

## CHAPTER 1

### *GENERAL LITERATURE REVIEW*

Malaria (literally meaning "bad air", which refers to the old theory of the miasmatic origin of the disease) is one of the serious environmental diseases of man (1). Malaria is caused by a unicellular parasite of the genus *Plasmodium* and is biologically mediated through the bite of a female *Anopheles* mosquito (2). During the complex life cycle in man, the malaria parasite enters the the blood stream, infiltrate the liver and eventually infects the erythrocyte and undergoes rapid growth and development. The release of merozoites leads to a new wave of infection. The asexual blood stages are responsible for the clinical manifestations and complications such as anemia, disseminated intravascular coagulation syndrome (DIC), electrolyte disturbances and multi-organ failure (3).

#### *1.1 Diagnosis of malaria*

The first detection of the malarial parasite in an infected patient's blood was reported by Lavaren, a French surgeon in 1880 (4). Optical diagnosis was improved by Romanowsky in 1891 and the thick film technique was introduced by Ross in 1903 (5). Presently a microscopic examination of thick films, stained with giemsa can detect a 0,0004% parasitemia at 1000 times magnification in 30 to 60 minutes (5). A newly developed rapid diagnosis is based on staining the parasites with acridine-orange and detection of the parasites using fluorescence microscopy (6). This technique may replace the thick blood smears if a fast diagnosis is needed and no species identification of the parasite is required. Microscopical diagnoses are not always practical in a Third-world context due to lack

of microscopes and experienced technicians to use them. Alternative methods, like DNA probe-hybridization may be useful, particularly if large scale screening is required (7). However, the expense problems with disposal, poor field applicability and the fact that final results are available only after a few days (if hybridizations are visualized on a photographic plate) renders this method impractical (7). Serology, e.g. using the ELISA method, can be used for the screening of a large number of samples. This technique can be used in a well-equipped laboratory as well as under field conditions. The antigenic diversity within the same parasite species and the fact that few antigenic determinants are shared in geographically distinct parasites, leads to standardization problems which have not yet been solved (8). A good test should be specific, sensitive, accurate, rapid, simple to learn and cheap. Presently, no tests comply to all of these criteria (8).

## *1.2 Epidemiology*

Presently more than 2,5 billion people are living in malaria areas, mainly in Third-world countries with little or no measures to control the disease. *Plasmodium falciparum* is annually responsible for more than one million deaths of children under 5 years of age in Africa alone (9). In South Africa the geographic range of malaria embraces the tropical and sub-tropical regions. There is a small endemic area on the border next to Mozambique. The rest of the region stretches from Mtubatuba next to the Great Umfolosi on the east coast at the border of Mozambique. East from the Drakensberg and north from the Soutpansberg malaria is unstable with rapid seasonal epidemics. The area around the Limpopo in the north-west and around Upington are epidemic malaria areas. In Fig.1.1 the distribution of malaria in Southern Africa is illustrated.

