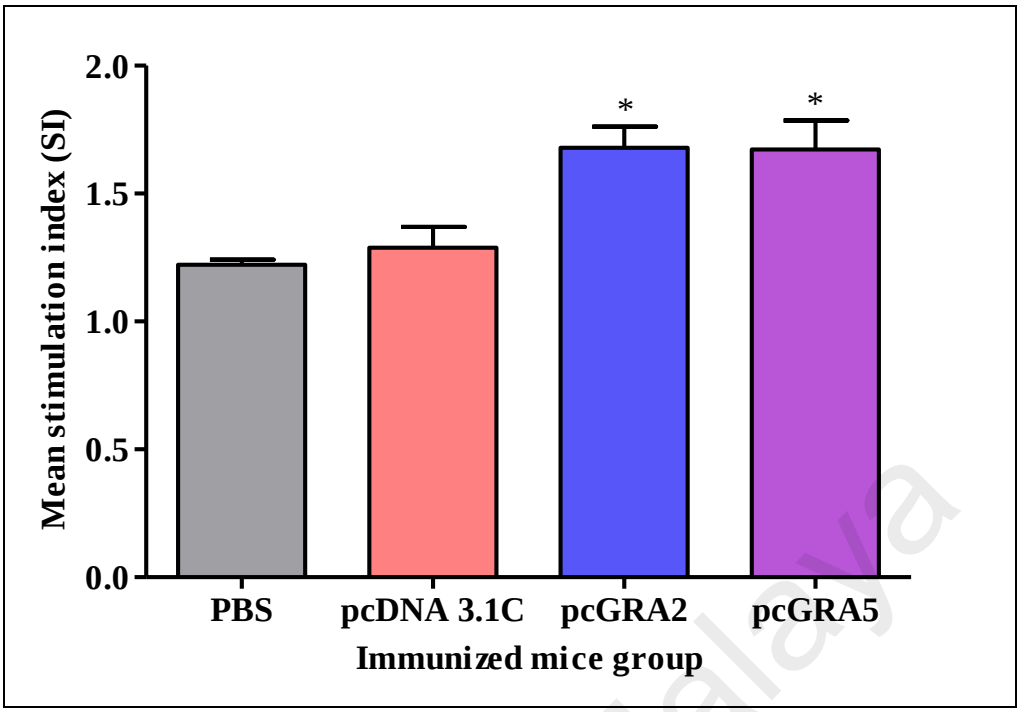

Figure 4.31: *In vitro* splenocytes proliferation response in mice. Spleen lymphocytes were harvested from mice immunized with pcGRA2, pcGRA5, pcDNA 3.1C and PBS three weeks after last injection. The splenocytes were cultured and stimulated with TLA. Proliferative response was measured by MTT assay. Data are expressed as mean stimulation index (SI) ±SD (n=3). Statistical difference is represented by * ($p<0.05$) in comparison with the control groups (PBS or pcDNA 3.1C).

Table 4.5: Characterization of cellular-mediated immunity in the vaccinated mice

Group (n=3)	Proliferation (SI)	Cytokine level (pg/ml)			
		IFN-γ	IL-2	IL-4	IL-10
pcGRA2	1.679 ±0.0828*	5341 ±79.7*	359.1 ±74.51*	21.51 ±11.28*	57.27 ±25.29*
pcGRA5	1.672 ±0.1136*	4669 ±453.2*	360 ±63.48*	15.12 ±2.738	51.47 ±20.06*
pcDNA 3.1C	1.289 ±0.0812	757.5 ±365.4	179.9 ±42.14	Undetectable	Undetectable
PBS	1.222 ±0.0189	625.1 ±367.8	143.1 ±34.39	Undetectable	Undetectable

SI stands for stimulation index.

IFN-γ activity was assayed at 96 h, IL-2 and IL-4 activities were assayed at 24 h, and IL-10 activity was assayed at 72 h.

Undetectable IL-4 and IL-10 level was observed in the stimulated splenocytes culture supernatant of the negative control mice groups.

Data are expressed as mean ±SD (n=3). Statistical difference is represented by * ($p<0.05$) in comparison with the control groups (PBS or pcDNA 3.1C).

4.13.2.2 Cytokine production assay

Stimulated spleen T lymphocytes of the vaccinated mice were evaluated for their cytokine production level. Cytokines (IFN-γ, IL-2, IL-4 and IL-10) secreted and released into the supernatant of the culture of TLA-stimulated splenocytes were collected (96 h, 24 h, 24 h and 72 h, respectively) and assayed by ELISA. The assay was performed in order to determine the type of immunity being polarized, either Th1- or Th2-type cellular immune response. The results obtained were demonstrated in Figure 4.32-4.33 and Table 4.5.

Results showed that the vaccinated mice produced significantly higher level of IFN-γ and IL-2 compared to the control groups ($p<0.05$) as depicted in Figure 4.32 and Table 4.5. No statistical difference was observed between two vaccinated groups ($p>0.05$) and between two control groups ($p>0.05$). Relatively low levels of IL-4 and IL-10 were secreted by the stimulated splenocytes of the mice immunized with pcGRA2 and pcGRA5 (Figure 4.33 and Table 4.5). In contrast, these two cytokines levels were undetectable in the control groups (Table 4.5). These results showed that predominantly Th1 immune response was favored in the vaccinated mice.

