# *IN VITRO* ANTIMALARIAL EFFICACY ENHANCEMENT OF SELECTED ANTIBIOTICS WITH PHEROID™ TECHNOLOGY

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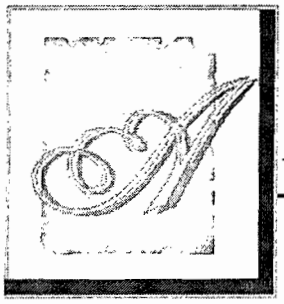
*I hope that my achievements in life shall be these ~ that I will have fought for what was right and fair, that I will have risked for that which mattered, and that I will have given help to those who were in need, that I will have left the earth a better place for what I've done and who I've been.*

*~ C. Hoppe ~*



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# ABSTRACT


***IN VITRO* ANTIMALARIAL EFFICACY ENHANCEMENT OF SELECTED ANTIBIOTICS WITH PHEROID™ TECHNOLOGY.**

The *Plasmodium falciparum* parasite, carried by *Anopheles* mosquitoes, is currently a global problem due to the rising incidence of resistance of the parasite to available antimalaria drugs. Resistance and difficult treatment groups, including pregnant woman and young children, are pressing for the development of new, safe and effective prophylactic and treatment antimalarials. Because of the extensive process of developing new drugs, researchers and health care professionals have turned to combination therapy where a fast acting antimalarial is combined with slower acting drugs, such as antibiotics.

The macrolide antibiotics, erythromycin and azithromycin, have been studied to a limited extent for their potential antimalarial effect. Certain advantages, such as their safety profile (especially that of azithromycin) in pregnancy and administration to young children, motivates continual research into the advancement of the effect these drugs exude on malaria. Drug delivery systems contribute to the efficacy of medicines, conquering several difficulties of treatment with oral medication. Pheroid™ technology is a patented drug delivery system, mainly consisting of plant and essential fatty acids, and has been demonstrated to entrap, carry and deliver pharmacologically active compounds and other useful molecules.

This study compared the *in vitro* effects of the macrolide antibiotics on the growth of a chloroquine-resistant strain (RSA 11) of *Plasmodium falciparum* to the effects of the macrolides entrapped in Pheroid™ vesicles on the same strain over and extended observation period of 144 hours. ELISA assays were conducted by analysing the HRP II (histidine-rich protein) levels on a pre-coated microtitre plate. The effects of the type of formulation, concentration and time were compared.

The *in vitro* difference between erythromycin alone and entrapped in Pheroid™ vesicles were found to be statistically significant (P = 0.000000) while the effects of both formulations did not seem to be concentration dependant (P = 0.628424). Prolonged exposure was also statistically meaningful (P = 0.008268), though it seems that exposure need not exceed 96 hours. The type of formulation, in the case of azithromycin (azithromycin alone vs. azithromycin entrapped in Pheroid™ vesicles), proved statistically significant (P = 0.002572), while neither formulation seemed concentration dependant (P = 0.427731). Prolonged exposure was found to be statistically insignificant for azithromycin (P = 0.221941).

**Keywords:** *Plasmodium falciparum,* malaria, Pheroid™ technology, erythromycin, azithromycin, RSA 11, ELISA assay, HRP II

# UITTREKSEL


## *IN VITRO* ANTIMALARIA EFFEKTIWITEITS VERBETERING VAN GESELEKTEERDE ANTIBIOTIKUMS MET PHEROID™ TEGNOLOGIE.

Die *Plasmodium falciparum* parasiet, oorgedra deur *Anopheles* muskiete, is tans 'n wêreldwye probleem weens die verhoogde insidensie van weerstandbiedendheid van die parasiet teen beskikbare antimalaria middels. Weerstandbiedendheid en moeilik behandelbare groepe, soos swanger vrouens en jong kinders, dryf die behoefte vir die ontwikkeling van nuwe, veilige en effektiewe profilaktiese en behandelings middels. Die ontwikkeling van nuwe geneesmiddels is 'n tydsame proses, en daarom het navorsers en gesondheid-sorgwerkers die gebruik van kombinasie terapie geïmplementeer, waar vinnig werkende antimalaria middels met stadig werkende geneesmiddels soos antibiotikas gekombineer word.