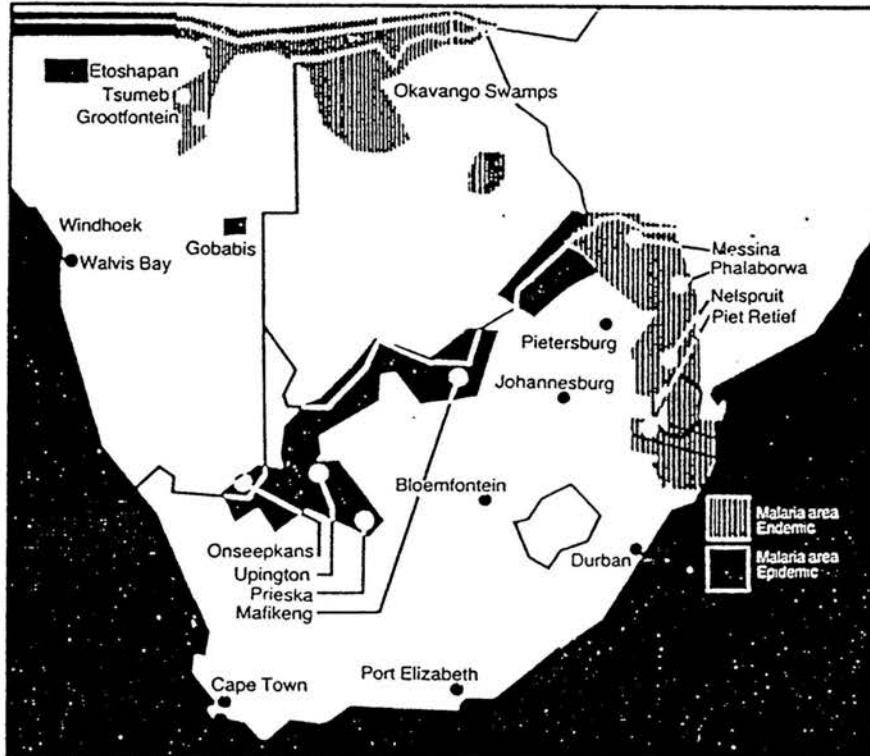


Figure 1.1 Malaria areas in Southern Africa (8).

Over the last decade (1980-1990) there has been an upsurge in malaria which can be attributed to a number of factors. Natural factors such as increased rainfall, increases the *Anopheles* mosquito's breeding sites, while the influx of refugees who harbor the malaria parasite, the increased incidence of drug-resistant malaria parasites and resistance of the vectors against insecticides are man-made problems (10). The number of districts that have incidence rates greater than 10 % have increased

yearly since 1986 (10). This increase in malaria notifications is shown in Fig 1.2.

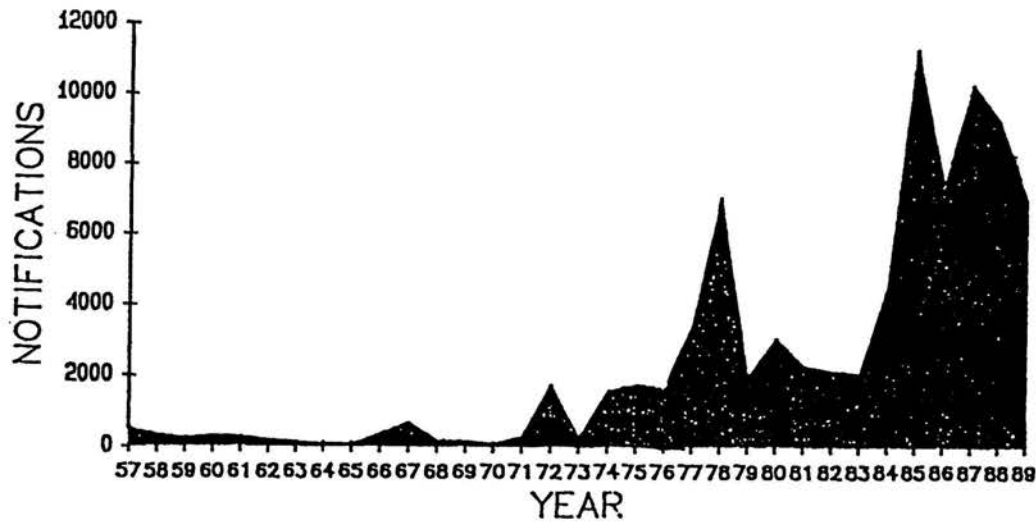


Figure 1.2 Malaria notifications in South Africa from 1957 to 1989 (10).

The re-organising of the active surveillance in 1975 led to a higher recorded incidence rate, but over the last decade in particular, a major increase was observed.

Malaria mostly occurs naturally through the exposure of a person to the feeding of an infected female *Anopheles* mosquito which transfers the malaria parasite among human victims (2).

### ***1.3 Life cycle of the malaria parasites.***

More than a hundred *Plasmodium* species exist of which only four are known to infect man (11). The malaria parasites are protozoa from the class sporozoa and genus *Plasmodium*. The life cycle of the four malaria

species are identical. Two processes, schizogony and sporogony, are distinguished. Schizogony occurs in the human host and can be divided into the liver (tissue) and the blood stages. Sporogony starts in the host but ends in the vector (11).

