Designing a Multi-epitope Vaccine based on MSP1, AMA1, and CSP Proteins of *Plasmodium falciparum* 3D7 with TLR4 as Adjuvant using Immunoinformatic Approach

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

~	About
≥	Greater than and/or equal
α	Alpha
β	Beta
ɣ	Gamma
ϕ	Phi
ψ	Psi
h	hour
min	Minute
MSP1	Merozoite surface protein 1
AMA1	Apical membrane antigen 1
CSP	Circumsporozoite protein
LBL	Linear B-lymphocyte
CTL	Cytotoxic T-lymphocyte
HTL	Helper T-lymphocyte
IL	Interleukin
IFN	Interferon
Ig	Immunoglobulin
TNF	Tumor necrosis factor
RBC	Red blood cell
pI	Isoelectric point
MEV	Multi-epitope vaccine
GRAVY	Thermostable grand average of hydropathicity

IRS	Indoor residual spraying
ITN	Insecticide treated net
kDa	Kilodalton- atomic mass unit
WHO	World Health Organization
CDC	Centre for disease control and prevention
TLR	Toll like receptor
HMGB1	High mobility group box protein 1
RAS	Radiation attenuated sporozoite
GAP	Genetically attenuated parasite
MHC	Major histocompatibility complex
PDB	Protein data bank

Membangunkan Vaksin Multi-Epitop berdasarkan Protein MSP1, AMA1, dan CSP *Plasmodium falciparum* 3D7 dan TLR4 sebagai Adjuvan menggunakan Pendekatan Imunoinformatik

ABSTRAK

Banyak kematian akibat malaria telah dilaporkan di seluruh dunia terutamanya disebabkan oleh *Plasmodium falciparum*, spesies yang paling merbahaya dalam kalangan *Plasmodium spp*. Pelaksanaan langkah-langkah pengawalan malaria menjadi kurang berkesan kerana kebolehan *P. falciparum* meningkatkan kerintangannya terhadap kebanyakan ubat anti-malaria. Vaksin malaria yang paling berkesan pada masa ini ialah RTS, S / AS01 dimana telah berjaya mencapai tahap percubaan fasa III. Namun begitu vaksin ini dikatakan kurang berkesan kepada kanak-kanak. Oleh itu pembangunan vaksin yang lebih efektif yang mampu melindungi semua peringkat umur sangat diperlukan segera. Dalam kajian ini, pendekatan imunoinformatik digunakan untuk merancang pembangunan vaksin multi-epitop yang mendorong penghasilan antibodi terhadap eritrosit *P. falciparum*. Ia mensasarkan protein permukaan merozoit 1 (MSP1), antigen membran apikal 1 (AMA1) dan antigen circumsporozoite (CSP). MSP1 dan AMA1 merupakan protein yang disintesis oleh parasit Plasmodium dan terlibat dalam pencerobohan dan gangguan terhadap RBC eritrosit. CSP pula merupakan salah satu protein yang terdapat pada permukaan sporozoit dan berperanan dalam pencerobohan hepatosit serta jangkitan lebih lanjut terhadap eritrosit. Daripada analisi yang dilakukan, 9 epitop linear B-lymphocyte (LBL), 11 epitop helper T-lymphocyte (HTL) dan epitop cytotoxic T-lymphocyte (CTL) yang sangat antigenik, bukan-alergi, bukan-homolog dan tidak beracun dipilih untuk konstruk vaksin ini. Semua epitop HTL yang dipilih mampu merangsang

penghasilan sitokin IL-4, IL-10, dan IFNγ. Dua adjuvant, Hp91 dan RS09 dimasukkan ke dalam konstruk melalui penghubung yang sesuai untuk merangsang tindak balas imun sel Th dan CTL. Analisis fizikokimia dan imunologi mendapati bahawa vaksin yang direka adalah antigenik (antigenisiti 0.7725), mempunyai sifat semula jadi (pI-9.16), bersifat larut (0.905932), hidrofilik (GRAVY-0.842) dan bukan alergen. Ciri struktur sekunder dan struktur tersier telah diramal menggunakan beberapa perisian bioinformatik dalam talian. Model vaksin yang direka kemudian dinilai daripada segi sifat asas protein. Perkaitan yang baik antara TLR-4 (Rantai A dari 3FXI) dan vaksin multi-epitop ini ditentukan oleh sambungan molekul. Ringkasnya, analisis imunoinformatik yang dilakukan menunjukkan bahawa calon vaksin yang dibangunkan mempunyai ciri-ciri vaksin yang dikehendaki. Walau bagaimanapun, penilaian secara *in vivo* dan *in vitro* perlu dilakukan terhadap vaksin yang dibangunkan ini untuk menilai keberkesanan dan keselamatannya.

Designing a Multi-epitope Vaccine based on MSP1, AMA1 and CSP Proteins of *Plasmodium falciparum* 3D7 with TLR4 as Adjuvant using Immunoinformatic Approach

ABSTRACT

A significant portion of severe malaria cases and deaths are reported all over the world mainly due to *Plasmodium falciparum,* the deadliest species among all *Plasmodium spp*. Established and implemented malaria controlling measures becoming less effective as *P. falciparum* gradually increasing its resistance to most of the primary line anti-malarial drugs. Until so far, the foremost effective vaccine RTS, S/AS01 which was succeeded to reach the phase III trial stage showed less effectiveness in young infants. Therefore, the development of a more effective malaria vaccine that can protect all individuals is urgently required. In this study, an immuno-informatics approach is used to design an effective and long-lasting antibody-inducing multi-epitope vaccine against the erythrocyte stage of *P. falciparum* targeting merozoite surface protein 1 (MSP1), apical membrane antigen 1 (AMA1) and circumsporozoite antigen (CSP). MSP1 and AMA1 can be synthesized in the mature stage of *Plasmodium* parasites and involved in the invasion and disruption of red blood cells (RBC) of erythrocyte stage. CSP is one of the most highly expressed proteins on the surface of sporozoites and plays role in hepatocyte invasion as well as further infection toward erythrocytes. After numerous analyses, highly antigenic, non-allergic non-homolog and non-toxic 9 linear B-lymphocyte (LBL), 11 helper T-lymphocyte (HTL) and cytotoxic T-lymphocyte (CTL) epitopes were selected for the final vaccine construct. All the selected HTL epitopes were IL4, IL10, and IFNγ inducer. Two

