DESIGNING A MULTIEPITOPE VACCINE

AGAINST *Toxoplasma gondii*

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by

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LIST OF ACRONYMS, ABBREVIATIONS AND SYMBOLS

T. gondii	*Toxoplasma gondii*
CD8+	Cytotoxic T cell
CD4+	Helper T cell
IFN	Interferon
ILN	Interleukin
LBL	Linear B cell lymphocytes
CTL	Cytotoxic T cell lymphocytes
HLT	Helper T cell lymphocytes
TLR	Toll like receptor
ROP2	Rhoptry protein 2
MIC3	Micronemal protein 3
GRA7	Dense granule protein
PRRs	Pathogen recognition receptors
NK	Natural killer cell
ILCs	Innate lymphoid cells
DNA	Deoxyribonucleic acid
SAGs	Surface antigen
MHC	Major histocompatibility complex
GRAVY	Grand average hydropathicity
PDB	Protein data base
α	Alpha
γ	Gamma

PEMBANGUNAN VAKSIN MULTI-EPITOP TERHADAP *Toxoplasma gondii*

ABSTRAK

Toxoplasmosis merupakan penyakit yang mengancam nyawa yang disebabkan oleh *Toxoplasma gondii*. Penyakit ini telah memberi kesan kepada bidang perubatan, penternakan dan ekonomi. ~~*Toxoplasma*~~ *gondii* menjangkiti lebih kurang 25-30% populasi manusia di seluruh dunia. Terdapat–pelbagai usaha untuk mengawal dan mengekang penularan penyakit ini. Pada masa ini, satu-satunya vaksin yang digunakan ialah strain S48 tachizoite yang dilemahkan yang dikenali sebagai Toxovax. Vaksin ini digunakan untuk mengawal jangkitan kongenital pada biri-biri, dan dilaporkan berjaya mengurangkan kadar pengguguran pada haiwan tersebut. Walau bagaimanapun, vaksin itu agak mahal dan dalam keadaan tertentu berpotensi untuk berubah menjadi patogen, oleh itu ia tidak sesuai digunakan pada manusia. Pada masa ini, tidak ada vaksin yang berkesan untuk mencegah pembentukan tisu sista kronik pada perumah yang dijangkiti. Oleh itu, penyelidikan ini bertujuan untuk merancang pembangunan satu vaksin multiepitop terhadap ~~*Toxoplasma*~~ *gondii* menggunakan kaedah *in sillico* bagi meramal dan menganalisis epitop sel B-dan sel T antigen ROP2, MIC3, dan GRA7. Keputusan análisis menujukkan epitop yang dipilih adalah antigenik, bukan alergen, tidak beracun dan bukan homolog manusia yang menjadikannya sesuai untuk dibangunkan sebagai vaksin. Struktur kedua dan ketiga serta sifat fisiokimia calon vaksin tersebut telah ditentukan dan eksperimen validasi seperti *molecular docking* dan simulasi imun telah dilakukan. Proses validasi yang dilakukan meramalkan calon vaksin ini adalah stabil dan larut dalam persekitaran biologi. Sebagai kesimpulan, kaedah *in silico* boleh digunakan untuk merancang pembangunan vaksin terhadap ~~*Toxoplasma*~~ *gondii*.

DESIGNING A MULTIEPITOPE VACCINE AGAINST *Toxoplasma gondii*

ABSTRACT

Toxoplasmosis is a significant, life-threatening disease with medical, veterinary, and economic importance caused by *Toxoplasma gondii*. *T. gondii* infects about 25-30% of human population globally. There have been noteworthy efforts to control and limit the disease incidence. At present, the only approved vaccine for use in veterinary is attenuated tachyzoites of strain S48 that control congenital infection of ewe known as Toxovax, which has significantly reduced the rate of abortion in sheep. However, the vaccine is expensive and has the probability of changing into a pathogenic form thus, it is inappropriate for human use. At the moment, there is no effective vaccine for preventing the formation of chronic tissue cysts in an infected host. Therefore, this research aims to design a multiepitope vaccine against *T. gondii* using *in silico* method to predict and analyze B-cell and T-cell epitopes of ROP2, MIC3, and GRA7. The result showed that the selected epitopes were antigenic, non-allergen, non-toxic, and non-human homology which makes them appropriate for the construction of vaccines. The secondary and tertiary structure, as well as the physiochemical properties of the vaccine construct, were determined and validation experiments such as molecular docking and immune simulation were conducted. This validation process predicted that the candidate vaccine is stable and soluble in the biological environment. In conclusion, *in silico* method can be used to design a good vaccine for *T. gondii*.

CHAPTER 1

INTRODUCTION

1.1 Study Background

Toxoplasmosis is a significant, life-threatening disease with medical, veterinary, and economic importance (Silva et al., 2014; Sun et al., 2014) caused by *Toxoplasma gondii (T. gondii)*, a member of the phylum Apicomplexa. *T. gondii* is a widely spread parasite because it has been isolated from different locations throughout the world but not Antarctica (Murat, 2019). Toxoplasmosis produces a variety of clinical syndromes in human, sea, and land mammals, as well as various bird species and the clinical sign, depending on the host animal species. Toxoplasmosis can be fatal in some species of sea mammals and marsupials as the parasite have largely separately evolved.

T. gondii infects about 25-30% of the human population globally (Montoya & Liesenfeld, 2004) and it is estimated that at least one-third of the human population is infected (Kasper et al., 2004). According to Hoffmann et al. (2012), over one billion are estimated to be infected by this parasite worldwide. Also, in animals, toxoplasmosis is of great economic concern as it affects all types of livestock especially, goats and sheep, and infection occurring in animals during pregnancy often causes abortion, stillbirth, and neonatal loss (Tenter et al., 2000; Guang-Yuan et al., 2009). Congenital infection is the most common in human and other livestock animals which causes disease in the developing fetus. Therefore, *T. gondii* is significant in medicine and veterinary worldwide (Figure 1.1).