Infection begins when a malaria-parasite-carrier mosquito injects sporozoites into the host during a bloodmeal. Sporozoites inoculated from the salivary glands of the mosquito, migrate to the liver where differentiation is initiated inside the hepatocytes and completed within 10-48 hours (11). Merozoites are released from the hepatic cells into the cardiovascular system. After infection of erythrocytes, the parasite will differentiate into the ring form, a name derived from the appearance of the Giemsa-stained parasite due to a ring of cytoplasm surrounding a large vacuole next to the deeply-stained nucleus. The parasite, feeding upon the hemoglobin of the host erythrocyte, deposits grains of pigment (haemozoin) in the cytoplasm and develops in an uninucleate trophozoite (12). The nucleus subsequently divides several times giving rise to a schizont. The erythrocyte containing mature schizonts ruptures and merozoites emerge. The merozoites have a short extra-cellular life of a few seconds during which they invade new erythrocytes by attachment to and invagination of the erythrocyte membrane. After a few cycles of schizogony the merozoites released from the erythrocytes changes into micro- or macrogametocytes (male or female). The rest of the cycle is completed inside the vector where macrogametocytes are fertilized by microgametocytes and form zygotes. The zygotes differentiate into ookinetes and eventually into sporozoites which migrate to the mosquito's salivary glands. Infection of a new host is now possible with the next bloodmeal (13). In Fig 1.3 the malaria life cycle is summarized.

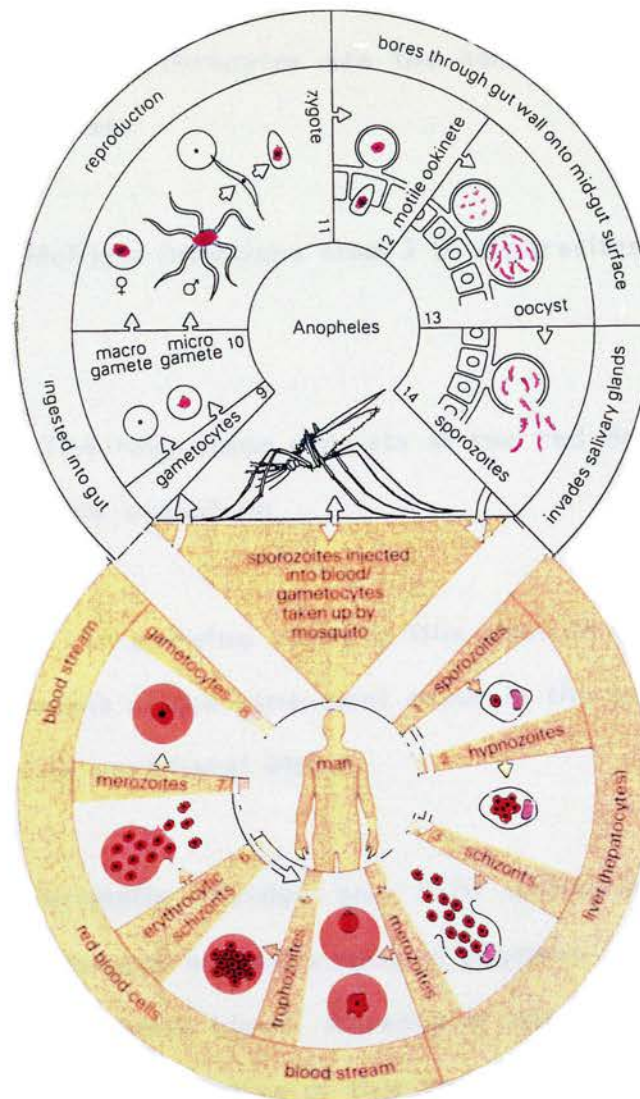


Figure 1.3 Life cycle of *Plasmodium* species (13).

#### 1.4 The different malaria species.

Although the malaria parasites all belong to one genus, there are morphological differences in the life cycles of the different species. Even in the same species genetic variants are possible. The main differences between the 4 most important strains are as follows:

**(A) Plasmodium falciparum.**

**Erythrocytes:** Infected erythrocytes are the same size and colour as non-infected erythrocytes.

**Multiple infections:** Multiple infections from 2 to 6 parasites per cell have been recorded.

**Young trophozoites:** The ring stage consists of two red dots of chromatin and a thin circle of blue cytoplasm.

**Growing trophozoite:** The growing parasite fills the cell and Schuffner's dots (small, red pigments of the same size) occur in the cytoplasm. This stage is rarely found in peripheral blood.