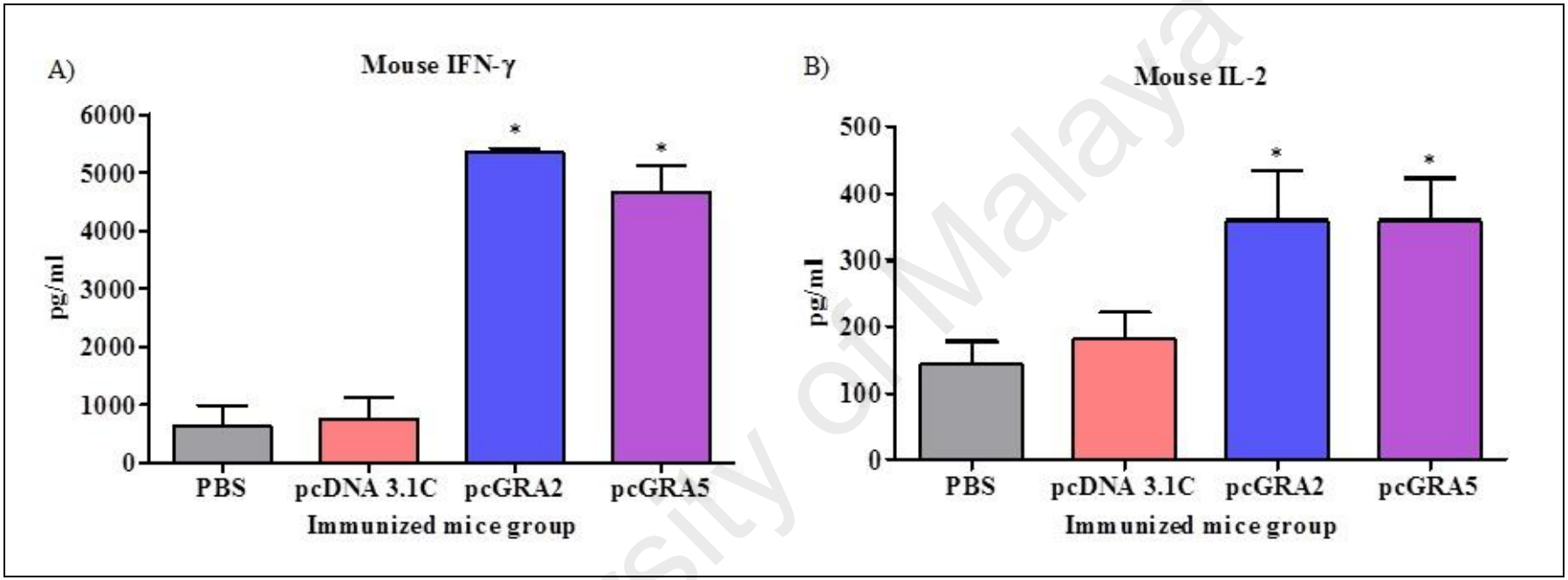

Figure 4.32: IFN-γ and IL-2 production by the stimulated splenocytes of the immunized mice. Culture supernatants from the TLA-stimulated immunized mice splenocytes were collected at 96 h and 24 h post-incubation for the evaluation of A) IFN-γ (96 h) and B) IL-2 (24 h) production respectively via ELISA. Data are expressed as mean±SD (n=3). Statistical difference is represented by * ($p<0.05$) in comparison with the control groups (PBS or pcDNA 3.1C).

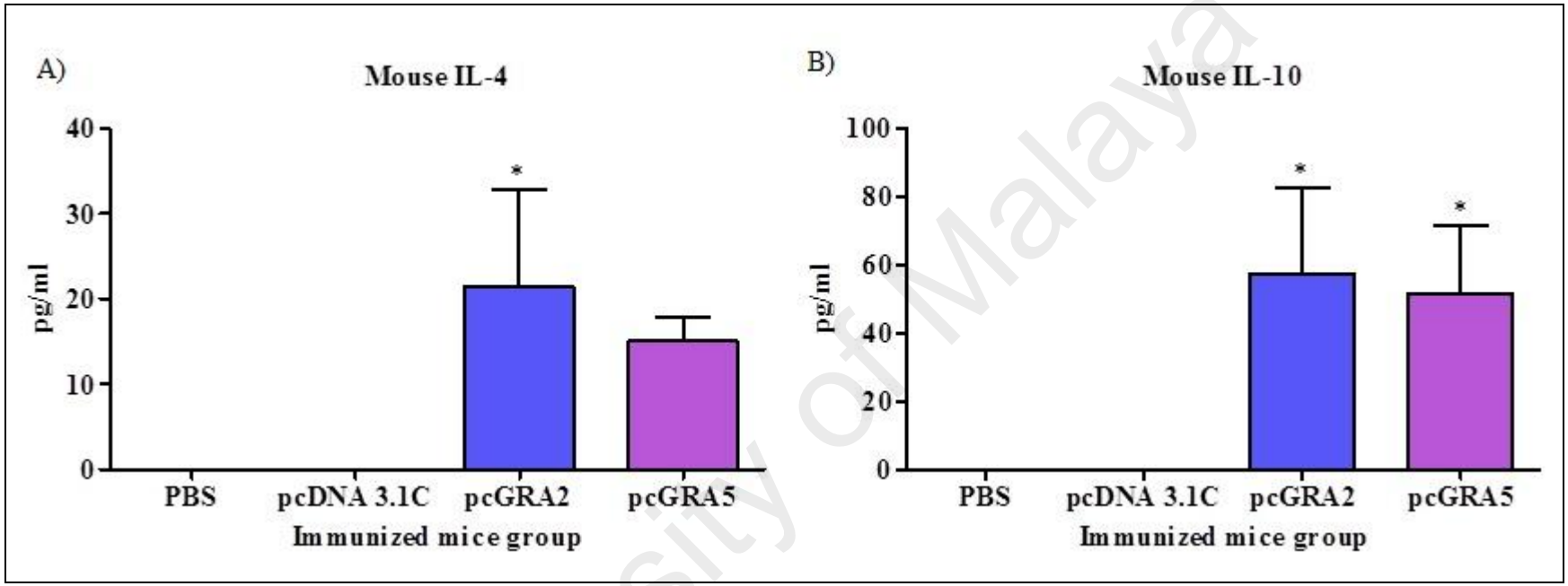

Figure 4.33: IL-4 and IL-10 production by the stimulated splenocytes of the immunized mice. Culture supernatants from the TLA-stimulated immunized mice splenocytes were collected at 24 h and 72 h post-incubation for the evaluation of A) IL-4 (24 h) and B) IL-10 (72 h) production respectively via ELISA. Data are expressed as mean±SD (n=3). Statistical difference is represented by * ($p<0.05$) in comparison with the control groups (PBS or pcDNA 3.1C).