Die makrolied antibiotikas, eritromisien en azitromisien, is voorheen tot 'n mindere mate ondersoek vir die potensiële effek wat die middels op malaria parasiete uitoefen. Sekere voordele, soos hul veiligheids profiel (veral azitromisien) vir toediening tydens swangerskap en in jong kinders, dien as motivering vir verdere navorsing in die bevordering van die middels se effek op malaria. Geneesmiddel afleweringsisteme dra by tot die effektiwiteit van geneesmiddels en oorkom menig probleme wat ondervind word met orale dosering. Pheroid™ tegnologie is 'n gepatenteerde geneesmiddelafleweringsisteem wat hoofsaaklik uit plant en essensiële vetsure bestaan. Die sisteem het bewys dat dit farmakologies aktiewe verbindings en ander bruikbare molekules kan vasvang, oordra en versprei.

Hierdie studie het die *in vitro* effek van die makrolied antibiotikas op die groei van 'n chloroquine-weerstandbiedende vorm (RSA 11) met die effek van die antibiotikas vasgevang in Pheroid™ vesikels oor 'n uitgebreide waarnemings tydperk van 144 uur met mekaar vergelyk.

ELISA analise is uitgevoer deur die HRP II (histidien-ryke proteïen) vlakke op voorafbedekte mikro-plate te analiseer. Die effek van die tipe formulering, konsentrasie en tyd was vergelyk. Die *in vitro* verskil tussen eritromisien alleen en vasgevang in Pheroid™ vesikels, was statisties betekenisvol (P = 0.000000) terwyl die effek van beide formulerings nie geblyk het om konsentrasie afhanklik te wees nie (P = 0.628424). Verlengde blootstelling was ook statisties betekenisvol (P = 0.008268) maar dit wil voorkom of blootstellingstyd nie nodig het om 96 uur te oorskrei nie. Die tipe formulering, in azitromisien se geval (azitromisien alleen teenoor azithromisien vasgevang in Pheroid™ vesikels), was statisties betekenisvol (P = 0.002572), terwyl nie een van die twee formulerings gebleik het om konsentrasie afhanklik te wees nie (P = 0.427731). Verlengde blootstelling was nie van statistiese betekenis vir azitromisien nie (P = 0.221941).

**Sleutelwoorde:** *Plasmodium falciparum,* malaria, Pheroid™ tegnologie, eritromisien, azitromisien, RSA 11, ELISA assay, HRP II

# INTRODUCTION AND AIM OF STUDY

The term malaria is derived from the Italian 'mal'aria' which means 'bad air'. The disease was earlier associated with swampy areas (Tuteja, 2007). Presently, malaria remains, together with tuberculosis and AIDS, one of three major communicable diseases (Lewison & Srivastava, 2008). In Africa, this killer disease has been ranked as the second highest contributor to the Disability Adjusted Life Year (DALY). The DALY is an estimation of a disease's direct cause of mortality and morbidity (Snow *et al.*, 2003; Snow *et al.*, 2004). Four species of *Plasmodium* is responsible for the spread of malaria, of which *P. falciparum* is the most dangerous (Tracy & Webster, 2001). Annually, millions of people succumb to malaria and roughly half a billion people are afflicted by it (CDC, 2007). Susceptible groups, resembling young children, pregnant woman, and nonimmune individuals, are at high risk of developing severe malaria and also constitute a high percentage of the mortality rate (Lagerberg, 2008; Tracy & Webster, 2001).

Efforts to eradicate and control this infectious disease have repeatedly faltered, partly due to the rising incidence and spread of drug resistant *P. falciparum* parasites (Ekland & Fidock, 2008). Resistance is dependable upon the spontaneous mutation or gene amplification (genetic change) in a malaria parasite which interferes with the parasite's susceptibility to an antimalarial drug (Chiyaka *et al.,* 2008). Drug pharmacokinetics (Hastings *et al.,* 2002), over-usage of antimalarials (Simpson *et al.,* 2000), cross-resistance (Iyer *et al.*, 2001), and inadequate treatment by means of inapt prescribing patterns, incorrect administration, meager absorbance and noncompliance (Simpson *et al.*, 2000; White, 1999) have all been considered to aggravate the emergence of resistant parasites.

When studying this parasitic disease several factors ought to be considered. Malaria is perceived as a multi-factorial problem stretching over a large domain, including; medical, health, ecological, agricultural, social, political, and economic aspects (Temel, 2005).