adjuvant motives, Hp91 and RS09 were adjoined via a suitable linker to stimulate the immune response of helper T cell and cytotoxic T cell, respectively. The physicochemical and immunological analysis found that the constructed vaccine was antigenic (antigenicity 0.7725), basic in nature (pI-9.16), soluble (0.905932), hydrophilic (GRAVY-0.842) and non-allergen. Secondary structural features and tertiary structure were predicted using several bioinformatics-based online software. The modeled vaccine was then refined and validated to evaluate the basic nature of proteins. A good binding affinity between TLR-4 (Chain A of 3FXI) and our multi-epitope vaccine was determined by molecular docking. In summary, findings of the immunoinformatic-based analysis had shown acceptable results however further *in vivo* and *in vitro* assessments are required to perform on constructed vaccine to confirm its efficacy and safety.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria is a life-threatening zoonotic disease caused by the *Plasmodium* parasite-infected female *Anopheles* mosquito bites. Due to the high morbidity and mortality rate, malaria becomes a public health concern all over the world (Abdikarim *et al.,* 2019). Malaria-causing *Plasmodium* parasite can survive in multiple hosts in which *Anopheles* mosquito known as 'malaria vectors' play the role of definitive host and human along with other vertebrates plays the role of secondary host. Besides mosquito bites, in some rare cases malaria can also be transmitted from mother to new born using placenta or via organ transplant, blood transfusion, using the same syringes or needles for both infected and non-infected patients (Malhotra *et al.,* 2006). Until so far five known species of *Plasmodium* parasite- *P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale*, *P. knowlesi* have been invented which are responsible for causing malaria disease in humans. Among them, *P. falciparum* is the most vulnerable group (World Health Organization, 2021). Normally, *P. knowlesi* is known for causing malaria in animal but recent studies have found that it also can cause malaria in human (Antinori *et al.,* 2013).

According to World Health Organization (2021), 229 million malaria cases and 4,09,000 deaths due to *Plasmodium* infections had been recorded worldwide in 2019 in which 94% of both malaria cases and deaths were reported from the African region. Children less than five years, pregnant women and immune-compromised patients are considered as a vulnerable group according to the report.

Various measures to prevent and cure this disease have been implemented however the prevalence of malaria is increasing due to various factors such as surviving ability to multiple hosts, genetic diversity, emerging multidrug-resistant strains, climate change and so on (Mohamad *et al.,* 2014). Besides preventive and curative measures, antimalarial drugs such as Chloroquine, Primaquine, Quinine (quinidine, mefloquine, doxycycline), Artemisinin (ART) along with lumefantrine, halofantrine, malarone have been recommended by CDC for providing treatment against malaria. Unfortunately, *P. falciparum* resistant to many of these drugs along with artemisinin derivatives which is widely used for malaria patients worldwide (Cravo *et al.,* 2015).

Numerous merozoite and sporozoite surface proteins have been studied for the development of a vaccine against the erythrocytic and pre-erythrocytic stages of malarial infection. Merozoite surface protein 1 (MSP1), Apical membrane antigen (AMA1) are highly expressed merozoite surface protein and Circumsporozoite protein (CSP) is the sporozoite surface protein. Among them merozoite is an effective vaccine target that may capable of reducing the spread of drug-resistant strains of *P. falciparum*, controlling and eradicating the malaria disease that has become a matter of concern for researchers. In the present study, MSP1, AMA1 and CSP surface antigens are targeted to construct an effective vaccine to develop humoral and cellular immunity against erythrocytes by complete invasion or blocking the parasite transmission pathways from hepatocyte to erythrocytes. The antigenic epitopes of these proteins, vaccine design, vaccines' secondary and tertiary structures, immunological and physicochemical features, and vaccine-receptor binding affinity will be predicted and evaluated utilizing computational immunology and immunoinformatic approach.

1.2 Rationale of the study

Strong and effective immunity inducing vaccine against malaria is required as *Plasmodium* is a genetically complex parasite that can invade red blood cells as well as hepatocytes. Mainly, the merozoites of *P. falciparum* invade and replicate inside the erythrocytes of the host, resulting in cell burst and spread via infecting new red blood cells. Consequently, pathogenesis and symptoms of the malaria diseases have risen from this stage. The invasive erythrocytic stage has become one of the attractive vaccine targets as this infection placing method can directly modulate hosts' humoral immune response (Bemani *et al.,* 2020).

MSP1, AMA1 and CSP are the surface antigens of *Plasmodium falciparum* 3D7 that are responsible for inducing malaria infection. So, they are potential to be developed as vaccine candidates targeting invasion of merozoites and reducing disease severity and transmission. MSP1 is one of the major proteins which bind to the receptor of the surface of erythrocytes to induce growth of the parasite and cause cell invasion (Bemani *et al.,* 2020). Similarly, AMA1 can be synthesized in the mature stage of *Plasmodium* parasites and involved in the invasion and disruption of RBC of the erythrocyte stage (Hodder *et al.,* 2020). CSP is one of the most highly expressed proteins of the surface of sporozoites and plays a role as a whole-sporozoite vaccine candidate targeting the pre-erythrocytic sporozoite stage of *Plasmodium falciparum* (Chaudhury *et al.,* 2017). A toll-like receptor (TLR) 4 agonist Hp91 is 18 amino acids long peptide derived from endogenous high mobility group box protein 1 (HMGB1) was included in the vaccine construct as an adjuvant to stimulate the immune response of helper T cells (Saenza *et al.,* 2014). Another TLR4 agonist RS09 has also been

included in the vaccine construction to stimulate the immune response of cytotoxic T-lymphocyte (CTL) epitopes (Rehman *et al.,* 2021).

By the combination of invasive antigens of different stages of *Plasmodium* parasites life cycle can be able to produce antibodies against *Plasmodium falciparum* 3D7 and can inhibit the growth of other parasites. The identification of multiple epitopes of these targeted proteins following immunology-based bioinformatic approach is less time-consuming, and helps to reduce the risk of autoimmune disease. To design epitope-based vaccine utilizing immunoinformatic technique, multiple highly antigenic epitopes are adjuvanted that help to improve immunogenicity of the vaccine construct. It also predicted that, a multi-epitope vaccine capable of inducing both humoral and cell-mediated immunity due to the combination of overlapping HTL, CTL, and B-cell epitopes (Oli, *et al.,* 2020). The overall study is designed depending on these beneficial aspects of immunological approaches.

1.3 Objectives of the study

1.3.1 General objective-

To design a multi-epitope vaccine (MEV) against *Plasmodium falciparum* 3D7 using functional and structural immunoinformatic approach.

1.3.2 Specific objectives-

i. To identify the immunodominant linear B-lymphocyte, cytotoxic T-lymphocyte, and helper T-lymphocyte epitopes of MSP1, AMA1 and CSP antigens.
ii. To evaluate immunological properties of the identified epitopes
iii. To design a multiepitope vaccine using the most immunogenic epitopes.

iv. To evaluate the effect of a TLR4 agonist (Hp91 and RS09) on the immunological properties of designed vaccine.

v. To determine the physicochemical and immunological properties of the designed vaccine.