4.13.3 Protective efficacy of recombinant DNA plasmid vaccination in BALB/c mice

Protective efficacy of recombinant GRA2 and GRA5 DNA plasmids in the immunized BALB/c mice were evaluated against lethal challenge with *T. gondii*. The survival rates of the four challenged mice groups were illustrated in Figure 4.34. It was shown that the two vaccinated mice groups were only managed to prolong the survival days up to 2–3 days as compared to the two control mice groups (PBS and pcDNA 3.1C) ($p<0.05$). All PBS-injected mice died on day 6 (median survival of 6 days) while pcDNA 3.1C-injected mice died within 6-7 days (median survival of 7 days). On the other hand, pcGRA2- and pcGRA5-immunized mice succumbed to the parasite infection on 6-9 days post-infection with the median survival of 8 days.

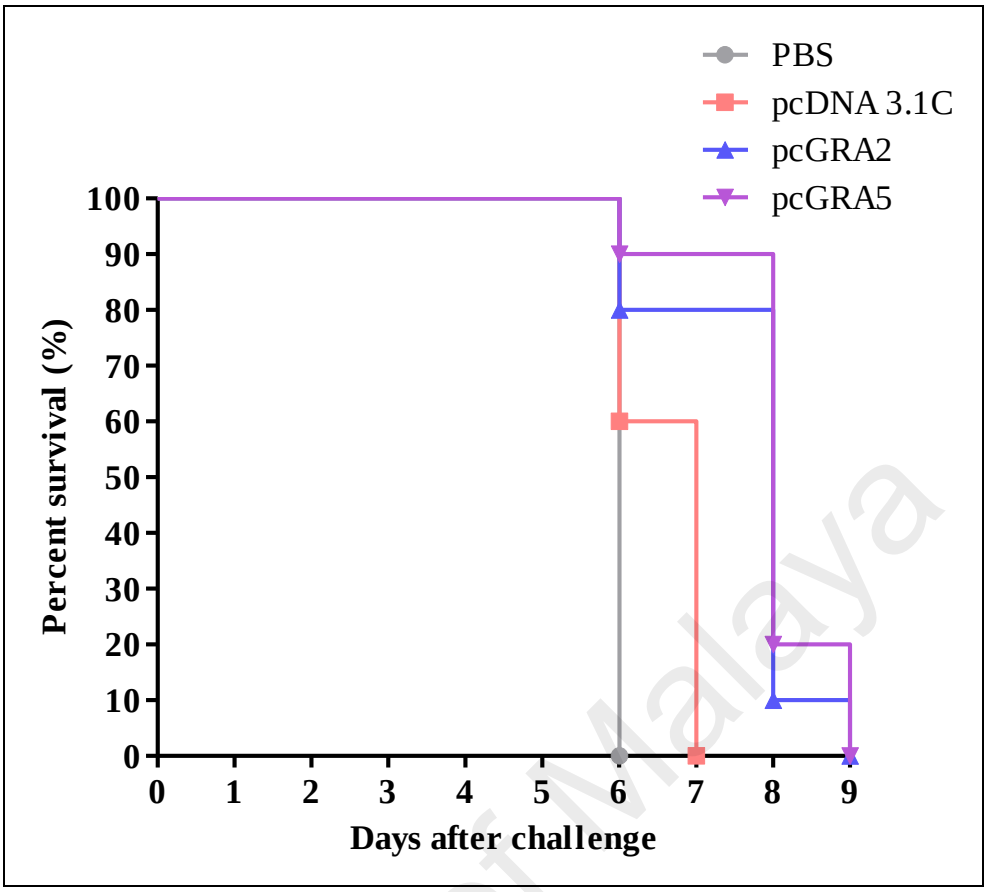

Figure 4.34: Survival rate of the immunized mice. All four groups of the immunized mice (PBS, pcDNA 3.1C, pcGRA2 and pcGRA5) were subjected to lethal challenge with 1000 live tachyzoites of *T. gondii* virulent RH strain 3 weeks after the last immunization. Mice immunized with pcGRA2 and pcGRA5 demonstrated a minor increase in their survival days (median survival of 8 days) in comparison to that of the control mice injected with PBS and pcDNA 3.1C (median survival of 6 and 7 days respectively). Each group consisted of 10 mice.

CHAPTER 5: DISCUSSION

5.1 Overview

Toxoplasma gondii infection or toxoplasmosis is a widespread disease affecting up to one-third of the world's human population (Jackson & Hutchison, 1989). This disease is of major clinical, veterinary and economic concerns. Immunoprophylaxis such as vaccination is considered one of the means to prevent and control the infection.

GRA2 and GRA5 play a critical role in maintaining the parasite's survival in the host cells. Previous studies have demonstrated that native GRA2 and GRA5 could protect vaccinated female rats against congenital challenge with *T. gondii* (Zenner *et al.*, 1999). The main aim of the present study was to produce substantial quantity of purified recombinant GRA2 and GRA5 for evaluation in diagnostic and vaccine development. This recombinant DNA approach would overcome the potential hazard of handling live *T. gondii* cells and impurity of native GRA2 and GRA5 samples.

5.2 Generation of recombinant plasmids of GRA2 and GRA5

As *T. gondii GRA2* contains a single intron (Mercier *et al.*, 1993), this gene was amplified from total RNA extracted from *T. gondii* tachyzoites using RT-PCR amplification yielding a product size of 486 bp. Meanwhile, the *GRA5* was amplified using genomic DNA as template because this gene has no intron. Amplification yielded a product of 285 bp. Both amplified gene fragments were successfully cloned into prokaryotic expression vector, pRSET B and eukaryotic expression vector, pcDNA 3.1C, harbouring the T7 promoter and were then transformed into BL21 (DE3) pLysS and TOP10F' respectively. It was important to ensure that the ligated GRA2 or GRA5 inserted in the expression vectors was in a correct orientation as only a single restriction site (*Eco*RI) was involved in the cloning process. This was confirmed by directional PCR amplification and sequencing verification using T7 forward primer.

5.3 Protein production in prokaryotic system

Two examples of prokaryotic expression vector containing strong bacteriophage T7 promoter which permits high-level expression are pRSET and pET. The latter has a larger plasmid size (>5 kb) as compared to pRSET (2.9 kb) which makes the cloning process more difficult. After several failed attempts to insert the target genes into pET vector, the pRSET was eventually selected for cloning and expression of *GRA2* and *GRA5*.