Therefore, when implementing strategies to control and minimise the morbid effect of malaria on communities, information from various fields of work need to be scrutinised and evaluated in order to implement optimal control measures (AAAS, 1991). Regarding the medical and public health research, results should aim to provide information critical for prevention, early diagnosis and treatment of malaria (Temel, 2005).

Some antibiotics have been studied for their antimalarial properties and are generally combined with antimalarials in an effort to overcome the global challenge of drug-resistant *P. falciparum* (Nakornchai & Konthiang, 2006). The slow onset of antimalarial action of antibiotics, excludes their use as single agents (Ohrt *et al.,* 2002). Antibiotics, like azithromycin, seem to exude better activity with prolonged exposure to *Plasmodium* parasites (Yeo & Rieckman, 1994). Treatment optimisation is dependant upon continuous research and development of safe and effective medicinal interventions. The relative safety profile of the macrolide antibiotics, erythromycin and especially azithromycin in high risk groups, was one of the aspects taken into consideration in the decision to study these test compounds.

The Pheroid™ drug delivery system is a patented, colloidal system, comprising of a unique submicron emulsion type formulation consisting mainly of plant and essential fatty acids. The Pheroid™ has been proven to entrap drugs with high efficiency and deliver these with significant speed to target sites in the body. Furthermore, Pheroids ™ are able to extravascate from the vascular system and are ideal for rapid release of active compounds. This characteristic of Pheroid™ technology may increase the antimalarial effect of slow acting antibiotics. The Pheroid™ also protects drugs from metabolism and inactivation in the plasma and other body fluids. The volume of distribution is decreased by this drug delivery system ultimately increasing the concentration at the target site, thereby achieving an enhanced but narrow therapeutic index (Grobler, 2004).

In this study the *in vitro* antimalarial effects of erythromycin and azithromycin, respectively, were compared to the effects of the test compounds entrapped in the Pheroid™ carrier system on a chloroquine resistant (RSA 11) *P. falciparum* strain. The effect of concentration and prolonged incubation periods were also investigated.

The specific objectives of this study were:

1. To develop an acceptable formulation of Pheroids™ together with the macrolide antibiotics, erythromycin and azithromycin, to apply in the *in vitro* drug efficacy assay (HRP II).
2. To evaluate the HRP II method in the applicability of *in vitro* drug efficacy assays with Pheroid™ technology.
3. To evaluate the *in vitro* efficacy of azithromycin and erythromycin alone and in combination with Pheroid™ vesicles against a chloroquine resistant strain (RSA 11) over an extended observation period of 144 hours.

Chapter 1 summarises malaria as infectious disease over viewing the problematic nature of the infection and in Chapter 2 the test compounds utilized in this study is discussed. Chapter 2 further elaborates on the advantages of Pheroid™ technology. Chapter 3 elucidates the materials purchased and methods applied in this study where after the results and discussion follows in Chapter 4.

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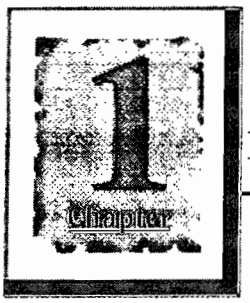


# MALARIA

## 1.1 INTRODUCTION

Malaria, an infectious disease caused by obligate intracellular protozoan parasites of the genus *Plasmodium*, is presently one of the foremost causes of global mortality and morbidity (Tracy & Webster, 2001; Tuteja, 2007). In humans, four species are known to cause malaria, namely; *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* (Pasvol, 2005; Tuteja, 2007). All of the above mentioned parasites have the ability to cause serious illness, though *P. falciparum* remains the mediator of rigorous, potentially terminal malaria (Rosenthal, 2004; Tuteja, 2007). In 1880, a French army surgeon Charles Louis Alphonse Laveran was the first to observe parasites in the blood of a patient infected with malaria. He was granted the Nobel Prize for his discovery in 1907. At the time, Laveran had believed that there were only one causative species of malaria; *Oscillaria malariae.* However, in 1890, the Italian investigators Giovanni Batista Grassi and Raimondo Filetti introduced the names *P. vivax* and *P. malariae* for two of the malaria parasites concerning humans. An American, William H. Welch, named the malignant tertian malaria parasite *P. falciparum* in 1897 (CDC, 2004a). The vector of this parasite is the *Anopheles* mosquito (Lewison & Srivastava, 2008). Dr. Ronald Ross, a British officer in the Indian Medical Service, established that malaria could be transmitted from infected humans to mosquitoes in 1897 and was awarded the Nobel Prize in 1902 (CDC, 2004a; Tuteja, 2007). Finally, the fourth human malaria parasite was described in 1922 by John William Watson Stephens (CDC, 2004a). Chapter 1 will briefly discuss malaria as an infectious disease, summarising key points such as the spread of the infection, risk groups, causative agents, treatments and associated problems like drug- and insecticide-resistance.