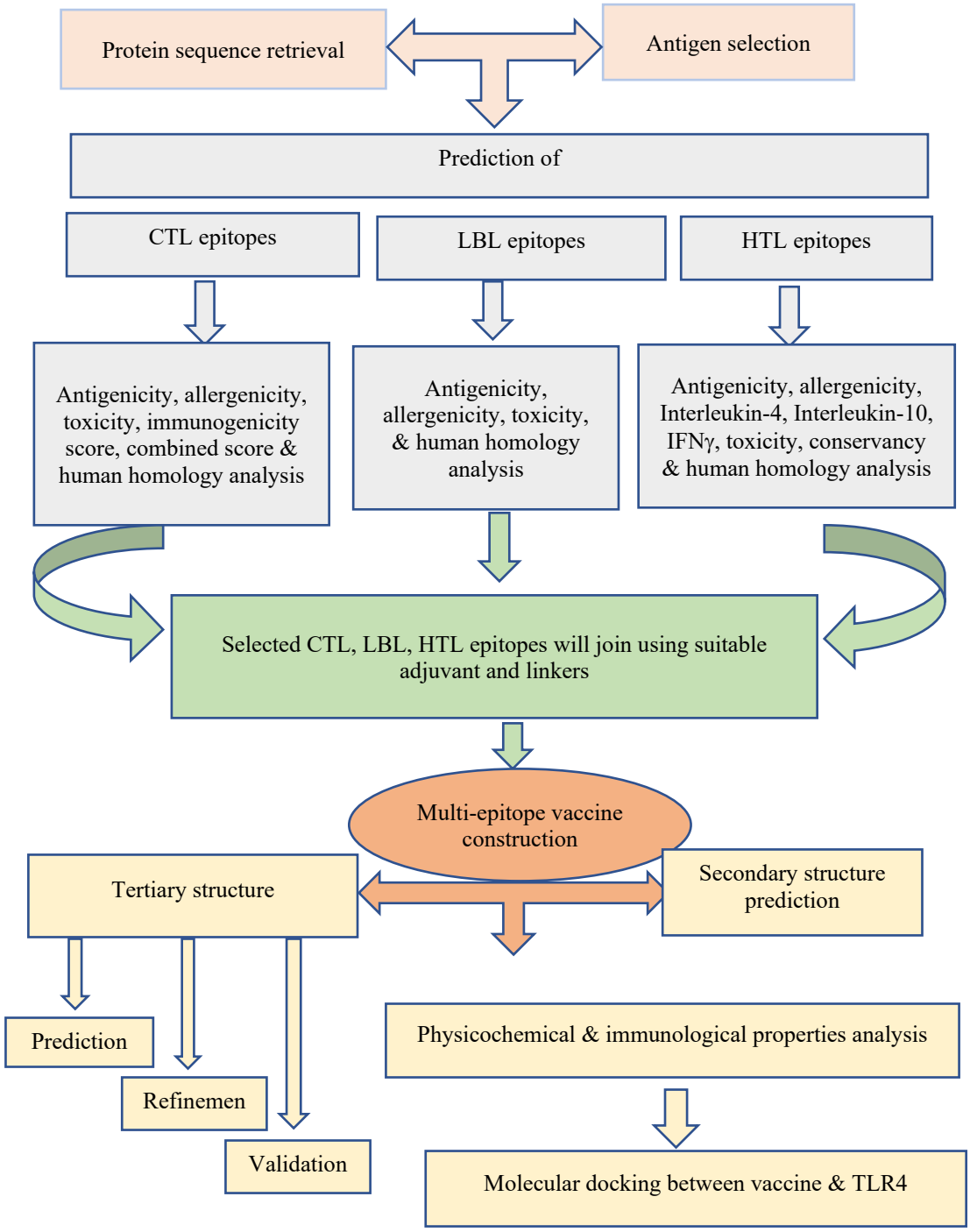


Figure 1.1 Flow chart of the study.

CHAPTER 2

LITERATURE REVIEW

2.1 Malaria

2.1.1 History

In the 20th century, mortality and morbidity rate are higher in sub-Saharan Africa, Asia, the Amazon basin and other tropical region but Neolithic dwellers, early Chinese and Greeks, princes and paupers were counted as malaria victims over the millennia. Egyptian papyri from 1570 BC, cuneiform script in clay tables from Mesopotamia from 2000 BC, and Chinese document from about 2700 BC had shown similar findings regarding the occurrence of malaria. Dhanvantari, an Indian sage wrote about malaria parasites transmission occurred possibly via the bites of mosquitoes which resulted in diseases, fever and shivering in 800 BC. Malaria was called the "King of Diseases" by some Indian writings during the Vedic period of 1500 to 800 BC. In 270 BC, Nei Chin made mention fever with spleen enlargement, headaches, chills as malaria's symptoms. From 384 to 750 BC these ancient speculations and hypotheses, features of poor health, malarial fevers, and even enlarged spleens in people living in swampy areas were quoted by the early Greeks poets and philosophers, such as Homer, Aristophanes, Aristotle, Plato, Sophocles, Empedocles of Agrigentum and Hippocrates (Institute of Medicine (US) Committee on the Economics of Antimalarial Drugs, 2004).

Malaria parasite infectious patterns and physical signs were later found to be pathognomonic and splenomegaly. According to history, malaria had arrived in the European region in the first century of AD and disease spreading started from African

rain forests, the Nile to the Mediterranean, east to the Fertile Crescent, north to Greece and Greece to Italy via soldiers, merchants, traders and colonists. It is attributed by most of the early scientists that malaria caused by contaminated air coming from swampy areas (Institute of Medicine (US) Committee on the Economics of Antimalarial Drugs, 2004). Based on this hypothesis the word malaria was named by the Italian word mal'aria meaning spoiled air. Many Poets and explorers who believed in the old bad-air theory of malaria have also contributed in describing symptoms that could be those of malaria. They felt that the vapours given off by marsh vegetation might be the cause of malaria (Cox, 2010).

After many decades, in 1717 a conflict had risen by the thought of Giovanni Maria Lancisi who stated that the causative agent of malaria can be some small bugs or worms which can enter blood stream via mosquito bites, open wounds on the surface of the human body and inoculate their salivary fluid to cause disease in his monograph titled Noxious emanations of swamps and their cure. In 1816, a century later Giovanni Rasori wrote about different species of microorganisms manifesting different patterns of fever based on their nature and reproduction pattern at variance with bad-air theory. A similar finding like Rasori and Lancisi was discovered by German anatomist Johann Heinrich Meckel via examining the blood and spleen of a patient with fever after three decades of Rasori's findings. In 1848, Meckel had discovered some round, ovoid, or spindle-shaped structures containing black pigment granules in protoplasmic masses. Virchow also described and pictured some pigmented bodies in the blood of a patient who died of chronic malaria in 1849. Virchow became the first to connect the pigments with malaria (Kakkilaya, 2015).