Expression of the target genes produced rGRA2 and rGRA5 with 6xHis tag at the N terminus, allowing single step affinity purification. The rGRA2 protein was expressed in soluble form, therefore native purification was carried out. Low range concentration (10-20 mM) of imidazole were applied during binding and washing steps to ensure strong binding of the 6xHis tag to the nickel ions as well as to reduce the nonspecific binding of contaminant proteins originating from the host cells. The target protein was eventually eluted with high imidazole concentration (250 mM).

However, rGRA5 was produced as inclusion bodies, accumulating intracellularly in the insoluble aggregates Purification of such bodies requires a denaturing agent (e.g., guanidine hydrochloride) in the buffer to facilitate solubilisation. Elution buffer with low pH (pH 4.0) (Terpe, 2003) was used to elute rGRA5 protein. Protonation of the histidine residues (pKa = 6.0) in the 6xHis tag occurred in low pH which would further help rGRA5 to dissociate from the positively-charged nickel ions attached to the NTA matrices. Washing and elution buffers contained 8 M urea to maintain rGRA5 in a soluble form. Eluted rGRA5 protein which was in denatured form was subjected to dialysis against phosphate-buffered saline (PBS) overnight in order to renature and refold the protein by removing the denaturant/solubilizing agents such as urea. SDS-PAGE and western blot analysis demonstrated that the renatured rGRA5 retained its antigenicity.

Poly-histidine tag is one of the common short affinity tags fused to the recombinant protein produced with varying number of histidine ranging from 2 to 10. The expression vector that was used in this study consisted of 6x histidine (6xHis). The small size of poly-histidine tag makes it more efficient compared to the larger peptide tags as it will not interfere with the structure and function of the target protein. It is also less immunogenic compared to larger peptide tags and thus the fusion protein produced is suitable for inducing antibody production without removing the short affinity tag beforehand. Even though large peptide tag can help to enhance solubility of the target protein but the tag has to be cleaved before the target protein can be utilized (Terpe, 2003). Tag removal is a tedious process and may encounter unspecific cleavage which subsequently leads to structural as well as functional defects in the protein of interest.

Heterologous expression and purification of rGRA2 and rGRA5 produced protein of about 30 and 20 kDa respectively, instead of the predicted molecular size of 23 and 16 kDa respectively. The size discrepancy can be attributed to the presence of the 6 histidine residues in the recombinant proteins and the intrinsic error ($\pm$10%) of molecular mass determination by SDS-PAGE. Besides, it is also possible that the difference stemmed from the high proline composition of GRA (Mercier *et al.*, 2005). The deduced proline content in rGRA2 and rGRA5 was 8.6% and 1.1% respectively. The presence of peptidyl-prolyl cis-trans-isomerase in the *E. coli* (Liu & Walsh, 1990) may contribute to the catalysis of proline isomerization during protein-folding activity (Lin *et al.*, 1988) which eventually may affect protein migration leading to the observed differences in sizes. The same phenomenon was noticed in other *Toxoplasma* proteins such as GRA3, GRA6 and GRA7 (Bermudes *et al.*, 1994; Jacobs *et al.*, 1998; Lecordier *et al.*, 1995).

Regardless of the size discrepancy, the identities of the purified recombinant proteins were verified by western blot analysis with monoclonal anti-Xpress^{TM} antibody

as well as MALDI-TOF MS analysis. Besides identity confirmation, western blot analysis also confirmed the antigenicity of both recombinant proteins produced.

5.4 Evaluation of sensitivity and specificity of the recombinant proteins

5.4.1 rGRA2

Evaluation of the immunoreactivity of rGRA2 protein against human sera of toxoplasmosis-positive, toxoplasmosis-negative and other infections revealed moderate to high sensitivity and specificity values. In brief, 100.0%, 100.0% and 61.5% sensitivity were observed with serum samples from patients with early acute, acute and chronic phase of toxoplasmosis infection respectively. Specificity of 90.0% and 83.3% were seen with human sera of toxoplasmosis-negative and of those infected with other pathogens respectively. Results of this study are in agreement with previous studies in France and Iran which used TRX-(Hisx6)-GRA2, a thioredoxin tagged fusion protein through IgG ELISA (Golkar *et al.*, 2007a). Results from the study in France revealed 95.8% and 65.7% sensitivity for sera of acute infection and of chronic infection respectively. Results in Iran showed 100% sensitivity for sera with acute infection and 71.4% sensitivity for sera with chronic infection (Golkar *et al.*, 2007a). Another similar study using *E. coli*-expressed rGRA2 also reported 100% and 22.5% sensitivity for sera with acute and chronic infection respectively (Holec-Gasior *et al.*, 2009).

In this study, cross-reactivity was not observed with serum samples from patients infected with amoebiasis, cysticerosis and filariasis (Table 4.1). However, two of the three toxocariasis-positive sera samples reacted with the rGRA2 antigen. These two toxocariasis-positive serum samples were IgG positive but IgM negative for toxoplasmosis based on Novalisa^{TM} *Toxoplasma gondii* IgG and *Toxoplasma gondii* IgM ELISA kits (NovaTec, Germany). This may suggest co-infection of *T. gondii* and *Toxocara* spp. in the patient (Jones *et al.*, 2008). Both *T. gondii* and *Toxocara* can be

acquired through soil ingestion. Therefore, the possibility of co-infection with these two parasites is highly possible (Jones *et al.*, 2008).

One of the key findings in this study is the ability of the purified rGRA2 antigen to detect *Toxoplasma* infection according to three main phases of infection – the early acute, acute and chronic. Immediate treatment is possible for the infected hosts, either humans or animals with this additional ability of detecting early acute phase of toxoplasmosis, hence preventing worsening of medical condition.

5.4.2 rGRA5

A previous study using immunodominant epitopes only of GRA5 failed to show any immunoreactivity with a pool of *T. gondii*-positive human sera (Dai *et al.*, 2012). Therefore, in this study, the full-length rGRA5 was constructed and produced. Evaluation of rGRA5 immunoreactivity revealed high specificity when tested with sera from toxoplasmosis-negative patients and those infected with other parasites (100.0% and 91.7%, respectively). In addition, sensitivity of 46.8% and 61.2% were obtained with sera of patients with acute and chronic *Toxoplasma* infection, respectively. However, none of the serum samples from the early acute phase patients reacted with rGRA5 protein. This is similar to the finding of a study on rGRA5 antigen-mediated detection of IgG antibodies using ELISA (Holec-Gasior & Kur, 2010). Specificity of the aforementioned study was shown to be 100.0%, whereas sensitivities of 63.0% and 75.0% were reported for sera from acute and chronic infection, respectively. Thus, it is likely that rGRA5 yields a much higher reactivity towards IgG antibodies in sera from chronically infected patients compared to that with acute infection. Notably, this protein showed no reactivity towards IgM antibodies in the sera of early acute stage patients.