**Schizonts:** The chromatin divides and 8-20 merozoites may form. The merozoites are arranged around a mass of pigment. These forms are also rarely seen in peripheral blood. Absence of late stage parasites in thick smears of patients is indicative of this type of parasite.

**Gametocytes:** The immature gametocyte is oval-shaped and lies to one side of the erythrocyte. The chromatin is pink and scattered among the pigment granules in the ectoplasm. As the mature gametocyte becomes concavely shaped the pigment granules gather in the middle half of the parasite. The immature macrogametocyte is spindle-shaped and only a small part of the erythrocyte can be seen at the edges of the parasite. The mature macrogametocyte is long and thin with chromatin granules concentrated in the middle of the parasite.

**(B) Plasmodium vivax.**

**Erythrocytes:** The infected erythrocyte is usually larger and irregular in shape compared to non-infected erythrocytes. The cell may be paler than non-infected erythrocytes, and Schuffner's dots can be seen in these cells.

**Multiple infection:** Common and easily seen in the ring stage.

**Young trophozoites:** The "signet" ring stage consists of a large red stained chromatin dot on a blue ring of cytoplasm. The ring is usually one third of the diameter of the erythrocyte. The cytoplasm is slightly broader opposite the chromatin dot. Small pseudopoda may develop in this stage and extend in various directions.

**Growing trophozoites:** The cytoplasm and the pseudopoda expand and small yellow-brown pigment granules form in the erythrocyte as the parasite digests the hemoglobin.

**Schizont:** The chromatin material divides and produces 12-24 separate particles. The pigment in the erythrocyte forms one or two conglomerates.

**Gametocytes:** Young gametocytes are round and the chromatin is centrally situated near a vacuole. The mature microgametocyte has a pale cytoplasm, a mass of chromatin that stains pink to dark red and pigment granules which are scattered inside the parasite. The macrogametocyte is larger and oval-shaped. The dark red chromatin lies near the edge of the parasite membrane and is free of vacuoles.



**(C) Plasmodium malaria.**

**Erythrocytes:** Infected erythrocytes are the same size or smaller than non-infected erythrocytes. Occasionally small red dots known as Ziemann's dots (irregular shaped red pigment) may be seen in the cytoplasm.

**Multiple infection:** This is rarely seen in this species.

**Young trophozoites:** In the ring stage the red chromatin dot is round or oval and the cytoplasm is small. The ring is about one fourth of the diameter of the erythrocyte. As the parasite ages, the cytoplasm elongates and forms a band across the erythrocyte with only a few pigment granules.

**Growing trophozoite:** The parasite grows larger and has a characteristic band form. The pigment may be scattered or arranged along the edge.

**Schizont:** The chromatin divides into 6-12 small chromatin masses in a rosette around a pigment conglomerate.

**Gametocytes:** The mature microgametocyte almost fills the erythrocyte and contains a round or an oval-shaped chromatin mass. The macrogametocyte is larger and has an oval-shaped chromatin mass which lies to one side of the erythrocyte with scattered pigment granules.

**(D) Plasmodium ovale**

**Erythrocytes:** The erythrocytes are larger than non-infected erythrocytes and are oval- or egg-shaped. The edges of the erythrocyte are commonly fimbriated and Schuffner's dots are abundant.

**Multiple infections:** This only occurs if the infection rate is high, but it is not a common phenomenon.

**Young and growing trophozoites:** The rings are small, about one third of the erythrocyte diameter. The cytoplasm is dark and pigment granules may form inside the erythrocyte.

**Schizont:** The roundly shaped parasites usually divide into 8 merozoites which rosette around the pigment mass in the center.

**Gametocytes:** They are similar to *Plasmodium malaria* although they appear smaller in the enlarged infected erythrocyte. A large amount of Schuffner's dots are present which assists with the classification of the parasites.

Fig.1.4 shows the bloodstages of the four stains of malaria that infects man.