In 1880 a French army surgeon, Charles Louis Laveran postulated that the term bad-area related to malaria was scientifically meaningless as he was able to connect the pieces after observing pigmented parasites in the blood of an Algerian soldier. Laveran's findings were confirmed by William Osler later in 1887 with a clearer description of blood film examinations showing a pigmented structure inside a red corpuscle. After a decade, in 1890, Golgi cultivated malaria-parasite in the mosquitoes with blood meal from patients having crescents and gained the photograph of the pigmented malaria parasite. By the early 1890s Celli, Marchiafava, Bignami, and Bastianelli identified the three malaria parasites, presently known as *P. vivax, P. malariae, and P. falciparum* along with their specific pathological and clinical features. Thayer, Hewetson, and Barker, on the other hand, compiled 616 well-documented malaria cases complete with fever curves and microscopy and four autopsies in 1895. Having identified the causative agents of malaria different stages of their life cycle had been recognized by Laveran. Grassi in 1893 was the first to suggest that the organism must have some developmental stage in cells other than red blood cells, possibly white blood cells (Institute of Medicine (US) Committee on the Economics of Antimalarial Drugs, 2004).

A further inroad into the life cycle of the malaria organism was made by William MacCallum, and Eugene Opie who observed some flagellated, motile structures which they described as the male gametes and the non-motile forms, as the female gametes and the fusion of the two forming the vermiculus; the zygote in the blood of crows infected with a haematozoan closely related to the malaria parasites called *Haemoproteus columbae*. MacCallum further opined that the same could be obtained in humans. The exoerythrocytic stage suggested by Grassi in 1893 was further

investigated by Henry Shortt and Cyril Garnham in 1947 while working on *Haemamoeba kochi*, a protozoan related to malaria parasites. They demonstrated that a phase of the division of the parasite in the liver preceded their development in the blood. Shortt, Garnham and their co-workers also found exoerythrocytic forms of *P. vivax* and *P. falciparum* in human volunteers (Institute of Medicine (US) Committee on the Economics of Antimalarial Drugs, 2004).

2.1.2 Global epidemiology of malaria

In 2019, 229 million infection and 409,000 death cases due to malaria had reported in 87 malaria-endemic countries by the World Malaria report published by WHO (World Health Organization, 2021) whereas in 2018, 228 million infection and 411,000 death cases were recorded. It represented the increasing number of malaria cases in comparison to 2018 but a drop of 9 million cases from the year 2000 (Al-Awadhi *et al.,* 2021). Between 2000 to 2019 global mortality rate due to malaria fell by 60% specifically, between 2000 and 2015 27%, and between 2015 and 2019 <2%, giving a hint of declining the rate since 2015 (World Health Organization, 2020). 95% of world malaria cases had reported from 29 countries however six of them including the African region (94%), Nigeria (27%), the Democratic Republic of the Congo (12%), Uganda (5%), Mozambique (4%) and Niger (3%) were accounted for approximately 51% of overall malarial deaths. 99% of Infections reveal in the African region are mostly due to the most virulent strain *P. falciparum* (Figure 2. 1).

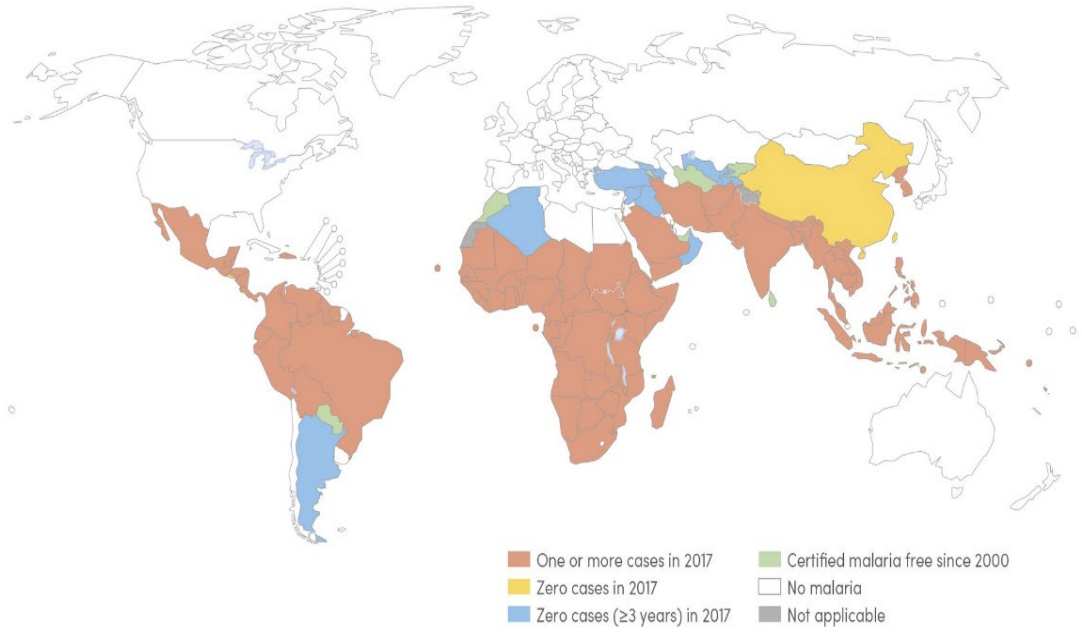
According to a WHO report, an estimated 3% of malarial cases were disclosed from the South-East Asian Region. Endemic malarial transmission has been observed in most of the region of Asia Pacific mostly due to *P. vivax*. Also, 80% of global malaria

cases are reported due to this strain. In 2019, a decline of 3 million to 1.7 million cases from 2000 to 2019 was witnessed in Western Pacific Region. Similarly, a 40% reduction of malaria cases had reported in American regions (World Health Organization, 2020). Since 2015, the European region counted as malaria-free state and study found that the majority of patients with severe malaria in Europe were tourists or migrants acquiring the infection in West Africa (Kurth *et al.,* 2017).

Figure 2.1 Global distribution of Plasmodium strain (Fisher, 2021)

Despite having both success and failure in controlling malaria, the important fact is that the disease is no longer considered as endemic; it has gone beyond its traditional enclaves. Increased globalization and population dynamics have increased the risk of malaria for many people from non-endemic areas of the world making it a global menace (Gowda, 2007) (Figure 2.2).

Countries with indigenous cases in 2000 and their status by 2017 Countries with zero indigenous cases over at least the past 3 consecutive years are considered to be malaria free. All countries in the WHO European Region reported zero indigenous cases in 2016 and again in 2017. In 2017, both China and El Salvador reported zero indigenous cases. *Source: WHO database.*



WHO: World Health Organization.

Figure 2.2 Countries with indigenous malaria cases (World Health Organization, 2017).