Cross-reactivity was not observed with sera samples from patients infected with amoebiasis, cysticerosis, and filariasis (Table 4.2). One out of three toxocariasis-

positive sera samples reacted with the rGRA5 antigen. However, two of these toxocariasis-positive serum samples were shown to be IgG positive but IgM negative for toxoplasmosis based on findings from Novalisa^{TM} *Toxoplasma gondii* IgG and IgM antibodies ELISA kits. These two toxocariasis-positive sera samples reacted with rGRA2 antigen (Table 4.1), indicating that rGRA5 was less sensitive in the detection of anti-*Toxoplasma* IgG compared to rGRA2.

5.4.3 Advantages of western blot over ELISA

Although ELISA is a sensitive quantitative assay, it however is less specific due to the higher probability of acquiring false-positive results leading to inaccuracy (Gamble *et al.*, 2004; Nockler *et al.*, 2009). Therefore, western blot which has higher specificity was chosen to evaluate both rGRA2 and rGRA5 in this study. Also, the chances of obtaining false-positive results via western blot are much lower compared to ELISA (Nockler *et al.*, 2009). In fact, it has been reported that western blot analysis is superior to ELISA for screening serum samples because it is more informative, is less affected by sample degradation, produces results of high confidence with direct visualization of the binding between antibodies in the test samples and their respective specific diagnostic antigens as well as offering improved determination of diagnostic antigen purity (Anderson *et al.*, 2007).

As for the future development of diagnostic tests for *T. gondii*, the western blot results obtained in this study should be reliable for predicting the efficacy of using rGRA2 or rGRA5 antigens in immunochromatographic tests (ICT) due to similarities between the two assays. Western blot and ICT are both immunoassays utilizing nitrocellulose membranes and direct visualization of results. Indeed, ICT is a better format for diagnosis of infections compared to ELISA, which is commonly used due to its simplicity. Furthermore, ICT is a rapid test with high accuracy that costs less than the

time-consuming and laborious ELISA (Huang *et al.*, 2004). Another advantage of ICT is that it can be used in the field (Huang *et al.*, 2004), especially for the diagnosis of farm animals.

5.5 Immunoprotective study with the recombinant proteins

Subunit vaccination is generally known for its efficacy in inducing humoral immune response against extracellular pathogens through antibody generation which favors T helper 2 (Th2) related response. This is proven by a shift from the initially Th1 to Th2 immunity by B cells (humoral) as observed in malaria infection (Langhorne *et al.*, 1998). However, *T. gondii* is an intracellular parasite, thus cellular mediated immune response especially by $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells are the main components required to combat this parasitic infection (Denkers & Gazzinelli, 1998). Immunization using animal model with recombinant expressed protein alone is weakly immunogenic (Sloat *et al.*, 2010) and often elicited a mixed Th1/Th2-like response with higher tendency of IgG1 isotype production, driving predominantly Th2-like response (Dziadek *et al.*, 2009; Dziadek *et al.*, 2012; Echeverria *et al.*, 2006; Sun *et al.*, 2014). As a result, addition of Th1-directing adjuvant such as Complete Freund's adjuvant (CFA) was used in this study. This was to enhance the immunogenicity of the subunit vaccine as well as directing the immune response towards Th1.

Immunogenicity and protective efficacy of several *T. gondii* recombinant antigens produced in bacteria have also been performed against *Toxoplasma* infection in experimental mouse models (Dziadek *et al.*, 2009; Dziadek *et al.*, 2012; Martin *et al.*, 2004). It was reported that alum adjuvant-formulated rGRA4 was a potential multi-antigen vaccination candidate against chronic *T. gondii* infection either alone or in combination with rROP2 which were both produced in pQE expression vector. It protected the vaccinated C57BL/6 and C3H mice against challenge with ME49 strain

through brain cyst reduction (Martin *et al.*, 2004). Meanwhile, vaccination of C3H/HeJ mice with rROP2 and rROP4 which were expressed in pHis vector has been shown to elicit mixed Th1/Th2-type immune response with specific IL-2 production. These two antigens conferred partial protection against challenge with DX strain (low virulent) with 46% brain cysts reduction. It was also reported that combination of the same rROP2 and rROP4 antigens with either rGRA4 or rSAG1 triggered both humoral (generation of high levels of IgG1 and IgG2a) and cellular- (secretion of IFN-γ and IL-2) associated immunity. The brain cysts loads in the vaccinated BALB/c mice were greatly decreased (84% and 77% reduction respectively) compared to PBS-injected mice (Dziadek *et al.*, 2009; Dziadek *et al.*, 2012).

In this study, immunization of BALB/c mice with rGRA2 and rGRA5 mixed with complete Fruend's adjuvant (CFA) successfully triggered both humoral and cellular mixed Th1/Th2-like immune responses, predominantly Th1 in the vaccinated mice. The triggered immune response eventually prolonged the mice's survival rates against lethal challenge with the virulent RH strain of *T. gondii*. These results confirmed the antigenicity and immunogenicity of the two recombinant proteins.

Analysis of IgG antibody through western blot and ELISA indicated that anti-GRA2 and anti-GRA5 IgG antibody was produced in the immunized mice two weeks after prime injection. The antibody levels increased with successive immunization where antibody titres ranged from 1:409,600 to 1:819200 at six weeks post-prime injection. Besides, relatively high levels of IgG1 and IgG2a were detected in the immunized mice serum. The levels of these two antibody isotypes are almost similar with slightly higher IgG1 than that of IgG2a. Production of IgG1 is Th2 related, while IgG2a is associated with Th1-driven immunity (Germann *et al.*, 1995).