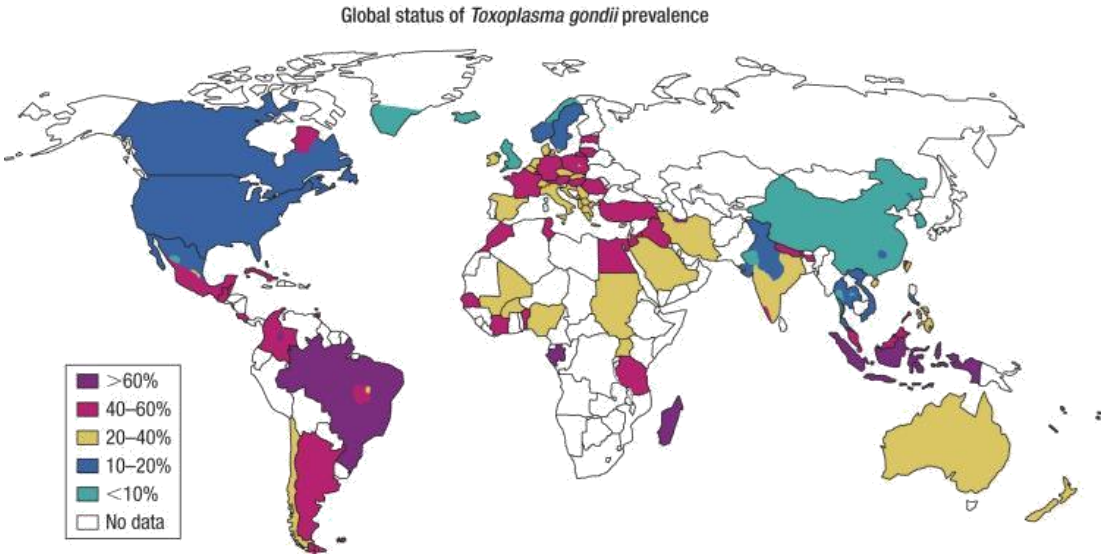

Figure 1.1 Global Status of *Toxoplasma gondii* prevalence

There have been different efforts in developing vaccine against *T. gondii*, such as the use of inactivated, killed and crude antigen. These are probably not sufficiently efficacious in the infection model used (Wang et al., 2019). Live attenuated vaccine on the other hand are capable of inducing MHC class I-restricted CD 8+ T-cell response, a major response needed for clearing parasitic intracellular infection. However, immune response elicited by live attenuated vaccine can be affected significantly by the degree of attenuation. Concerns have also been raised on the safety of the attenuated vaccine because of the probability of reverting to pathogenic strain (Wang et al., 2019). Also, subunit vaccine seems to lack appropriate immunogenicity, hence requires the use of appropriate adjuvants for the enhancement of their potency. Subunit vaccine can also cause allergic reactions in individuals because the protein is being expressed in another organism like bacteria or yeast (Qi et al., 2012). Furthermore, DNA vaccines compared the traditional vaccine elicits both cellular and humoral immune responses. The major mediator induced by DNA vaccine, CD8+ T cell, is the major mediator of immunity *T gondii* infection thus, makes it promising.

The potency of the DNA vaccine can be enhanced by using different delivery systems like gene gun approach, modification of the vaccination site microenvironment. It has been established that epitope- based vaccine have been used to control quite several infections by stimulating both cellular and humoral immunity using antigenic epitopes. Developing an effective vaccine against *T. gondii* has been faced with the complex life cycle exhibited by the parasite involving multiple host with diverse protein form to express and variety of invasion pathway.

1.2 Problem statement and rationale of the study

Many antiparasitic chemical drugs are available to prevent, treat toxoplasmosis, and control the *T. gondii* parasite spread in an infected host. However, most prescribed drugs are only effective against tachyzoites phase of the parasite while not effective against encysted bradyzoites which may remain alive throughout the life span of the infected host (Nosrati et al., 2020). Also, these drugs have limited efficacy and are not safe absolutely as they may provoke side effects (Yang et al., 2017; Zhang et al., 2018; Hajissa et al., 2019), like hypersensitivity and bone marrow suppression (Antezak et al., 2016). The entire human population is exposed to *T. gondii* infection risk and vaccine against this parasite could be of benefit to every individual (Robert et al., 2002). Also, immunization against *T. gondii* is necessary because of the possibility of reactivation of latent infection in immunocompromised individuals and the primary infection of pregnant women, leading to abortion and other complications in the fetus (Rezaei et al., 2019). Hence, it is essential to have an effective vaccine to control the effect of toxoplasmosis in both human and animal populations (Hajissa et al., 2019). There have been noteworthy efforts to control and limit the disease incidence, but no vaccine is available to prevent the human disease (Hajissa et al., 2019). Also, at

present, the only approved vaccine for use in veterinary is the attenuated tachyzoites of strain S48 that control congenital infection of ewe known as Toxovax. it has significantly reduced the rate of abortion in sheep but still does not eradicate the parasite (Pinzan et al., 2015, Rezaei et al., 2019). However, the vaccine is expensive and has the probability of changing into a pathogenic form thus, it is inappropriate for human use (Kur et al., 2009). Hence, developing effective vaccine for protection against toxoplasmosis using bioinformatics that explore the potential of B and T cell epitopes is being employed lately. For this reason, it is highly suggested that the parasite's antigen with high immunogenicity should be recognized (Saadatnia and Golkar, 2012).

Bioinformatics as a field of science involves several disciplines such as biology, computing, and information technology. It involves organizing huge amount of biological data generated from advances in genetics, molecular biology, and biotechnology (Lesk, 2002) and the main goal of bioinformatics is to effectively and timely streamline and interpret information from genome, transcriptome and proteome (Brusic and Flower, 2004). This discipline aims at promoting health benefits which includes area of vaccine development (Soria-Gurra et al., 2015). Bioinformatics tools development in conjunction with advances in recombinant DNA technology as well as knowledge about immune responses and the genetic knowledge about the pathogen give rise to developing a new vaccine against disease which currently have few or no control measures in just 1 or 2 years employing in silico predictions in defining the target (Jackwood et al., 2008). Vaccine developed using bioinformatics are safer, have more efficacy and/or less expensive than vaccines developed using the conventional method (Soria-Gurra et al., 2015). In vaccine development, epitope-based vaccines are

of particular interest in both clinical and basic biomedical research because they have huge potential in the design of vaccines, prevention of disease, as well as diagnosis and treatment of disease (Soria-Gurra et al., 2015). Specific epitopes can be isolated using recombinant DNA technologies replacing the use of the whole pathogen in a vaccine. Recombinant DNA technology has also allowed the use of multiepitope vaccines with advantages such as many immunoprotective epitopes present in a single molecule, as well as inclusion of immunodominant and epitopes exerting adjuvant effects like promiscuous T cell epitopes to improve immunogenicity (Almeida et al., 2012). Also, because *T. gondii* has a complex life cycle and antigenic variability, employing a multiepitope vaccine is a promising strategy for preventing the parasitic infection. MIC3, GRA7 and ROP2 are protein that are expressed in all the three stages of *T. gondii,* have high pathogenicity and stimulate high immune response.