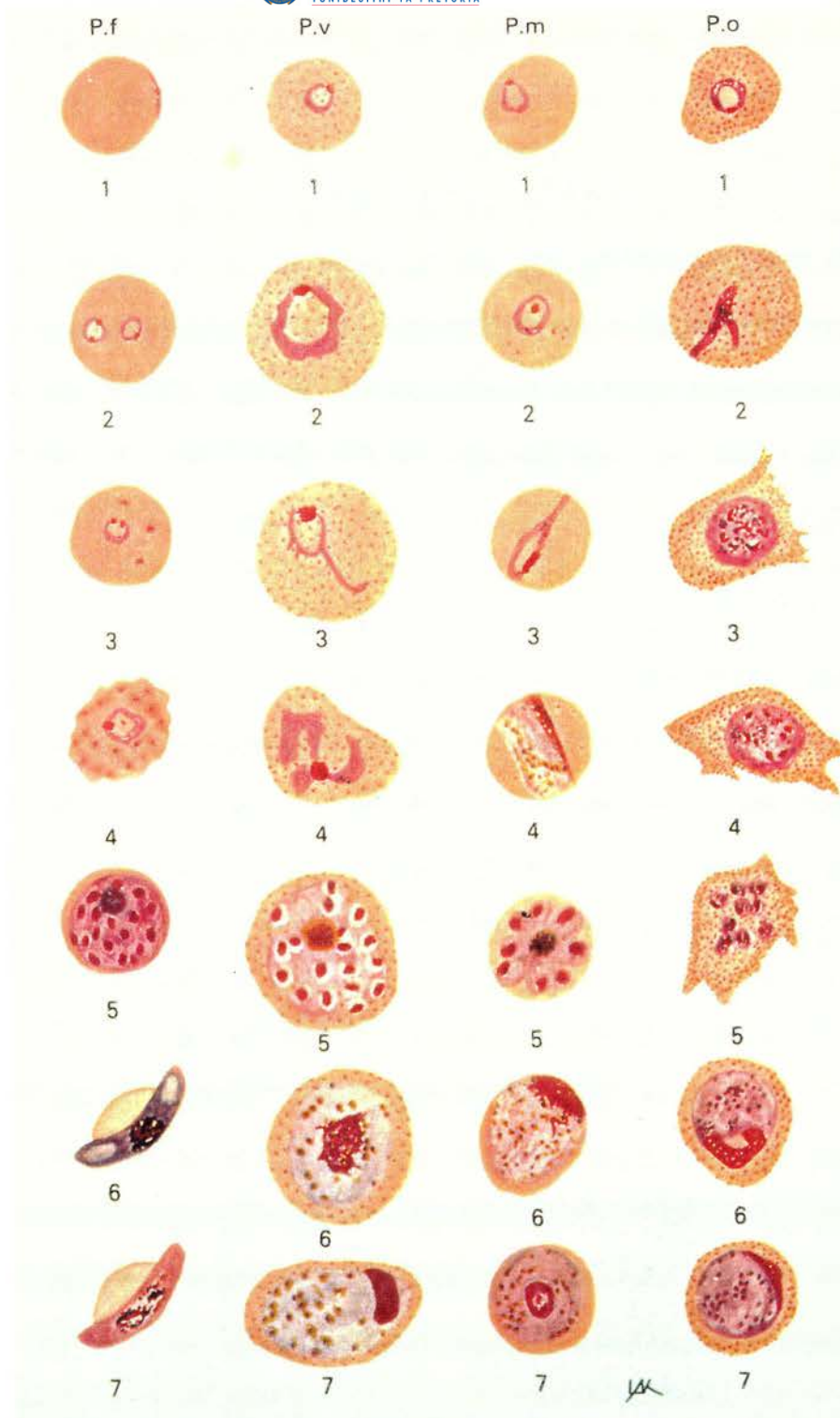


Figure 1.4 Comparison between the bloodstages of human malaria parasite strains after Giemsa staining.

(*P.f.*) *Plasmodium falciparum*. (*P.v.*) *Plasmodium vivax*  
(*P.m.*) *Plasmodium malariae*. (*P.o.*) *Plasmodium ovale*..

1. Ring stages
2. Young Trophozoite stage
3. Trophozoite stage
4. Young schizont stage
5. Schizont stage
6. Mature microgametocyte
7. Mature macrogametocyte

Taken from (39).