## 1.2 EPIDEMIOLOGY

One definition of the term epidemiology is depicted in Dorland's Illustrated Medical Dictionary, (1988) as: 'the science concerned with the study of the factors determining and influencing the frequency and distribution of disease, injury, and other health-related events and their causes in a defined human population for the purpose of establishing programs to prevent and control their development and spread'. The following sections will give an overview of the epidemiological factors that play an important role in malaria.

### 1.2.1 Statistics

To emphasize the complexity of malaria it is essential to enumerate the effects these parasitic infections convey on the population as well as on the economy. Estimates are derived from the statistics made available by the World Health Organization (WHO) in the malaria report of 2008, data from Centers for Disease Control and Prevention (CDC) and the Medical Research Council of South Africa.

#### 1.2.1.1 Malaria cases and mortalities

According to WHO, (2008) estimates there were 230 million cases of *P. falciparum* infections worldwide during 2006. Malaria claimed an estimated 881 000 lives globally of which 90 % were in the African Region. Data suggests that only 1 in 5 malaria deaths were reported internationally in 2006, thus concluding that these estimates were expected to be much higher. An estimated 85 % of mortalities due to *P. falciparum* infection occurred in young children under the age of five. CDC, (2007) reported that malaria was the fourth cause of death in children in developing countries, in 2002, subsequent to birth complications, lower respiratory infections and diarrheal diseases, consequently accountable for 10.7 % of children's deaths in developing countries. South Africa experienced a malaria epidemic in 2000 with a reported 64 622 malaria cases. Newer statistics indicate that there had been a 15 % decrease in the number of reported malaria cases during January - December 2003, although the number of malaria deaths displayed a 21 % increase when compared to the same period in 2002 (DOH, 2003b). Nevertheless, the number of reported malaria cases have declined steadily since the epidemic in 2000, but malaria remains a threatening disease even in countries where a measure of control through interventions like combination drug therapy have led to a decrease in the number of malaria cases.

#### 1.2.1.2 The economy

Malaria has a considerable effect on the economical development of a country (Tren, 2008). Economists believe that malaria could cause a 'growth penalty' of up to 1.3 % per annum in some African countries (RBM, 2007). Data for 2006, compiled by the National Malaria Control Programme (NMCP), as reported by the WHO, (2008) indicated that there were more funds available for malaria control in the African Region than any other, but the total of US$ 688 million is certain to be underestimated because only 26 of 45 countries submitted reports. Therefore, the estimated US$ 4.6 available per malaria case in the 26 reporting countries is likely to be inadequate to meet targets for prevention and cure. According to CDC (2007) estimates the average costs for potentially life-saving treatments of malaria are expected to be US$ 0.13 for chloroquine, US$ 0.14 for sulfadoxine-pyrimethamine, and US$ 2.68 for a 7-day course of quinine. Direct costs of malaria consist of personal and public expenditures on both the prophylactic treatment and treatment of the disease. Indirect costs consist of loss of income and productivity due to illness or death (RBM, 2007).

### 1.2.2 Geographic distribution

It is estimated that one-third, or more than 40 %, of the world's population resides in malarial endemic areas and are thus at risk of contracting the disease (Pasvol, 2005; Tuteja, 2007). In 2008, the total number of countries endemic for malaria was 109 of which 45 was within the WHO African region (WHO, 2008). Figure 1.1 demonstrates areas where malaria transmission occurs.