1.3 Objectives

1.3.1 General objective:

To design a multiepitope vaccine for the prevention of Toxoplasmosis.

1.3.2 Specific objectives:

- To predict the B-cell & T-cell (CD8+ &CD4+) epitopes of MIC3, GRA7 and ROP2
- To design a multiepitope vaccine against *T. gondii* based on the predicted epitopes
- To determine the physicochemical properties, antigenicity, allergenicity, toxicity, and solubility of the designed vaccine
- To predict the secondary and tertiary structures of the designed vaccine

- To perform molecular docking with toll-like receptor (TLR) on the designed vaccine
- To perform an immune simulation on the designed vaccine

1.4 Flow chart of the study

The activities of the study are summarized in Figure 1.2 below:

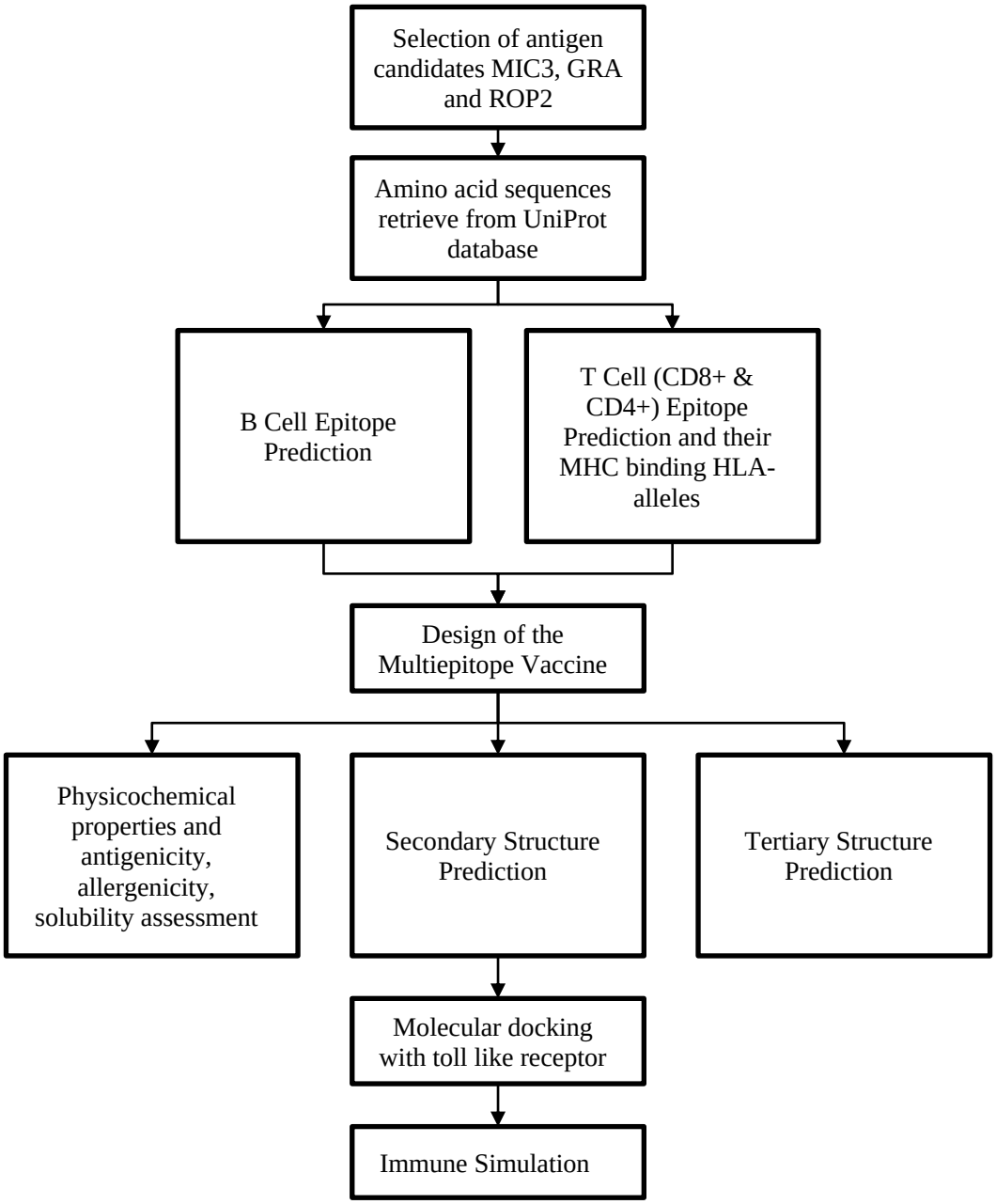

Figure 1.2 Flowchart of the study

CHAPTER 2

LITERATURE REVIEW

2.1 History of Toxoplasma

T. gondii was accidentally discovered in a North African rodent known as Ctendactylus gondii's blood, spleen, and liver by Nicolle and Manceaux in 1908 when searching for the natural reservoir of Leishmania in the native rodent and named the parasite Toxoplasma a crescent-shaped morphology observed in the tachyzoites and bradyzoites stage the scientist observed and gondii (after rodent) in 1909. At about the same time, 1908, a similar parasite was discovered in Rabbit by Alfonso Splendore working in Sao Paulo.