2.1.3 Prevalence of malaria in Malaysia

In 1960s with 240 000 malaria cases, the existence of malaria in Malaysia was initiated, however, by establishing and implementing effective preventive and controlling measures the drastic reduction of about 40000 cases were observed in 1980. Also, the majority of the cases were reported from rural and urban areas along with travelers and migrants from developed or under-developed countries (Ramdzan *et al.,* 2020). Over the last 30 years of strenuous and strategic efforts, a 7-fold reduction of malaria incidence was achieved (Rahim *et al.,* 2020). In Malaysia, 4630 malaria cases were identified due to *P. knowlesi* infection including 499 human malaria (Chin *et al.,* 2020) in 2018 had become a great concern. According to World Malaria Report published on 2018, a steady decline in the number of indigenous malaria infection from 5194 to 85 were reported between 2010 to 2017. In 2017, 69% of country-wide malaria cases resulted from *P. vivax*. Indigenous cases of *P. vivax* dropped to around 160 cases in 2016 from over 3000 cases recorded in 2010. The region of Sabah and Sarawak recorded a higher number of cases than Peninsular Malaysia due to the most vulnerable strain, *P. falciparum* (Hussin *et al.,* 2020). Malaysia aims to be positioned as malaria free country by 2020, and had reconstructed the malaria control plan from mere disease control to elimination, with a suitable strategic plan to eradicate and eliminate malaria in 2011-2020 (Figure 2.3) (Chin *et al.,* 2020).

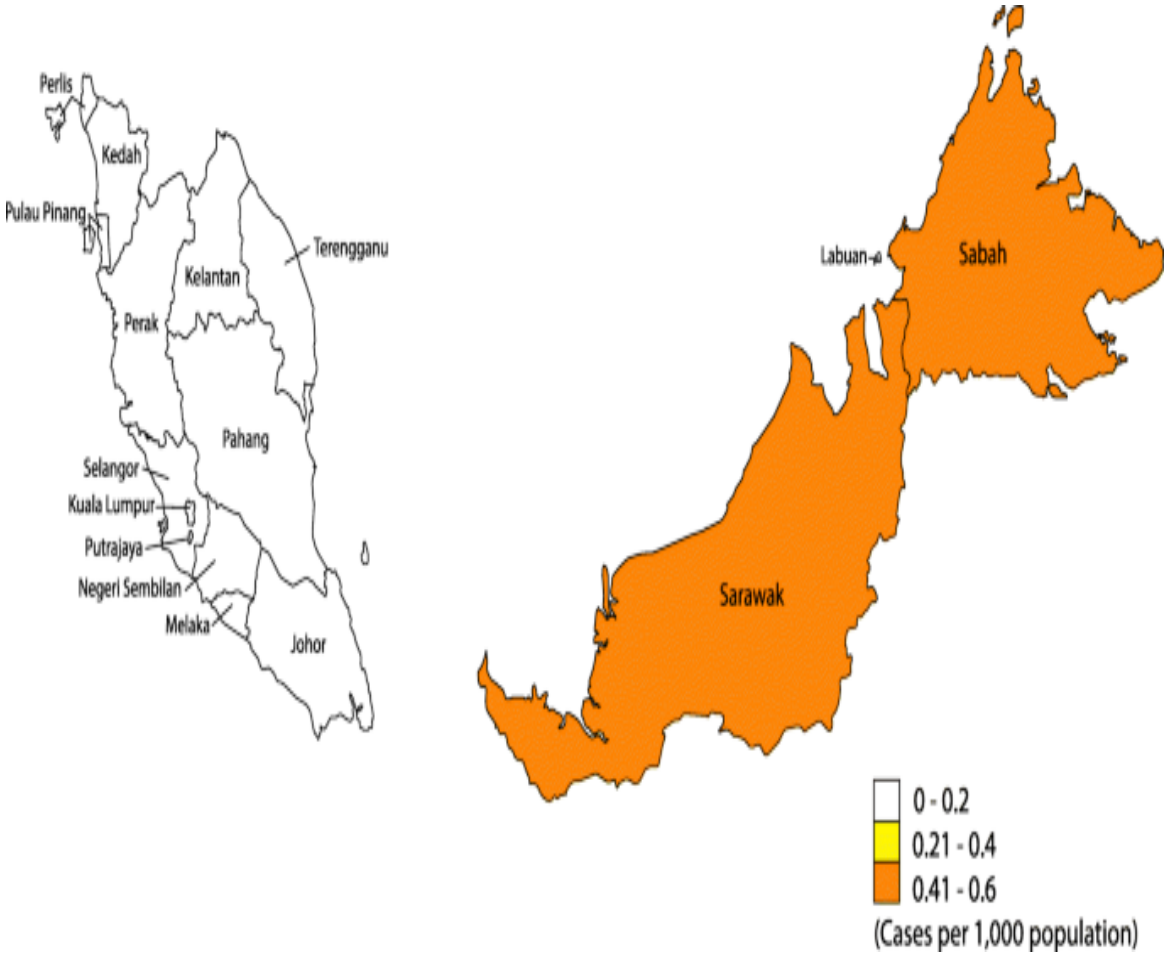


Figure 2.3 Distribution of Plasmodium knowlesi malaria cases by state in Malaysia, 2018 (Chin et al., 2020).

Probably due to its government's malaria intervention expenditure and strategy which focuses more on human resources development and provision of technical assistance presently, Malaysia is already in the WHO pre-elimination phase of malaria with only 299 reported cases and 2 recorded deaths from malaria (Rahim *et al.,* 2020). In the face of these achievements, the malaria picture in Malaysia and its neighbours is changing, with *P. knowlesi* becoming the predominant cause of human malaria and a threat to its elimination herein (Richards & Mueller, 2017).

2.1.4 Life cycle and infectious stages of *Plasmodium* parasites

As shown in Figure 2.4 *Plasmodium spp.* Has a complex life cycle due to their surviving capability in both blood-feeding mosquitoes and vertebrates including human and non-human primates. Female *Anopheles* mosquitoes perform the responsibility of transmitting malaria parasite from one host to another aiming to cause disease in the host. Three cycles, namely the Sporogonic cycle, exoerythrocytic cycle and erythrocytic cycle complete the whole life cycle of *Plasmodium* parasites (Global Health, Division of Parasitic Diseases and Malaria, 2020).