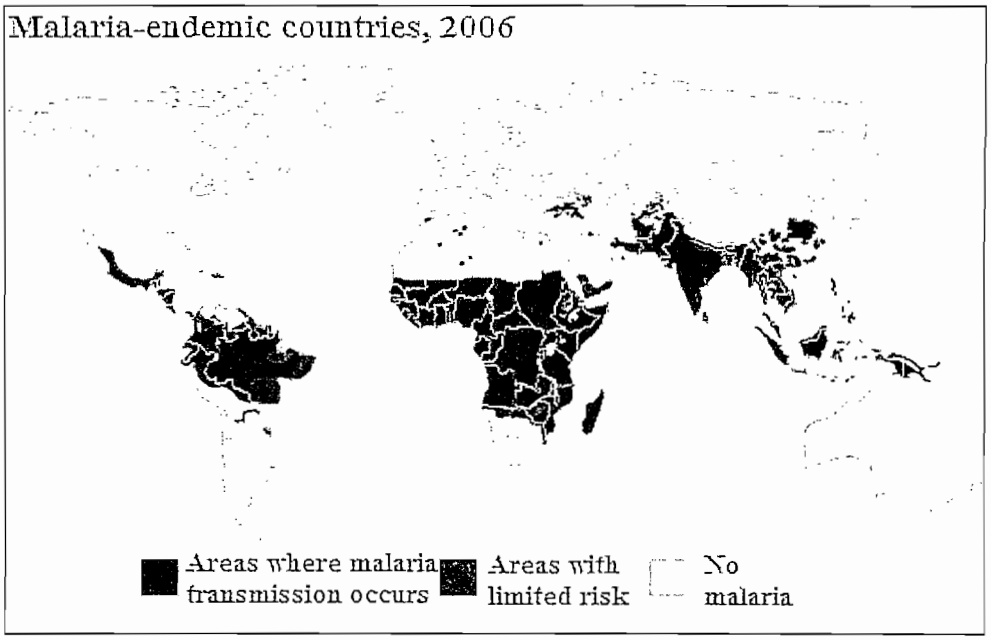


**FIGURE 1.1: Global regions where malaria was prevalent during 2006** (MVIPath, 2010).

In South Africa, malaria is mainly confined to the northern and eastern border areas with roughly 4.3 million individuals at risk of developing the disease (Blumberg & Frean, 2007). Malaria transmission is mostly seasonal in South Africa with an onset in October. Transmission season usually ends in May with malaria cases peaking between January and February (DOH, 2003b). Nearly every South African, including occupants of seasonal malaria transmission areas, is non-immune and is, as a result, at risk to develop severe malaria. A conclusion can be drawn that despite successes in malaria control since the epidemic in 2000, many challenges remain. Vector control through indoor residual spraying, case management, disease surveillance, epidemic preparedness and response, and public awareness are the key strategies implemented in the control of malaria in South Africa (Blumberg & Frean, 2007).

### 1.2.3 Climate

Various climatic factors add to the incidence of malaria in a specific region. During seasons with high rainfall, water build-up can create breeding sites where the mosquitoes deposit their eggs and larvae and pupae develop into adulthood. After 9 - 12 days, adult mosquitoes emerge and temperature, humidity, and rainfall will ultimately contribute to their survival. Temperature is especially important for *P. falciparum* cannot complete its growth cycle in the

*Anopheles* mosquito at temperatures below 20°C. The duration of the extrinsic cycle is shortened during warmer temperatures accordingly increasing the chances of transmission. Warmer temperatures also lead to human behavior such as sleeping without bed-nets or sleeping outside, thereby increasing the risk of infection (CDC, 2004b).

### 1.2.4 *Anopheles* mosquitoes

The intensity of malaria transmission will be dependent on which species of *Anopheles* mosquitoes are present in an area at a given time. *Anopheles* species may vary in selected behavioral traits, consequently impacting their abilities to act as malaria vectors. The females of some species favor a blood meal from humans ("antropophilic") while others prefer animals (zoophilic). Some species bite indoors ("endophagic") and others outdoors ("exophagic"). The antropophilic, endophagic species will encounter humans more frequently and will therefore be more effective malaria vectors (CDC, 2004b). Of the approximately 422 species of *Anopheles* mosquitoes, 60 are known to cause malaria (Service, 1993; Tuteja, 2007). The main mosquito vector of malaria in sub-Saharan Africa is *Anopheles gambiae*. In malaria-endemic regions, insecticide treated nets (ITNs) saturated in pyrethroid insecticides are commonly in use as a protective measure in vector control, but emergence of pyrethroid resistant mosquitoes has notably reduced their efficacy. A potential alternative, which could be safely developed as a new class of ITNs, is an acetylcholine esterase (AChE) inhibitor (Carlier *et al.*, 2008).