In 1920s and 1930s, there have been several reports about the pathogenicity of *T. gondii* in human. In 1923, the cyst of the parasite was isolated from the retina of an infant suffering from hydrocephalus, seizures, and unilateral microphthalmia (Innes 2009). In 1937, T. gondii associated with encephalomyelitis in developing fetus was described by Wolf and Cowen. They concluded that syndrome such as hydrocephalus, retinochoroiditis and encephalitis are of severe congenital *T. gondii* infection. Around the same period, fatal disseminating *T. gondii* infection was first reported in young adults. In 1953, the first case of eye disease from *T. gondii* was reported by Janku, where *T. gondii* was implicated in severe intraocular inflammation (Innes, 2018).

2.2 Toxoplasma infectious stage

A study of the life cycle of *T. gondii* shows that it passes its sexual stage in feline hosts and asexual stage in human and other intermediate hosts (Weilhammer and Rasley,

2000). There are three forms/stages of *T. gondii*, the tachyzoites (in groups or clones), the bradyzoites (in tissue cyst), and the sporozoites (in oocysts).

2.2.1 Tachyzoites

This stage describes a multiplying stage in any intermediate host cells and non-intestinal epithelial cell of the definitive host. It is often crescent-shaped and can move by gliding, flexing, undulating, and rotating. Tachyzoites actively penetrate the host cell through cell parenchyma or by phagocytosis (Dubey et al.,1998). Tachyzoites multiply asexually within the host infected by repeated endodyogeny (two progeny forms within the parent parasite). In a tissue culture strain rapidly dividing, Tachyzoites within the vacuole may divide synchronously but not the norm, and really, some strains divide by binary fission. The hosts' cells rupture when it can no longer support the growth of the tachyzoites. Also, the rate of invasion and growth depends on the strain of *T. gondii* and the host cell type (Dubey et al.,1998). Upon invasion into the host cell the parasite enters the lag phase, this varies depending partly on the parasite. The highly virulent strain has a shorter lag phase thus grows faster while avirulent have a longer lag phase thus grow slower. Also, it is characteristically found in acute infection and clinical manifestation.

2.2.2 Bradizoites

This also known as cystozoites, are tissue cysts that grow and remain intracellular since they divide by endodyogeny. They assume different shapes, cysts found in the brain are spherical while those found in the muscle are elongated. Though, tissue cysts may be formed in visceral organs like lungs, liver, and kidney but are more commonly found in neural and muscular tissues such as the brain, eyes, skeletal and cardiac muscles and develop in the cytoplasm of the host cell. Bradyzoites are characterized

by slow multiplication, originates in tissue cyst, and are responsible for the infection's chronic phase. Tissue cyst may persist throughout the life of the host without causing any inflammatory response (Dubey et al.,1998).

2.2.3 Sporozoites

This is also known as Oocyst, is a spore like stage in the life cycle of *T. gondii* and is produced only in the definitive host during sexual reproduction (Rezeal et al., 2019). It is a hardy thick-walled spore that can survive a lengthy period in the environment outside of the host. The zygote develops within the spore and is protected during transfer to the new host.

2.3 Life cycle of *T. gondii*

The life cycle of *T. gondii* can be summarized broadly into two components: the sexual (which occurs only in feniles, the definitive host: wild or domestic), and the asexual which occurs virtually in all warm-blooded animals (the intermediate hosts) (Weiss and Kim, 2011). Fenile is infected with *T. gondii* by ingesting mouse carrying tissue cyst and the parasite infects the epithelial cells of the small intestine after being able to survive through the stomach (Weiss and Kim, 2011).

Inside the intestinal epithelial cells, the parasites develop and reproduced sexually giving rise to millions of thick-walled zygotes known as oocysts. Eventually, the infected epithelial cell ruptures and the oocysts are released into the intestinal lumen then shed in the cat feces (Dubey, 2010). When an oocyst is ingested by an intermediate host, the resilient cyst wall is dissolved by proteolytic enzymes present in the stomach and small intestine releasing the sporozoites. The parasite then invades

the intestinal epithelial cells and the surrounding cells where they differentiate into motile, highly multiplying tachyzoites (Weiss and Kim, 2011). Tissue cysts such as those found in the brain and muscle tissues are formed about 7-10 days after the initial infection (Robert-Gangneux and Darde, 2012). During the invasion, the tachyzoites form a specialized vacuole known as the parasitophorous vacuole from the host cell membrane. The tachyzoites replicate inside the vacuole until the host cell dies and ruptures, therefore releasing and spreading the tachyzoites to all organs and tissues of the body including the brain via the bloodstream (Weiss and Kim, 2011). After the initial proliferation of tachyzoites, pressure from the immune system of the host causes the conversion of tachyzoites to bradyzoites, a slowly dividing cellular stage of the parasite (Miller et al., 2009). The cluster of bradyzoites inside the host is known as tissue cysts with the wall of the cyst formed by the parasitophorous vacuole membrane (Weiss and Kim, 2011). Although bradyzoites can be found in virtually all organs, it is predominantly found in the brain, eyes and striated muscle including the heart (Weiss and Kim, 2011). Tissue cysts can persist throughout the animal's lifespan and its persistence seems to be due to the periodic process of cyst rupturing and re-encysting and in a chronically infected host, only a little percentage of the tissue cysts are rupturing (Weiss and Kim, 2011) (Figure 2.1).