## 1.5 Pathology of malaria

### 1.5.1 Parasite induced membrane changes in the erythrocyte.

Electron microscopy of infected erythrocytes obtained from tissue culture reveal extensive changes of the membrane as well as lesions, depending on the infection stage (14). The lesions to the membrane can be attributed either to the merozoite penetration in the newly infected erythrocyte, or to the presence of mature trophozoites. The entry of the parasite into the erythrocyte is ordered and can be divided into 5 steps: (a) Merozoite recognition of the erythrocyte membrane. (b) Attachment of the parasite to a susceptible erythrocyte. (c) Junction formation. (d) Penetration of the parasite into the erythrocyte. (e) Sealing of the membrane after entry. These steps lead to a merozoite enclosed in a parasitophorous vacuolar membrane which differs from the erythrocyte membrane. The vacuolar membrane doesn't have spectrin and is initially almost devoid of intramembranous particles (15).

Export of malarial proteins from the parasitophorous vacuole membrane to the erythrocyte membrane involves diverse subcellular structures, or organelles (16). From electron microscopic evidence it seems that many of the membrane structures originate either from the parasite or the parasitophorous membranes via budding (16). According to Howard *et al.* (16) newly synthesized proteins from the intracellular *P. falciparum* parasite are translocated across the rough endoplasmic reticulum (RER) yielding asymmetrically-integrated proteins. The vesicle formed from the RER fuses with the parasitophorous plasma membrane (PPM) followed by subsequent blebbing from the PPM, fusion with the parasitophorous vacuolar membrane (PVM) and again blebbing from the PVM to reach the

erythrocyte cytoplasm on its way to fuse with the erythrocyte membrane (17). Parasite induced structures include single membrane clefts (Maurer's clefts) and electron-dense spheres. Maurer's clefts are diverse in shape and consist of erythrocyte membrane-bounded vesicles with a lumen of very low electron density (16). Spheres are electron-dense circular bodies which contain parasite proteins inside and on the surface of the organelle. These proteins are not expressed on the surface of the erythrocyte but play a structural and/or functional role in the protrusions found on the host erythrocyte membrane in certain *P. falciparum* species (16). PfHRP1 (*P. falciparum* histidine rich protein) are found inside the cytoplasm of the host erythrocyte which led to the hypothesis that it may be involved in the ferrying of other proteins from and to the erythrocyte surface. According to Sherman and Vinograd there is no biochemical evidence and little precedent for such trafficking, but it is not to say that it doesn't exist (18). If these structures are seen as part of the parasite organelles, then this is an extraordinary process in which an organism modifies its external environment, by releasing organelles into the host cytoplasm surrounding it (19).

*P. falciparum* and *P. malariae*-infected erythrocytes exhibit electron-dense excrescences or so called knobs on the surface of the erythrocyte membrane (20,21). Although there is much controversy surrounding this point, it is commonly believed that the knobs are proteins of parasite origin (22). The knob density increases during intracellular maturation of the parasite (21). It has also been observed that the distribution of knobs on the surface of the infected cell is not a random event. This implies that the knobs are produced in certain areas which may represent specific domains on the erythrocyte. The knobs are electron-dense,

conically-shaped, measuring 90-100nm in diameter and 30-40nm in height (21). It was also observed that not all the *P. falciparum* strains exhibit knobs on the erythrocyte surface (22). Loss of ability to produce knobs after lengthy culturing was noticed by Langreth *et al.* (22). By comparing the knob (K<sup>+</sup>) and knobless (K<sup>-</sup>) strains of *P. falciparum*, It was observed that the knobless parasites lacked a unique histidine-rich protein which occurs in the electron dense cone which is bound to the skeleton of the erythrocyte (23). Considering the fact that the knobs appear only in the late stages of trophozoite and schizont stages and that only ring forms can be seen in bloodsmears from infected patients, these knobs are thought to mediate the binding of the cells to the capillary endothelium (21). The role of the knobs in sequestration of infected erythrocytes is not understood. It may play a role in endothelial adherence, although not all strains with knobs adhere, while some knobless strains do when evaluated *in vitro* (21).