The sporogonic cycle has initiated when a female *Anopheles* mosquito takes infected blood meal via biting the host and transmits the parasites to the mosquito gut. Elaborately, during blood meal microgametocytes (male) and macrogametocytes (female), combinedly the gametocytes are ingested by the mosquitoes, matting and multiplication take place in the stomach of the mosquito and form zygotes via penetration. Later on, by undergoing some morphological changes zygotes become motile and elongated turning into ookinetes. Ookynates are transformed into oocysts by invading the mosquito's midgut membrane where they can replicate, mature, and transform into oocysts. After completing proper breeding of matured oocysts, they are ruptured to release sporozoites. Released sporozoites then travel to the salivary glands of mosquitoes to inoculate them in the next hosts body through female *Anopheles* mosquito bites. The sporogonic cycle is also considered as the sexual stage of *Plasmodium spp.*

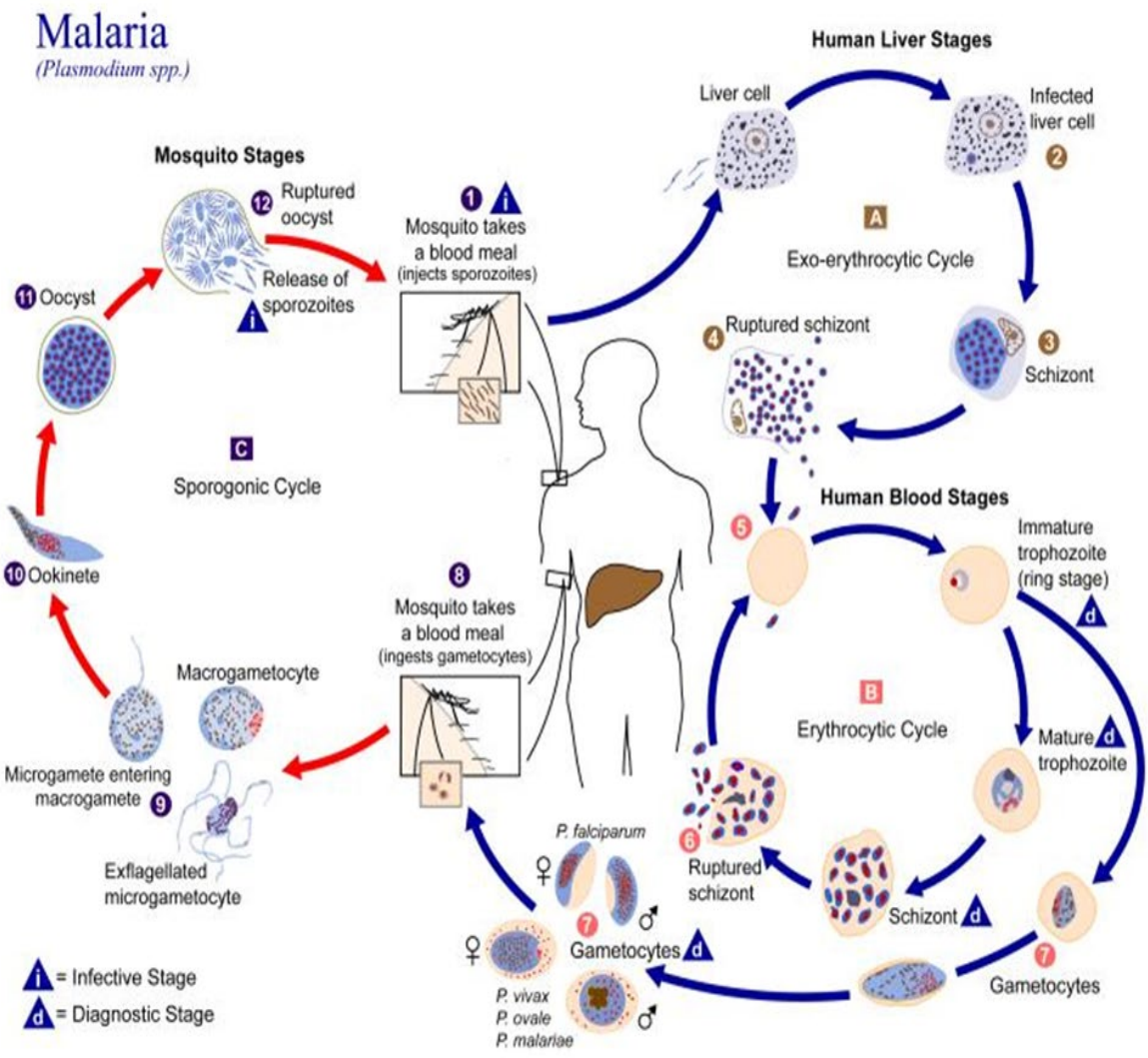


Figure 2.4 Life cycle of Plasmodium spp. (Global Health, Division of Parasitic Diseases and Malaria, 2020).

Skin infection, liver-stage development (exoerythrocytic cycle) and blood-stage development (erythrocytic cycle) are involved from the inoculation of sporozoites to malaria disease causation (Venugopal *et al.,* 2020). Itching and radish skin is observed as an immediate effect of mosquito bites. After injection, sporozoites start moving from blood capillary to sinusoidal capillary of liver aiming to invade hepatocyte by performing asexual replication inside the cell. As a result of initial asexual replication,

sporozoites become mature into liver schizont that release thousands of merozoites (Venugopal *et al.,* 2020) which are responsible for invading red blood cells causing RBC infection and the process of releasing merozoites is termed exoerythrocytic schizogony.

The liver development stage is also known as the asymptotic stage. Parasites undergo another asexual multiplication within the RBC which is known as erythrocytic schizogony (Global Health, Division of Parasitic Diseases and Malaria, 2020) and increase the trophozoites population 6 to 20 times per cycle as a result of transforming from immature or ring stage to mature trophozoites. Matured trophozoites then transform into schizonts, and release merozoites by rupturing schizonts to infect another erythrocyte and the release of erythrocyte and parasite debris. Thus, the blood-stage parasites are responsible for the clinical manifestations of malaria which can occur as early as three days from the beginning of the erythrocytic stage. At the third stage, the gametocyte, or sexual stage, in a small percentage of the merozoite-infected blood cells, the asexual reproduction would stop and the *plasmodia* now mature into sexual forms of the parasites known as male and female gametocytes (Figure 2.5) (Global Health, Division of Parasitic Diseases and Malaria, 2020).