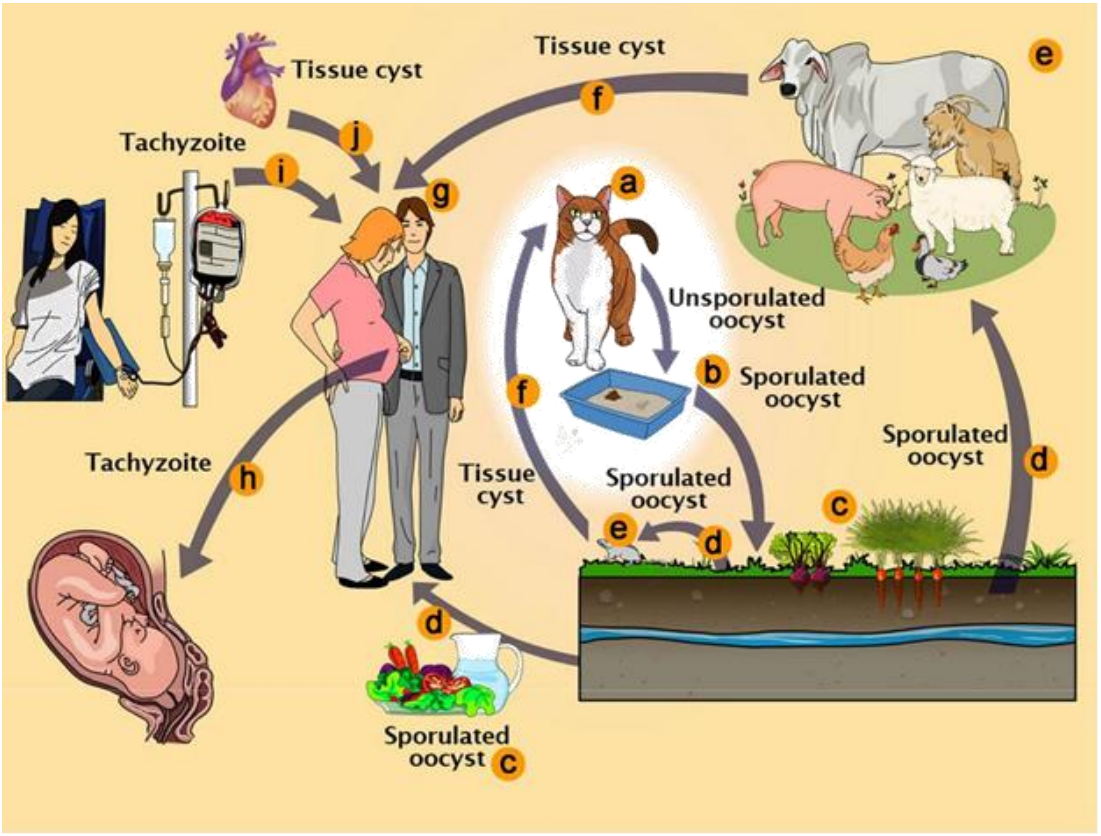

Figure 2.1 *Toxoplasma gondii* pathways of transmission

Note: **a** Feline definitive host (cat). **b** Unsporulated oocysts in cat feces. **c** Food contaminated with sporulated oocysts. **d** Oocysts may be ingested by intermediate hosts via water or raw vegetables. **e** Intermediate hosts (e.g. cattle, sheep, poultry and swine). **f** Ingestion of tissue cysts in uncooked meat. **g** Intermediate hosts (humans). **h** Tachyzoites transmitted through the placenta to the foetus. **i** Transmission by blood transfusion and organ transplant **j** (Attias et al., 2020).

2.4 Toxoplasmosis in human

Humans can be infected by ingestion of tissue-cyst, contamination with oocyst, and congenital infection (Black and Boothroyd et al., 2000).

2.4.1 Toxoplasmosis in immunocompetent individuals

Toxoplasmosis in humans has different implications. Individuals with competent immune systems are usually asymptomatic or with mild self-limiting symptoms such as fever, body aches, fatigue, headache, and swollen lymph nodes (Branko et al., 2019). However, it can be quite severe or even fatal in individuals with an underdeveloped immune system (fetus and newborn).

2.4.2 Toxoplasmosis in an immunocompromised individual

Toxoplasmosis can be found in those that are immunocompromised with HIV and other diseases associated with cellular immunity, depression, or as a result of medical treatment (Hajissa et al., 2017; Branko et al., 2019). Immunocompromised individuals may not effectively control the parasite's multiplication and persistent *T. gondii* infection in such individuals may cause severe brain lesion when the parasite within the tissue cyst becomes active and keep multiplying because of cell immune system dysfunctionality. Otherwise, it would keep the parasite in check (Inness et al., 2019). Patients undergoing immune-suppressing treatment like cancer treatment or organ transplant may be at the risk of toxoplasmosis (Wang et al., 2017). If the infection is left untreated, it can cause encephalitis (Coppens and Joiner, 2001).

2.4.3 Congenital toxoplasmosis

Pregnant women primarily infected with Toxoplasma gondii exposes the developing fetus to severe risk, presented as mild to the profound lesion shown during gestation, at birth, or later in life (Branko et al., 2019). Infection of newborn (fetus) is through vertical transmission from the mother. Also, acute infection during pregnancy or periconceptional period leads to congenital toxoplasmosis. Globally, the incidence of Toxoplasmosis is 1.5 cases per 1000 live birth approximately and estimated to be 190,100 cases globally which is equivalent to be a burden of 1.20 million Disability-Adjusted Life Years (Torgerson and Mastroiacovo, 2013). The rate at which maternal-fetus transmission without prenatal treatment occurs is estimated at 50% throughout pregnancy. However, the rate of transmission and clinical presentation of CT depends on the gestation maturity at the time of infection. The risk of fetal infection occurring during the early pregnancy is <10% and about 60-81% at the end of the third trimester (Wallon et al., 2013; Branko et al., 2019).

Furthermore, the consequences of the infection occurring at early pregnancy are intrauterine death, hydrocephalus, microencephaly, and seizure (Gilbert et al., 2006). Serious consequences such as chorioretinitis or neurological disorder may also occur in the second and third-trimester infections, but these are less common. Congenital toxoplasmosis in the neonate may also cause severe neurological congenital disabilities such as mental retardation and blindness (Robberts & Mcleod, 1999; Torrey &Yolken, 2003). Clinical symptoms are often absent at birth, but children infected may develop late sequelae (Villena et al., 2010). *T. gondii* in an immunocompromised patient typically causes ocular toxoplasmosis. Some researchers stated that *T. gondii* cysts in the brain might cause mental disorders (Dubey, 2009).