Nearly a dozen proteins have been localized in regions near the knob, but only PfEMP 1, a protein with a molecular mass of >240 kDa has been claimed to be exposed on the surface of the infected erythrocyte. According to Sherman and Winograd alterations in Band 3, the principle protein of the erythrocyte, contributes to changes in the antigenicity of knobby cells (18), as not a single monoclonal or monospecific polyclonal antiserum has been developed against the PfEMP1 protein and difficulty is experienced in cloning the gene for PfEMP1. Moreover Band 3 is clustered in the regions of the knobs and knobby cells show an increase in binding to naturally occurring anti-Band 3 auto-antibodies.

Sherman and Winograd thus claim that the changes to the erythrocyte membrane proteins are parasite-induced changes to host proteins (18). This is contradictory to Newbolt and Marsh who concluded that modified host and neo-antigens (contributed by the parasite) co-exist on the erythrocyte surface (19). However, alteration to the erythrocyte membrane after invasion by *P. falciparum* parasites leads to changes in morphological and physiological properties (20).

The extent and the nature of the parasite-induced changes in the host erythrocyte membrane and cytoplasm suggests that a new entity is created with the "fusion" of the two interacting cells (malaria parasite and host erythrocyte). This new entity has special properties of both cells lacking in the original component parts. When one considers the intimate relationships in nature among symbiotic viruses, unicellular prokaryotes and eukaryotes and multicellular organisms this concept is not unusual.

### *1.6 Drug resistance*

Using all the metabolic skills it has, the malaria parasite constantly adapts to its changing surroundings induced by the hosts immune system and the taking of antimalarial drugs as cure against the infection. While the seemingly limitless genetic ability of the parasite can be attributed for the multitude of drug-resistant mutants that have appeared, misuse of the drugs by man is much to blame (23).

In the 1960's chloroquine resistance in *P. falciparum* was reported for the first time (9). Twenty years later more than forty countries including South Africa face the same problem. In 1986 widespread resistance was

reported against sulphonamides and mefloquine, which is a second line drug treatment when chloroquine resistance is encountered (9). This led to the utilization of more toxic drugs ( Fansidar) giving a 1 / 20 000 mortality rate (9).

Resistance is " the ability of the parasite to survive and/or multiply despite the administration and absorption of a drug given in equal or higher doses than usually recommended but within the limits of tolerance of the subject" (23). Evaluation of drug resistance can either be tested *in vivo* or *in vitro*. In the *in vivo* method the levels of blood stage parasites are monitored after the administration of a normal curative dose of the antimalarial drug (23). In this thesis a survey of drug resistance was made using the *in vitro* assay.

### *1.7 Immunology and vaccine development*

Attempts to eradicate malaria using parasitocidal drugs and insecticides have only been partially successful and are now further hampered by the growing resistance against anti-malarial drugs (25). Inherent immunity against infection with *Plasmodium* does not exist, with one exception. Some rural Africans, lacking the Duffy antigen on the erythrocytes are highly resistant to *P vivax*. Certain enzyme deficiencies, eg. glucose-6-phosphate dehydrogenase and other genetic diseases like sickle cell anemia and thalassaemia, provide protection against infection with malaria (26). Experimental studies show that induced immunity against blood stages are short-lived, requires constant boosting for the maintenance of immunity and is much less effective against cloned than heterogeneous strains of parasites (25).



Protective immunity is usually parasite species specific, although cross reactions has been known to occur between rodent malaria species (27). The effectiveness of the response to an early infection with the malaria parasite may be limited to the intrastrain antigenic variants which are causing the infection (27).

Sporozoites entering the cardiovascular system of the host disappear rapidly, but their exact fate remains unknown in normal and immune hosts. However, the kuppfer cells appear to be the distal point of entry for

development in parenchyma cells (27). Circum-sporozoite protein (CS) the major protein on the sporozoite surface coat, can induce an immune response as is seen in endemically infected human populations. Vaccination with various attenuated sporozoites has been effective in avian malarias but not in human malaria. The reason for this is unknown (27). Research into the immune response to gametocytes has been largely neglected in comparison to the other parasite stages. Gametocytes may persist in the host for some time after clearance of the asexual stages, but their infectivity for mosquitoes decline (27).