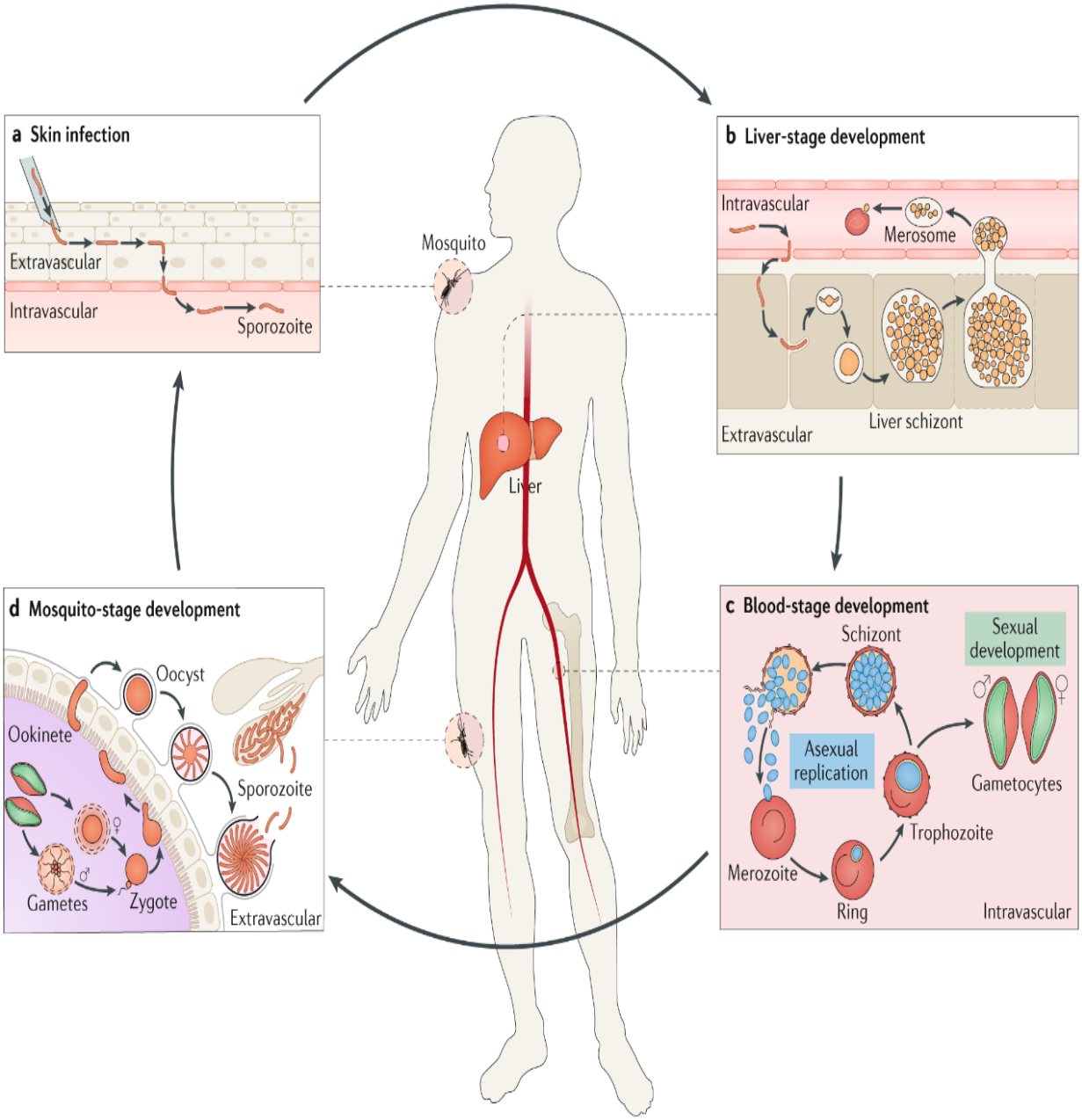


Figure 2.5 Development of P. falciparum in both Anopheles mosquito and human (Venugopal et al., 2020).

2.1.5 Preventive and control measures against malaria

Numerous international and nation malaria controls programs have been implemented aiming to control and eradicate malaria disease, but malaria is considered as the most important vector-borne parasitic disease. According to WHO, controlling vector transmission is one of the most prominent ways for the prevention and management of malaria, even though malaria can be transmitted via organ transplant, and blood transfusion. Studies found that high-income countries (HICs) succeeded to eradicate malaria, but low and middle-income countries (LMICs) are still facing difficulties to tackle the menace (Salam *et al.,* 2014).

Numerous preventive measures have been recommended by World Health Organization, among which using insecticide-treated net (ITNs) while sleeping, indoor residual spraying (IRS), implementation of artemisinin combination therapy (ACT), and maintaining other personal protection modalities (World Health Organization, 2021). Personal protective modalities of control include wearing those clothes that cover most parts of the body for avoiding mosquito bites in malaria-endemic regions, use of mosquito repellents, and try to be indoors during mosquito feeding hours for the avoidance of mosquito contact, which usually occurs between dusk and dawn, and also the clearing of the environment on mosquito breeding site (Fischer & Bialek, 2002).

As parasites and vectors are equally responsible for disease causation, only controlling vector transmission will not fulfill the quest of malaria-free world. Many antimalarial drugs have been recommended for reducing the severity of the disease and also used for preventing malaria. Chemoprophylaxis, an antimalarial drug that can suppress erythrocytic malaria infection, is recommended only for travellers and migrants from

malaria endemic regions. Treatment of sulfadoxine-pyrimethamine is provided maintaining regularity among pregnant women, infants, and children under five years living in high- and middle transmission areas (World Health Organization, 2021).

Unavoidable emergence and re-emergence of insecticide-resistant mosquitoes and antimicrobial drugs resistant Plasmodium parasites resistance become an unresolved issue continuing to worsen the situation and rendering malaria control measures virtually ineffective. In the quest for alternatives to the antimalarial drugs and vector control, researchers have shifted the emphasis to eradication as the answer to the question of control, elimination, and possible eradication of malaria lies on an effective antimalarial vaccine (Abbas & Suppian, 2019). Many researchers have beamed their search light towards the search for an efficient and effective malaria vaccine targeting many surface antigens such as merozoite surface protein 1 (Bemani *et al.,* 2020), apical membrane antigen 1 (Remarque *et al.,* 2008), circumsporozoite protein (Plassmeyer *et al.,* 2009) and so on.

2.2 Immunity against *P. falciparum*

The availability of the pattern of *Plasmodium* parasite's transmission and infections make it possible to study the dynamics of immune response initiated by hosts. Immunity to malaria can be acquired more rapidly in individuals living in moderate to high transmission areas even though complete immunity is rare, less potent, and effective for a shorter period. Studies found effective immune response against sporozoites, the first infectious stage which is responsible for hepatocyte invasion. Exposure to sporozoites modulates the cytokine secretions via antigen-presenting T cell specifically helper T cell. Recent studies found that as an innate immune response

in terms of primary exposure of *P. falciparum* to human hepatocyte, the activation of CD16+ dendrite cells and production of other immunoregulatory cytokines such as IL-10, TNFα, and so on (Loughland *et al.,* 2019). Destruction of sporozoites by the production of antibodies via complement fixation mechanism blocking the invasion pathways of red blood cells was also reported recently (Kurtovic *et al.,* 2018). Unfortunately, these defence mechanisms are generally acquired by the adult and comparatively slower as small number of sporozoites are inoculated during mosquito bites (Frimpong *et al.,* 2018). RTS, S- a sporozoite targeting recombinant vaccine has developed based on the observation of antibody production to circum-sporozoites antigen infection. This vaccine showed 29-36 % efficiency in children less than five years old (Kurtovic *et al.,* 2018).