2.4.4 Ocular toxoplasmosis

Ocular toxoplasmosis is a type of disease that was long thought to be caused by congenital infection, but recent evidence has supported postnatal infection and ocular manifestation are more common than congenital ocular toxoplasmosis. Evidence has also shown that ocular toxoplasmosis can occur in immunocompetent individuals due to chronic infection reoccurrence (Robberts and Mcleod, 1999; Torrey and Yolken, 2003; Hunter and Sibley, 2012). The hallmark of ocular toxoplasmosis is necrotizing retinochoroiditis (inflammation and death of the retina). This may occur primary or recurrent and the retina is the primary site of the multiplying parasite.

2.5 Pathogenesis of *T. gondii*

During toxoplasmosis pathogenesis, the pathogen's surface and secretory organelles such as Surface Antigen (SAG), Rhoptry organelle protein (ROPs), and Dense Granule Antigen (GRA) are released, and they all play a pivotal role. Microneme is important in the attachment, recognition, and penetration of the host cell by the parasite (Carruthers, 2006). At the infection stage, Rhoptry contents and its neck proteins (RONs) are released into the host cell membrane to enhance microneme protein and apical membrane antigen (AMA1) in motility (Liu et al., 2012; Alexandra et al., 2005). The parasite also releases the ROPs bulb into the cytosol to interact with the cellular organelles for the parasitophorous vacuole's biogenesis. GRAs, dense granules, which contain GRA proteins, modify the parasitophorous vacuole widely. They are also thought to contribute to gaining nutrients for intracellular survival and replication (Zhou et al., 2005 & Nam, 2009).

2.6 Treatment of *T. gondii*

The main drugs recommended for treating or prophylaxis of active toxoplasmosis are Sulfadiazine and Pyrimethamine. These drugs have been shown to cause side effects such as bone marrow suppression, hematological abnormalities, thrombocytopenia, leukocytopenia, neutropenia, increased serum liver enzymes and creatinine, and severe skin rashes (Foroutan et. al., 2019). Also, drugs such as Clarithromycin, Azithromycin, Atovaquone, Trimethoprim-Sulfamethoxazole and Dapsone are alternative drugs used for the treatment of Toxoplasmosis (Foroutan et. al., 2019). These routinely used drugs for Toxoplasmosis reduce the multiplication of tachyzoites during the initial infection phase. However, these drugs are unable to eliminate parasite encysts in the tissue and are poorly tolerated. These drugs are also reported to have weak antiparasitic activities as well as drug resistance. Also, the use of these drugs by pregnant women is faced with the probability of the drugs having a teratogenic effect on the fetus (Antezak et al., 2016). Also, because *T. gondii* highly adapts to environmental changes, makes combating Toxoplasmosis difficult, and most failed (Antezak et al., 2016). For these reasons, as well as due to the heavy burden of latent infection worldwide, it is of high priority to developing a vaccine against *T. gondii*.

2.7 Immune response to *T. gondii*

Innate immunity is the first line of defense that detects and immediately responds to a pathogen using pathogen recognition receptors (PRRs) such as Toll-like receptors, C-type lectins, and Nod-like receptors. Recognition of ligands by PRRs induces the production of proinflammatory cytokines such as tumor necrotic factors alpha (TNF-α), interleukin (IL) -6, and IL-12. These cytokines play role in the activation of immune responses (Sasai and Yamamoto, 2019).

Usually, infection of humans and animals by *T. gondii* is oral as a result of the presence of its cysts in undercooked meats or unwashed fresh vegetables. Once ingested, the intestinal mucosal encounters the cysts first and activates the three groups of innate lymphoid cells (ILCs) which are grouped based on their functional characteristics. Activation of ILC1 which includes ILC1 and natural killer (NK) cells, causes Th1 cytokines production like IFN-γ and TNF-α. Activation of ILC2 causes the production of Th2 cytokines including IL-4, IL-5, IL-9 and IL-13 (Sasai and Yamamoto, 2019).

Furthermore, the innate immune system cells, such as macrophages, neutrophils, dendritic cells, and natural killer cells, are responsible for the innate immune response against *T. gondii*. A cytokine, interleukin-12 (IL-12), important for regulating interferon-gamma (IFN-γ), is produced by natural killer and T cells. Cellular immune response controls both acute and chronic infections (Reazie et al., 2019). T helper cells such as CD4+ and CD8+ T cells and cytokines IL-2, IL-12, IFN- γ, and TNF-α are involved in protective immunity. Other cytokines involved in balancing immune responses are IL-4, IL-5, and IL-10 (Fillisetti et al., 2004 & Wagner et al., 2015). Also, Specific antibodies inhibit the parasite's attachment to the host cell and enhance antibody-coated tachyzoites killing via complement-dependent pathways as well as macrophages (Kang et al., 2000).

2.8 Vaccine development against *T. gondii*

Many types of research have been conducted on developing a safe and effective vaccine. Different forms of the parasite, or its antigens, such as inactivated or life attenuated vaccine, crude or recombinant antigen, subunit or multi-antigenic vaccines, and DNA vaccine have been used (Li et al., 2018) and results have shown achieving

an effective vaccine is attainable (Hajissa et al., 2019). Because the vaccine is not available, it calls for the need to explore other reagents that can be employed for immunization (Hajissa et al., 2019). Future research on vaccine development should be centered on multiepitope antigen, which entails various immunoreactive epitopes of a different antigen of *T. gondii* parasite. An ideal vaccine against Toxoplasmosis in the human host should include antigens that can elicit a protective T helper cell type (Th 1) immune response and generate long-lived IFN-γ-producing CD8+ T cells (Tan et al., 2011). It is noteworthy that developing a protective vaccine against the *T. gondii* parasite can essentially reduce the disease's high incidence and prevent clinical outcomes in humans and animals. Hence, it is expected that adequate immunization would reduce oocyst shedding and prevent the formation of a cyst. The vaccine would also significantly reduce parasite transmission to the intermediate host and ultimately control the disease (Haung et al., 2016). An effective vaccine will also reduce the livestock industry's economic losses (Gao et al., 2018).