$CD4^+$ Th1 cell populations are involved in B cells activation and subclass-switched antibody production, whereby its absence would lead to increased

susceptibility to *T. gondii* (Harris *et al.*, 2001; Johnson & Sayles, 2002). Induction of humoral immune response plays an essential role in the resistance against *Toxoplasma* infection in which most of the B cell-deficient mice survived from the infection post-treatment with immune serum (Frenkel & Taylor, 1982). Significant protection elicited through intraperitoneal injection of monoclonal anti-*T. gondii* surface antigen antibody into mice against moderately and highly virulent *Toxoplasma* infection further supports the importance of humoral immunity in fighting toxoplasmosis (Johnson *et al.*, 1983). Other than conferring resistance to *T. gondii* acute infection and controlling its chronic infection, humoral immunity has been demonstrated to be important in protecting rodents against other protozoa parasites as well, such as *Plasmodium berghei yoelii* (Weinbaum *et al.*, 1976) and *Trypanosoma cruzi* (Rodriguez et al., 1981).

A study on the protective and resisting roles of B cells response against lethal challenge with virulent strains of *T. gondii* demonstrated that antigen-specific antibody inhibited host cell active invasion by blocking the tachyzoites directly, preventing them from attaching to the host cell and thus restricting parasite propagation (Sayles *et al.*, 2000). On the other hand, antibody-coated tachyzoites could be destroyed by phagocytic cells such as macrophage through passive phagocytosis (Sibley *et al.*, 1993). Positive correlation was observed between the high titers of two IgG isotypes; IgG1 and IgG2a and the levels of phagocytosis which eventually protected immunized mice against *S. pneumonia* (Lefeber *et al.*, 2003).

Earlier findings reported that monoclonal antibody against *T. gondii* surface antigens successfully blocked tachyzoites invasion and *in vitro* propagation as compared to monoclonal antibody against antigens of *T. gondii* secretory organelles with little or no effect on invasion (Grimwood & Smith, 1996; Johnson *et al.*, 1983). However, another study showed that phagocytic cell (macrophage) invasion of *T. gondii* was inhibited by monoclonal anti-GRA2 in the presence of complement (Cha *et al.*, 2001).

At the same time, the monoclonal antibody partially protected mice against RH strain tachyzoite infection mediated by complement-dependent effector mechanism (Cha *et al.*, 2001; Sayles *et al.*, 2000). This highlights the protective role played by specific antibody.

Apart from humoral immunity, cell-mediated immunity is a major protective response against intracellular *T. gondii*. The cell-mediated immunity response is through specific T lymphocytes activation ($CD4^+$ and $CD8^+$), especially Th1 response coupled with IFN-γ production (Gazzinelli *et al.*, 1991; Parker *et al.*, 1991; Sibley *et al.*, 1993; Suzuki & Remington, 1988). Interferon-gamma-mediated cytotoxic T lymphocyte (CTL) response restraints the propagation and spreading of the parasite by impeding the growth of actively-dividing tachyzoites (acute phase) and limiting reactivation of the encysted bradyzoites (Dillon *et al.*, 1992; Pfefferkorn, 1984; Pfefferkorn *et al.*, 1986; Suzuki *et al.*, 1988; Zheng *et al.*, 2013).

In this study, stimulated T-lymphocytes in the spleen cells of rGRA2- and rGRA5-immunized mice proliferated significantly. Interferon-gamma and IL-2 were two pro-inflammatory cytokines that were secreted in large amount suggesting that $CD4^+$ Th1 and $CD8^+$ cytotoxic T-cells were being triggered. Meanwhile, IL-4 and IL-10 were also produced but in relatively low levels which are associated with $CD4^+$ Th2 cells induction. Besides fighting against *T. gondii*, these two anti-inflammatory cytokines play a vital role in balancing and reducing the deleterious inflammatory effect, especially of IFN-γ (Bessieres *et al.*, 1997; Gazzinelli *et al.*, 1991; Snapper & Paul, 1987).

Humoral and cellular immune responses are interrelated and synergistic instead of acting alone to mount protection against any pathogen. Activated cytokine-secreting Th cells are involved in the stimulation of antibody-producing B cells as well as determining the switching of antibody isotype, either IgG1 or IgG2a in T-cell dependent

immunity (Germann *et al.*, 1995; Snapper & Paul, 1987). Th1-related IFN-γ induces IgG2a generation and expression of the respective FcR1 on mouse macrophage (equivalent to human monocyte FcR), suppressing IgG1 synthesis at the same time. In other words, Th1 is responsible for macrophage activation through stimulation of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-2) and IgG2a antibody generation. The protective role of IFN-γ and IgG2a has been demonstrated through opsonisation, complement-mediated cell lysis and antibody-dependent cellular cytotoxicity (ADCC) (Johnson *et al.*, 1985; Petersen *et al.*, 1998; Snapper & Paul, 1987). On the other hand, Th2-related IL-4, which is also known as B cell stimulatory factor-1 (BSF-1), possesses the antagonistic effect of IFN-γ whereby it enhances IgG1 production and FcR2 expression on mouse macrophage (equivalent to natural killer cell FcR in human) but suppresses IgG2a production (Perussia *et al.*, 1983; Snapper & Paul, 1987). Th2 stimulates development of anti-inflammatory cytokines (IL-4, IL-5, IL-6 and IL-10) and IgG1 antibody which leads to down regulation of macrophage activity (Petersen *et al.*, 1998).

Interaction between antibody-producing B lymphocytes and cytokine-producing T lymphocytes (Th1 and Th2) had successfully increased the median survival time of *T. gondii*-challenged mice from 6-8 days (non-immunized mice; PBS and pRSET B-injected) to 16-16.5 days (immunized mice; rGRA5 and rGRA2-injected). It has been reported that sterile protective immunity to toxoplasmosis is possible if strong humoral and cellular immune responses are successfully elicited and acting synergistically whereby the survival rates of the infected mice will not significantly increase in the absence of either antibody production or T cell immunity (Frenkel & Taylor, 1982).