Clinical manifestations of malaria disease initiated in the erythrocytic stage due to merozoite infections. Evidence had been found regarding antibody production in response to the blood-stage infection caused by surface proteins along with many functional activities. Mechanisms of active antibodies direct against merozoites include blocking of RBCs invasion, removing parasites via opsonization, increasing lysis of infected cells by monocytes and macrophages via complement- fixation cascade, and prevention of further infection to vascular endothelium (Teo *et al.,* 2016). Disruption in expression and changes in antigenic properties of erythrocyte- binding antigens had been observed due to the production of invasion- inhibitory antibodies (Persson *et al.,* 2013). According to studies $FOXP3^{-}$ Th1 cells protect the prolongation of pyrogenic factors responsible for malarial clinical manifestations by releasing produce IFNγ, TNFα and IL-10. Less effective protective measures implemented by

regulatory T cells have found against *P. falciparum* erythrocyte infection in children for a shorter period (Frimpong *et al.,* 2018).

2.3 Promising approaches to develop malaria vaccine

2.3.1 Vaccine construction targeting infectious stages

As shown in Figure 2.6, there are three stages of malaria parasite life cycle that are potentially targeted as a vaccine candidate. Development of vaccine targeting pre-erythrocytic stages that will protect hepatocytic infection blocking the movement of sporozoites from hepatocyte to red blood cell limiting RBC invasion. In other word, this vaccine design approach involves the initiation antibody response preventing sporozoite infection to the liver cell or cytotoxic T-cell that will destroy the infected hepatocyte interrupting blood cell invasion (Frimpong *et al.,* 2018).

According to current studies, radiation attenuated sporozoite (RAS), genetically attenuated parasite (GAP), and sporozoite administered under drug coverages are the uncovered vaccine design approach targeting the pre-erythrocytic stage (Molina-Franky *et al.,* 2020). Eradication of sporozoites via vaccination to mice was first observed by Ruth Nussenzweig (Vanderberg *et al.,* 1968). Later on, using circumsporozoite antigens, a sporozoite protein as a vaccine candidate gave promising results showing 30-60% protection in phase III clinical trial. At present, RTS, S is one of the most promising licensed subunit vaccines following this approach (Frimpong *et al.,* 2018).

Based on targeting antigens that are responsible for invading red blood cells that are only expressed on the surface of merozoites, the malaria symptoms rising erythrocytic

stage of parasite's life cycle, the second approach naming erythrocytic vaccine have developed. The idea of this approach designed observing the pattern of infection and development immunity in malaria-endemic regions (Frimpong *et al.,* 2018). Erythrocyte-Binding Antigen-175, Apical Membrane Antigen 1 (AMA1), Glutamate-Rich Protein, Serine-Repeat Antigen 5 (SERA5) and Merozoite Surface Protein 1 (MSP1), MSP2, MSP3 are the blood-stage proteins that expressed on the surface of merozoite. Use of these antigens as erythrocyte stage vaccine candidates will provide protection limiting RBCs invasion due to the release of merozoites (Ellis *et al.,* 2010).

Erythrocytic stage vaccine candidates might not only be utilized as solo antigen vaccine candidates but also to give protection against breakthrough infections in a multistage vaccine with pre-erythrocytic stage vaccine candidate constituents, therefore they are considered valuable All of these candidates have not shown protection against clinical results till this day. The effectiveness of these candidates may be constrained by the variation of the genetic of the malaria parasite surface antigens, which is likely because of the selective pressure used by the human immune system (Ellis *et al.,* 2010).

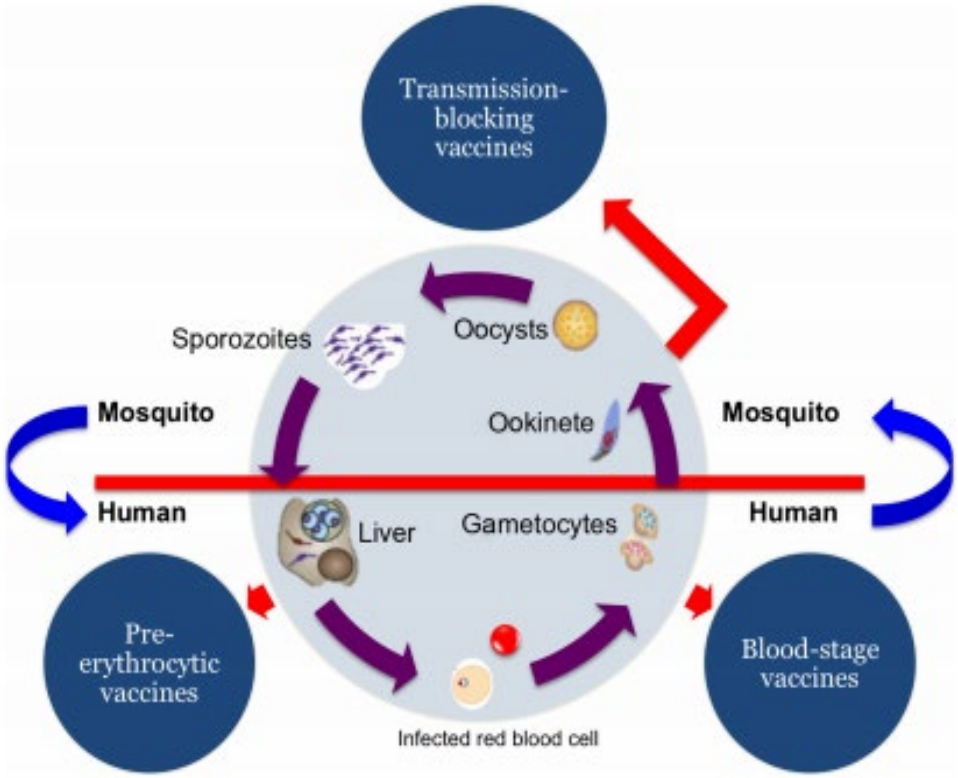


Figure 2.6 Possible infectious stages and vaccine approach targeting those stages (Arama & Troye-Blomberg, 2014).

The third approach for malaria vaccine development is built upon targeting those antigens that are mainly expressed during the sexual stage of the parasite's life cycle as vaccine candidates capable of blocking malaria transmission, namely the transmission-blocking vaccine (TBV) approach. The motivation of this design is to restrict the transmission of parasites from vectors to the human host and vice-versa destroying pre- and post-fertilization antigens of the gametocytes (Frimpong *et al.,* 2018). Gametocyte antigens such as Pfs48/45 and Pfs230 and ookinete/zygote antigens such as Pfs28 and Pfs25 of *P. falciparum* are considered as the most potential TBVs