2.9 Approaches used in the development of a vaccine against *T. gondii*

Developing a vaccine against *T. gondii* is highly prioritized and essentially crucial because of its high incidence and worldwide distribution. There are many approaches employed to develop a vaccine against *T. gondii* infection in the past. The protection levels have been evaluated with various immunogens such as live-attenuated parasites, killed vaccines, native parasite antigen, recombinant antigens, and DNA vaccine (Palatnik-De-Sousa et al., 2018; Fereig et al., 2018; Hajissa et al., 2019). Despite all the approaches, no safe and protective vaccine existed for humans and animals. All the available information on developing an effective and safe vaccine for *T. gondii* infection is that the vaccine should contain antigen with the potential to stimulate cell-

mediated immunity against the parasite, and the antigen should be expressed in all life stages of the parasite (Hajissa et al., 2019).

One of the strategies employed in *T. gondii* vaccine development is the use of live mutant bradyzoites, T-263 used in vaccinating kittens (Lu et al., 2018). Most of the vaccinated kittens were immunized, and about 84% of oocyst shedding was prevented. Unfortunately, some disadvantages are associated with T-263, such as the need for live bradyzoites, the high cost associated with its production, and the need for refrigeration (Li et al., 2018). DNA vaccine encoding rhoptry protein (ROP) 2 used in kittens did not reduce oocyst shedding (Lee et al., 2018), although DNA vaccine currently shows potential as an immunization tool.

Also, the use of live *T. gondii* in pigs shows mild protection against the parasite but with the risk of reverting to its virulence type, thereby causing disease (Supply et al., 1999). A recent study shows that intradermal immunization of pigs using DNA vaccine, which encodes GRA1-GRA7 of T. gondii antigen, stimulated the production of high humoral and cellular immunity (Suschak et al., 2017). Tachyzoites strain S48 has also been shown to reduce the number of cysts in pork (Burrells et al., 2015).

Recombinant DNA technology is another approach that shows great potential in developing vaccines (Lu et al., 2018). DNA vaccines have shown several advantages, such as the ability to induce high humoral and cell-mediated immunity. It is also easy to produce, administer, stable and has long-lasting immunity. DNA vaccines also show high flexibility as different gene types can be encoded in one DNA vaccine. DNA vaccine also has little risk of reverting to a virulent form or causing secondary infection

(Kim et al., 2012). However, there is the need to use the appropriate adjuvant to enhance their potency. This is evident in Toxoplasma recombinant antigens, such as surface antigen (SAGs), microneme protein, dense-granule protein (GRAs), and ROPs, which have been tested for their immunological effects. However, only a few have shown the capability of inducing strong and protective immunity without appropriate adjuvant (Liu et al., 2017; Lee et al., 2018; Wang and Yin 2014; Cao et al., 2015; Didierlaurent et al., 2014). The major problem associated with having a successful vaccine is antigenic variation and polymorphisms. Therefore, understanding these changes is essential in designing effective vaccines as well as vaccine and evaluation. It also enhances understanding parasite-host interaction (Thompson et al., 2018; Jalloh et al., 2009).

Advancement in recombinant DNA technology and improvement in bioinformatics knowledge has led to a new strategy in designing and producing novel epitope-based vaccines (Khan et al., 2006). Epitopes are the antigenic determinants. They form the minimum immunogenic part of an antigen capable of inducing specific immune responses (Shi et al., 2015). The epitope-based vaccine concept is mainly based upon predicting immunodominant T and B cell epitopes involved in eliciting a specific and protective immune response (Patronov & Doytchinova, 2013). Designing a proper epitope vaccine requires identifying both B and T cells and selecting a novel approach in delivering those epitopes. Studies have shown that epitope-based vaccine has the potential to stimulate effective, high and protective immunity against different pathogens such as Influenza Virus (Munoz-Medina et al., 2015), Hepatitis B virus (Comber et al., 2014), Human Immunodeficiency Virus (Sahay et al., 2017), Epstein-Barr virus (Comber et al., 2014), and Corona Virus (Bhatnager et al., 2020).

T. gondii life cycle is complex and has many antigenic compositions. Each antigen can induce distinct immune responses in the host. It has also been validated that monovalent vaccines are not ideal. Hence, developing a multiepitope vaccine is therefore important (Wang Yin, 2014). The multiepitope vaccine containing B-and T-cell epitopes, is a newly trending technique in vaccine development. Also, immunization with a multiepitope vaccine expressing T-cell and B-cell epitopes against various pathogens has shown a notable increase in cellular and humoral immune response and prolonged survival time (Zhang et al., 2013). An epitope-based vaccine has been used to control various infections, and it encourages stimulating protective cellular and humoral immunity (Alonso-Pailla et al., 2017; Thompson et al., 2018). Epitope-based vaccines have shown to be a potential candidate for developing a novel and effective *T. gondii* vaccine (Hajissa et al., 2019).

2.10 *T. gondii*'s potential vaccine candidates

Some *T. gondii* antigens have been widely tested in animal models. These are membrane-associated antigens (SAGs), secreted-dense granule protein (GRAs), micronemal protein (MICs), and rhoptry proteins (ROPs) (Henriquez et al., 2010: Hiszczynska- Sawicka et al., 2009). Each of these antigens is believed to have its unique antigenicity.

The life cycle of *T. gondii* has varied life stages and selecting multi-stage antigens will produce more protection (Chu et al., 2014; Zhou et al., 2016). There are a few antigens secreted in all three stages: tachyzoites, bradyzoites, and sporozoites. Therefore, these antigens can be considered appropriate as vaccine candidates (Dubremetz & Lebrun, 2012; Fritz et al., 2012; Mohammed et al.,2003). Antigens such as MIC 3, MIC 4, MIC