### 1.2.5 Control

Eradicating efforts need to be constantly revised and adjusted for their successes and failures may lead to the implementation of control strategies to manage malaria. In the past 50 years the WHO has led many a global effort to control this infectious disease (Olumese, 2005). The roll back malaria initiative was launched in 1998 and aimed to halve the malaria mortality by 2010 and again by 2015. According to the WHO Expert Committee on Malaria Control: twentieth report (as quoted by Olumese, 2005) the programme is based on a strategy of:

- early diagnosis and prompt antimalarial intervention,
- vector control,
- intermittent preventive therapy in pregnancy (IPT) (see section 1.4), and
- prevention and control of malaria epidemics.

Seasonal transmission, an effective national malaria control programme and the well-developed scientific, economic and health infrastructure contribute to the control of and decline in malaria cases in South Africa (Blumberg & Frean, 2007).

## 1.3 *PLASMODIUM FALCIPARUM*

Responsible for the most dangerous form of malaria, *P. falicparum* infection can produce extreme parasitaemia, sequestration of infected erythrocytes in the peripheral microvasculature, hypoglycemia, hemolysis, and shock with multiorgan failure. If detected early and treated appropriately infection generally responds within 48 hours; conversely, inadequate treatment may result in *recrudescence* of infection when parasites multiply persistently in the blood (Tracy & Webster, 2001). The following sections describe the life cycle of *P. falciparum* and the pathogenesis leading to the clinical symptoms.

### 1.3.1 Life cycle

The asexual stage of the parasite develops in the human liver and the sexual stage continues in the mosquito (Wolmarans, 2004), as demonstrated in Figure 1.2. Immediate schizogony is triggered once *P. falciparum* is injected into a human host (Tuteja, 2007).