The delayed onset of death observed in rGRA2 and rGRA5-immunized mice was partly due to the interplay between Th1 and Th2-driven responses as findings have shown that Th1-related cytokine primarily IFN-γ causes early mortality whereas Th2-

related IL-4 and IL-10 diminish short-term fatality by down regulating Th1 response and thus reducing the severe inflammatory effect provoked by IFN-γ at the early acute phase of toxoplasmosis (Neyer *et al.*, 1997; Petersen *et al.*, 1998; Roberts *et al.*, 1996). An increase in survival rate and decrease in necrosis of the small intestine was observed in C57BL/6 mice treated with monoclonal anti-IFNγ antibody (Liesenfeld *et al.*, 1996). It has also been determined that secretion of IFN-γ is directly proportional to the mortality rate of the infected mice (McLeod *et al.*, 1989). IL-4-deficient mice have higher susceptibility towards acute *Toxoplasma* infection compared to the wild type due to excessive IFN-γ secretion. In contrast, development of necrotic lesions with free living tachyzoites has been seen in wild-type mice but not in IL-4-deficient mice (Roberts *et al.*, 1996). One of the negative regulatory effects of IL-10 is to suppress macrophage killing activity mediated by IFN-γ (Sibley *et al.*, 1993). Infected IL-10-depleted mice died during acute *Toxoplasma* infection with relatively high levels of IFN-γ and IL-12 detected in their serum. These mice were believed to have succumbed to lethal immunopathology instead of parasitic infection as there was no sign of significant *T. gondii* propagation (Gazzinelli *et al.*, 1996b).

The overall results obtained in the present study are in agreement with the results of previous studies. The recombinant antigens triggered strong humoral and cellular Th1-dominating immune response by up-regulating the development of antigen-specific IgG antibody (IgG2a) and Th1-related cytokines (IFN-γ and IL-2) (Golkar *et al.*, 2007b; Zhou *et al.*, 2007). Monophosphoryl lipid A (MPL) adjuvant-formulated rGRA2 had been shown to reduce brain cysts formation significantly in the immunized CBA/J mice either alone or mixed with rGRA6, thereby protecting against chronic *T. gondii* infection (Golkar *et al.*, 2007b). Immunization of BALB/c mice with multiantigenic protein vaccine containing SAG1-GRA2 expressed in yeast host successfully increased

survival time of the vaccinated mice up to 15 days against lethal challenge with *T. gondii* RH strain (acute infection) (Zhou *et al.*, 2007).

Protective efficacy of GRA5 subunit vaccine against chronic toxoplasmosis has been indicated by intranasal immunization in combination with rGRA7 and rROP2 adjuvanted with cholera toxin by reducing brain cyst formation of VEG strain by 58.3% in BALB/c mice (Igarashi *et al.*, 2008b). Nevertheless, this is thus far the first report of evaluation of the immunity elicited by GRA5 as a single-antigenic either subunit or DNA vaccine candidate against acute toxoplasmosis in the mouse model.

5.6 Protein production in eukaryotic system

Heterologous expression of recombinant protein in mammalian cell involves several steps including foreign gene transfection into the host cell, delivery into host cell nucleus where transcription takes place followed by transportation of the transcribed mRNA into cytoplasm for protein translation (Kunert & Vorauer-Uhl, 2012). TurboFect™ Transfection Reagent that was used in this study is a water-based cationic polymer. It formed a positively-charged complex with the recombinant DNA plasmid of GRA2 or GRA5 at pH 7.4. The condensed and stable polyplex formed carrying the positive charges from the cationic polymer were essential for the uptake by CHO cell through endocytosis against the cell membrane which is negatively-charged. Other than providing the positive charges, the transfection reagent helped to protect foreign DNA from degradation (Kunert & Vorauer-Uhl, 2012). The two target genes of *T. gondii* were successfully transfected into CHO cells in the form of pcDNA 3.1C-GRA2 and pcDNA 3.1C-GRA5 followed by expression of the respective proteins as seen in the western blot analysis. However, the protein expression levels were too low that these proteins could not be seen in Coomassie stained SDS-PAGE gel. The transfection approach in this study is transient as the target genes were introduced into CHO cells as

foreign plasmids and were not integrated into the host cells genomes. Therefore the foreign genes will not be propagated but only present in the host cells for few days which would eventually be diluted upon host cells divisions, degraded by nucleases or other environment factors (Hartley, 2012).

Transient transfection study served as a preliminary experiment before performing the actual DNA vaccination in live animal models. Previous studies demonstrated that pGRA2- and pGRA5-transfected HEK 293-T produced 28 kDa and 18 kDa of GRA2 and GRA5 proteins respectively as detected in western blot analysis against their respective monoclonal antibody (Babaie *et al.*, 2011; Golkar *et al.*, 2005). The size of the two target proteins are almost similar to that obtained in this study. Another study reported that *in vitro* expression of pVAX1-GRA2 in HFF cells produced an antigenic protein at about 20 kDa (Zhou *et al.*, 2012). Meanwhile, *in vitro* expressions of several others *Toxoplasma* genes were also evaluated in mammalian cells and were detected either through western blot, indirect immunofluorescence or immunochemistry assay (Chen *et al.*, 2013; Hiszczynska-Sawicka *et al.*, 2010; Hiszczynska-Sawicka *et al.*, 2011; Ismael *et al.*, 2003; Qu *et al.*, 2013; Tao *et al.*, 2013; Wu *et al.*, 2012; Xue *et al.*, 2008; Yuan *et al.*, 2011a; Yuan *et al.*, 2011b; Zhou *et al.*, 2012).

5.7 Immunoprotective study with the recombinant DNA plasmids

DNA vaccination of BALB/c mice with pcDNA 3.1C-GRA2 and pcDNA 3.1C-GRA5 triggered mixed Th1/Th2-like cellular immune responses, predominantly Th1 in the vaccinated mice. Humoral immunity was not successfully elicited as anti-TLA IgG production was not detected either through western blot or ELISA analysis. The vaccinated mice were not protected against lethal challenge of *Toxoplasma* infection.

The results obtained here demonstrated that T-lymphocytes harvested from the spleen cells of pcDNA 3.1C-GRA2- and pcDNA 3.1C-GRA5-vaccinated mice were stimulated and proliferated significantly. Two pro-inflammatory cytokines; IFN-γ and IL-2 were secreted in huge quantity. However, relatively low levels of the anti-inflammatory cytokines IL-4 and IL-10 were produced. Collectively, it was shown that a predominantly Th1 immune response was elicited which prolonged median survival days of the injected mice 1 to 2 days.