13, RON 5, ROP 1, ROP 2, GRA 1, GRA 6, GRA 7, GRA 8, and GRA 14 are expressed in all three stages of the parasites (Daryani et al., 2013;Razaei et al., 2019; Amirreza et al., 2020). Furthermore, research has shown that this *T. gondii* antigen has high pathogenicity and effectively stimulates an immune response. It could, therefore, be suitable for vaccine candidates. According to Razaei et al. (2019), antigens such as MIC3, ROM1, ROM4, ROM5, ROP5, ROP16, ROP18, GRA6, GRA10, SAG1, SAG 5D, and ENO2 show higher pathogenicity than others. Therefore, this research will consider exploring MIC3, GRA7, and ROP2 as vaccine candidates. Micronemes are adhesive antigens commonly discharged by the parasite and are important for motility and invasion (Zhou et al., 2005). Among all the micronemes, MIC3 is a strong adhesion of T. gondii (Qu et al., 2009) and has high pathogenicity. Hence, it is a suitable vaccine candidate (Razaei et al., 2019). Also, GRA protein is secreted at all stages of *T. gondii* infection, and it is likely involved in intracellular survival and tracking (Daryani et al., 2013). GRA7, a subunit of GRAs protein, induces a strong immune response in the acute phase. It also contributes to the survival and rapid growth of the parasite and deficiency of this protein, resulting in impaired growth of the parasite (Seseleh et al., 2012). GRA7 is an attractive vaccine candidate because it is expressed in all *T. gondii* infectious stages and can stimulate both humoral and cellular responses significantly (Fergunson et al.,1999, Verhelst et al., 2011). Furthermore, ROP is secreted in the acute phase of the disease and aids invasion and active penetration of *T. gondii* into the host cell. The ROP2 is secreted in sporozoites, tachyzoites, and bradyzoites form. It forms part of the parasitophorous vacuole, has high pathogenicity and immunogenicity, and could be a promising vaccine candidate (Razaei et al., 2019).

CHAPTER 3

MATERIALS AND METHODS

3.1 Retrieval of amino acid sequence

The FASTA format of amino acid sequence for the selected proteins MIC3, GRA7, and ROP2, were retrieved from the uniport (https://www.uniprot.org/). They include ROP 2 protein of RH strain, accession number A0A7J6JZL4, link: https://www.uniprot.org/uniprot/A0A7J6JZL4.GRA 7, with accession number 17CQR1 with link: https://www.uniprot.org/uniprot/I7CQR1 and MIC3 protein accession number A0A7J6KDBO, link :https://www.uniprot.org/uniprot/A0A7J6KDB0. These protein sequences were chosen based on their length (the most complete sequence).

3.2 B cell epitope prediction of MIC3, GRA7, and ROP2

Linear B cell epitopes of our selected proteins are predicted using BCpred prediction tool **BCPREDS Server 1.0**. This prediction tool is based on Machine learning algorithms to distinguish experimental B cell from non-experimental B cell epitopes. The criteria such as 75% specificity and the use of overlap filters and the epitope length of 20 amino acids were used. The human homology of the selected epitopes was predicted using BLASTp server at https://blast.ncbi.nlm.nih.gov/ (Altschul et al., 2005). For BLASTp prediction, the parameters were kept at their default and Homo sapiens [taxid: 9606] were used for organism comparison. The e-value threshold was selected to be 0.05 and the epitopes below the e-value were selected as non-homologous peptides (Mehla and Ramana, 2016).

3.3 T-lymphocyte epitopes prediction

3.3.1 Cytotoxic T-lymphocytes (CTLs) epitopes

To predict cytotoxic T-lymphocytes epitopes, the retrieved sequence of the epitopes selected were submitted to NetCTL v1.2 server available at https://www.cbs.dtu.dk/services/NetCTL/ using FASTA format and a default score of the threshold of 0.75 (Larsen et al., 2007). The antigenicity of the predicted epitopes was further assessed for their antigenicity using VaxiJen v2.0 available at http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html (Doytchinova & Flower 2007). Major histocompatibility complex (MHC) class I immunogenicity was predicted using (http://tools.iedb.org/mhci/) link employing human and artificial neural network as a source of MHC species (Calis et al., 2013). To determine the toxicity, ToxinPred was employed and it is available at (http://crdd.osdd.net/raghava/toxinpred/) (Gupta et al., 2013), and AllergenFP V1.0 (http://ddg-pharmfac.net/AllergenFP/) was used to predict the allergenicity (Dimitrov et al., 2013) and http://tools.iedb.org/conservancy/ was used to check the conservancy. For all the above predictions, the default parameters of the sever were used.

3.3.2 Helper T-lymphocyte (Th) epitopes

Immune Epitope Database MHC class II binding allele prediction tool was used to predict Helper T-lymphocyte available at https://tools.iedb.org/mhcii/. Three different MHC class II alleles including HLA-DQA1*05:01, HLA-DQB1*02:01, and HLA-DRB1*03:01 were employed as target alleles. CONSENSUS 2.22 method (Wang et al., 2010) was used and epitopes were chosen based on 5% percentile rank. Toxicity and Allergenicity were predicted as in 3.3.1 above. For all the above predictions, the default parameters of the sever were used. Interferon-gamma was determined using http://crdd.osdd.net/raghava/ifnepitope/design.php.

3.4 Designing a multiepitope vaccine

The multiepitope vaccine was designed using the previously selected CTC epitopes, HTC epitope and LBC epitopes fused with AAY, GPGPG, and KK linkers reported in previous studies as aiding antigen processing and presentation (Abass et al., 2021). Human β defensins-3 was added as an adjuvant to the N-terminus of the designed vaccine using an EAAK linker. This is to enhance the amount of the antigen-specific immune response elicited (Abass et al., 2021).

3.5 Physiochemical properties, antigenicity, allergenicity, and solubility prediction of the designed vaccine

ProtParam web-server (http://web.expasy.org/protparam/) was used to predict the physiochemical properties using primary protein sequence (Gasteiger et al., 2005). The physiochemical properties are the number of amino acids, the molecular weight, the theoretical isoelectric point, amino acid composition, atomic composition, extinction coefficients, formula, estimated half-life, aliphatic index, instability index, and grand average of hydropathicity (GRAVY). The protein instability index determines the stability of the protein and protein with a stability index of <40 indicates that the protein is stable while >40 mean indicates that the protein is unstable. VaxiJen 2.0 server was used to predict the vaccine antigenicity. The antigenicity prediction method was solely based on the proteins' physiochemical properties with recourse to protein alignment with a precision rate between 70-89% (Abass et al., 2021). Also, an allergenic protein induces a harmful immune response, therefore AllergenFP servers were employed to predict allergenicity potential (Shey et al., 2021). Solubility was predicted (SOLpro, http://scratch.proteomics.ics.uci.edu/) (Mangnan et al., 2009).