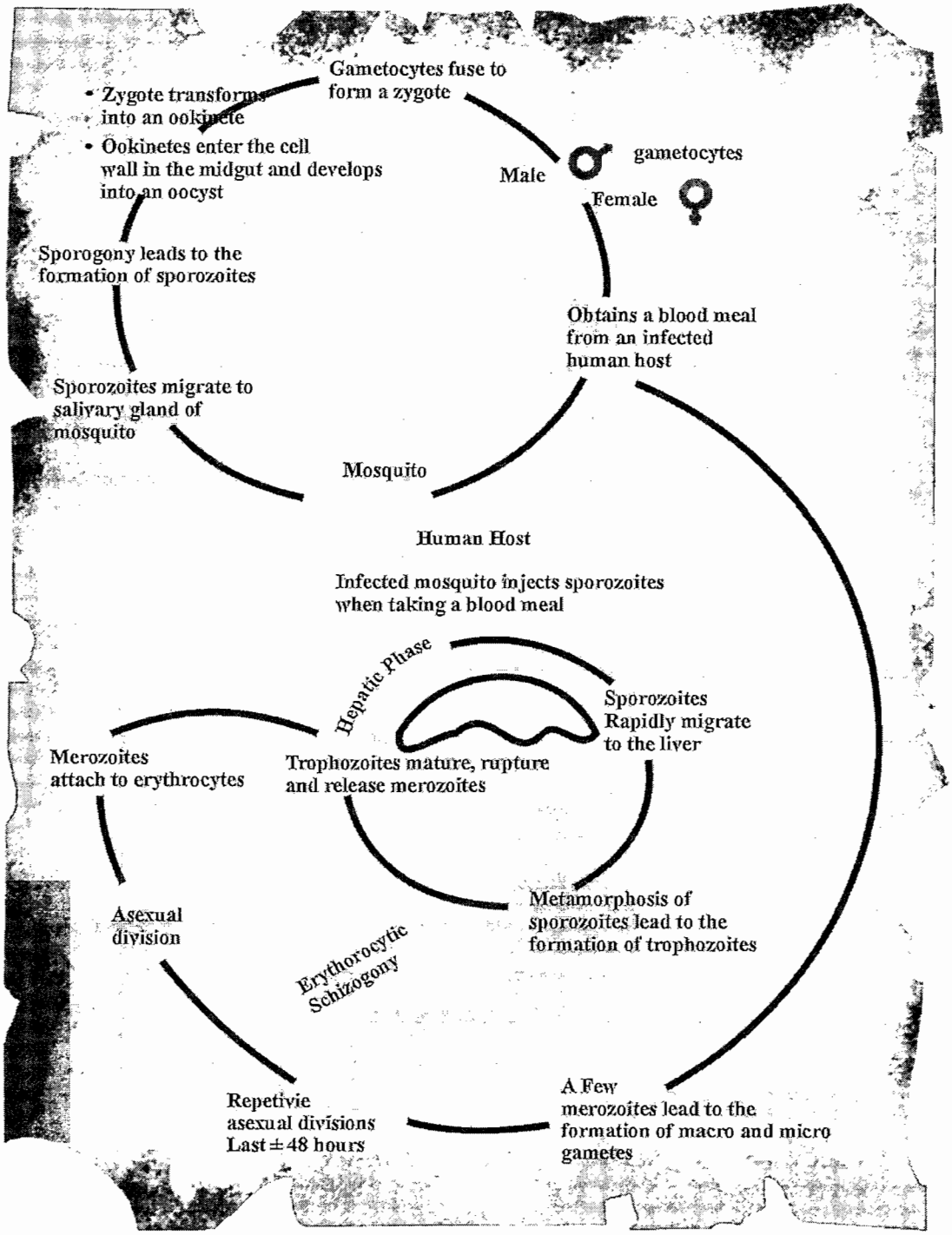


**FIGURE 1.2: Life cycle of the *P. falciparum* parasite**

(Adapted from Tuteja, 2007; Wolmarans, 2004).

### 1.3.1.1 Tissue schizogony (hepatic phase)

Mobile, infective sporozoites from the salivary gland of the *Anopheles* mosquito are injected into the human host once the vector obtains a blood meal (Tuteja, 2007; Wolmarans, 2004).

Within one to two days after invasion, these sporozoites reach the hepatocytes where they undergo metamorphosis and change into a trophozoit or tissue schizont (Tracy & Webster, 2001; Wolmarans, 2004). Pre-erythrocytic schizogony takes place when these trophozoites mature and give rise to tens of thousands of merozoites, each capable of invading a red blood cell once released from the liver (Tuteja, 2007; Wolmarans, 2004). The time taken to complete tissue schizogony in the case of *P. falciparum,* is 8 - 25 days (Tuteja, 2007).

#### 1.3.1.2 Erythrocytic schizogony

Specific receptors are involved during the attachment of the merozoites to the erythrocyte membrane (Tuteja, 2007; Wolmarans, 2004). Subsequent to attachment, asexual division is initiated and the parasite develops through different phases in the erythrocyte. The early trophozoit is frequently referred to as the 'ring form' (Tuteja, 2007). Multiple rounds of nuclear division lead to the formation of schizonts which, upon reaching maturity, contain around 20 merozoites each. After lysis of the erythrocyte these merozoites are released to invade further uninfected red blood cells (Tuteja, 2007). During lysis of the erythrocyte, metabolic waste of the parasite and hemosoin is also released. Hemosoin is the end product of hemoglobin digestion by the parasite. The released metabolic waste is one of the features responsible for the clinical manifestations of malaria (Wolmarans, 2004). Following repetitive asexual divisions, lasting approximately 48 hours in the case of *P. falciparum*, some merozoites once again invade erythrocytes and lead to the formation of macro-and microgametocytes (Tuteja, 2007; Wolmarans, 2004).

#### 1.3.1.3 Sexual phase of *Plasmodium falciparum* in the mosquito

When obtaining a blood meal from an infected human host, a female *Anopheles* mosquito may ingest male and female gametocytes into its midgut. After combining, the gametes undergo fertilization and form a zygote (Ashley *et al.,* 2005; Tuteja, 2007). The zygote finally transforms into an ookinete, which enters the wall of a cell in the midgut and ultimately develops into an oocyst. Within the oocyst, sporogony leads to the formation of multiple sporozoites which migrate to the salivary gland for onward transmission when the oocyst bursts. The mosquito remains infective for 1 - 2 months and when biting a susceptible host sets the *Plasmodium* life cycle into motion (Tuteja, 2007).