The predominant Th1-like response in the vaccinated mice were most likely contributed by the presence of unmethylated CpG motifs in the pcDNA 3.1C; a bacterial DNA plasmid that was used to construct recombinant DNA vaccines (Rosenberg *et al.*, 2009; Sun *et al.*, 2011). The immunostimulatory CpG motifs possess Th1-directing adjuvant activity and are associated with IFN-γ secretion as well as enhancing the immunogenicity of the respective DNA vaccines (Chu *et al.*, 1997; Klinman *et al.*, 1997; Roman *et al.*, 1997; Sato *et al.*, 1996).

The DNA vaccines of GRA2 and GRA5 were constructed without their respective signal peptide at the N-terminal to ensure the translated proteins remained in the cytosol of the vaccinated host for subsequent antigen processing and presentation through the major histocompatibility (MHC) class I molecule to trigger cellular-mediated immune response (York *et al.*, 1999). The exclusion of signal peptide from these two DNA vaccine constructs might be the reason contributing to the failure to develop antigen-specific antibody response as the translated proteins were not signaled out from the cells for the formation of peptide-MHC class II complex (Corr & Tighe, 1997). A similar phenomenon was observed in previous studies whereby DNA vaccination candidates encoding MIC2-MIC3-SAG1 and GRA3-GRA7-M2AP lacking the signal peptides stimulated only weak antibody production (Rosenberg *et al.*, 2009) while no antibody was elicited by DNA vaccines expressing MIC2 and MIC3 antigens

(Beghetto *et al.*, 2005). In contrast, full-length recombinant DNA vaccine constructs have been shown to produce strong antigen-specific antibody response (Dautu *et al.*, 2007).

In contrast to the findings of this study, several earlier investigations showed GRA2 DNA vaccine success against acute infection of *T. gondii*. Both as multi- or single antigen elicited IgG antibody production with high ratio of IgG2a/IgG1 and conferred partial protection to the vaccinated BALB/c mice (Liu *et al.*, 2009; Xue *et al.*, 2008; Zhou *et al.*, 2012). Nonetheless, the pcDNA 3.1C-GRA2 in present study, triggered Th1-favored immunity associated with significant lymphocytes proliferation, increased IFN-γ secretion as well as relatively low production of IL-4 and IL-10. These results were similar to those of Xue *et al*. (2008), Liu *et al*. (2009) and Zhou *et al*. (2012). Xue *et al*. (2008) used GRA2 fragment in combination with SAG1-ROP2 plus DNA plasmid encoding IL-12 as the additional adjuvant, whereas Liu *et al*. (2009) incorporated small segment of GRA2 with other small gene segments inclusive of SAG1, GRA1 and GRA4. In place of pcDNA 3.1, Zhou et al. (2012) used pVAX. These discrepancies may account for the overall different results acquired in this study.

Based on the overall results obtained, it was presumed that cell-mediated immunity alone could not combat acute *T. gondii* infection effectively in the absence of B cell responses. As such, mortality of vaccinated mice may be mainly attributed to the immunopathology exerted by the overwhelming amount of IFN-γ and IL-2 followed by parasitic infection upon lethal challenge study. It has been postulated that deficiency in B cells would actually increase Th1-related cytokines and eventually succumbed to the inflammatory effect of the cytokine (Sayles *et al.*, 2000). In contrast, subunit vaccination with same target genes (GRA2 and GRA5) conferred partial protection against the same parasitic challenge because both humoral and cellular immunity were

successfully mounted with relatively lower IFN-γ and higher IL-10 level in comparison with DNA vaccination.

5.8 Summary of findings and limitations

Overall, the antigenicity and immunogenicity of GRA2 and GRA5 had been tested and evaluated as single-antigen in this study. The sensitivity and specificity of the rGRA5 were much lower than rGRA2. Protein immunization with the recombinant antigens was able to provide partial protection against *T. gondii* lethal infection. Although DNA vaccination with recombinant plasmid of GRA2 or GRA5 was able to induce Th1-like cell-mediated response, no protection against *T. gondii* was observed. Antibody production was also not detected in the DNA-vaccinated mice.

5.9 Future work

It will be worthwhile to construct and evaluate the efficacy of recombinant multi-antigen GRA2-GRA5 in serodiagnosis assay and vaccination study. Parasite challenge of vaccinated mice should be tested using intraperitoneal injection with virulent *T. gondii* tachyzoite RH for acute infection, and oral feeding with tissue cysts of low-virulent strain (ME49 or VEG) for chronic infection. Prime-boost strategy vaccination should be tested, in which it begins with subunit vaccination, and followed by DNA vaccination for subsequent boosters. As for DNA immunization regime, several modifications can be incorporated such as inclusion of full-length sequence with the inclusion of signal peptide and addition of better adjuvant. Recombinant protein expression encoded by DNA vaccine could be assessed through Real Time-PCR of splenocytes or muscle cells of the vaccinated mice upon sacrifice.

CHAPTER 6: CONCLUSION

The *Toxoplasma gondii GRA2* and *GRA5* genes were optimally expressed in *E. coli*. The recombinant proteins were affinity purified and their identity was verified by western blotting and MALDI-TOF MS. rGRA2 protein was proven to have the ability to detect early (acute) stage *Toxoplasma* infection as well as differentiating past (chronic) infections. On the other hand, rGRA5 lacked sensitivity for detecting IgM antibodies and displayed much lower reactivity towards IgG antibodies in sera from patients with acute infection compared to those with chronic toxoplasmosis. These findings should contribute to the future development of an ICT for diagnosis of *T. gondii* infection.

Subcutaneous injection of mice with subunit vaccines rGRA2 and rGRA5 successfully triggered humoral and cellular responses which resulted in partial protection to the vaccinated mice against parasitic lethal challenge. A combination of Th1/Th2-related responses primarily Th1 was obtained with significant increased production of IgG2a, IFN-γ, IL-2 and IgG1 but relatively low level of IL-4 and IL-10. Intramuscular vaccination of mice with DNA vaccine also triggered Th1/Th2-response with predominant Th1-directed response associated with significant elevation of IFN-γ and IL-2 level, but relatively low level of IL-4 and IL-10. Humoral immunity was not elicited in the vaccinated mice, which subsequently succumbed against *Toxoplasma* challenge.

The encouraging findings obtained in this study provide a basis for further investigation into the development of a recombinant multi-antigenic candidate using combination of GRA2-GRA5 for serodiagnosis and immunization against *T. gondii* infection.

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