The antigenicity of erythrocytes infected with the asexual blood stages of the parasite are demonstrated by the opsonization, agglutination and possible sensitization for destruction by antibody-dependent lymphocytes (27). At present it is not known how intra-erythrocytic parasites are killed *in vivo*, although phagocytosis of parasite infected erythrocytes by splenic and liver macrophages are evident in the immune host (28). CD8<sup>+</sup> T cells are activated by CD4<sup>+</sup> T cells in response to the malarial antigens and these CD8<sup>+</sup> T cells are either directly cytotoxic, and/or

secrete toxic cytokines. In doing so, CD4<sup>+</sup> T cells control the parasite numbers, ensuring the survival of both the host and the parasite (27). However, the appearance of crisis forms of parasites in the blood could not be accounted for, until Pouvelle *et al.* observed that IgG molecules have access to the intra erythrocytic parasite via a parasitophorous duct (28). Until this discovery trophozoite and schizont surface antigens were never considered important as potential antigens for vaccine development. Using these parasitophorous ducts, toxins may be coupled to anti-parasitic antibodies, to home in at the parasite inside the erythrocyte (28).

The malaria parasite undergoes adaptive antigenic variation (29). Antigenic variation is divided into four categories: (a) The parasite is able to successively produce large quantities of different immunodominant surface antigens, possessing no shared determinants, to evade the immune response. Examples are the Circumsporozoite protein (CSP), merozoite surface antigen 1 (MSA1) and MSA2. An unusual finding in *Plasmodium* is that most antigens are immunodominant tandem repeats of oligopeptide sequence (30,31). (b) The parasite is able to switch the presenting variant specific antigen before the entire parasite population are wiped out by antibodies. This switch can be antibody-induced or spontaneous, resulting in a constant low rate of switching to create a small heterotype of parasites that will survive the specific host response better than homotypic parasites (30). This implies that the introduction of new forms of immune pressure (anti-malarial vaccines) may induce new variants, as has already been observed under laboratory conditions (31). (c) Expression of surface antigen genes in a sequential order to avoid gross population heterogeneity and rapid induction of antibody formation

against all the surface antigens. Parasites will continue to express the variants in the same sequence, even if they are transferred to another host. However transfer to an antibody-containing, variant specific host induces switch to the next variant in the sequence. This implies that the expression of antigens of a particular variant is predetermined, but is modulated by the immune milieu of the host. Chronically infected animals acquire an overall parasite immunity and their sera appear to recognize the transcended range of variant antigen expression. By sequentially expressing different gene products, a single infection produces a series of serologically distinct phenotypes of parasites. This strategy is used by the malaria parasite apparently to outpace the immune system of the host (31). (d) The parasite needs to feed (inside the erythrocyte) mate (in the vector) and absorb to specific targets without exposure of it's non-variant antigens (32). Furthermore by presenting immunodominant antigens on the surface that can be alternated, the parasite creates decoys to divert the immune response away from the constant inner-surface elements (31).

Various surface antigens like circumsporozoite protein (CS) from sporozoites, *Plasmodium* merozoite major surface antigen (MSA) and ring-infected erythrocyte antigen (RESA) are presently used to develop vaccines against the malaria parasite (33). None have yet proved to be effective for use (34). It is important to guard against the impression that an effective vaccine will eradicate malaria. Many good vaccines presently available are not widely used, especially where they are needed most, due to their cost and some Third world traditions (33).

### 1.8.1 Carbohydrates

Glucose is the main source of carbohydrate used by the malaria parasites (35). Constant supply of this metabolite is important for parasite survival because none is stored. Infected erythrocytes utilize 10 to 100 times the amount of glucose compared to non-infected erythrocytes (35). The plasma membrane of the asexual stages of *Plasmodium* seems to possess an active transport system for D-glucose consisting of a carrier mediated D-glucose co-transport mechanism which utilizes ATP (35). Other carbohydrate substrates utilized by *Plasmodium* include mannose and fructose by rodent parasite species.

The metabolites from glucose utilization vary between species and in many cases 10-20 % of the end products are not accounted for (35). The amount of glucose conversion to lactic acid seems to depend on the species and the culture conditions. Results indicate that no complete citric acid cycle exists in *Plasmodium* for only malic acid dehydrogenase could be identified in rodent and avian malaria parasites. No pentose phosphate cycle enzymes were identified either and it seems that ribose-1-phosphate and free base are obtained from ATP catabolites in the host erythrocyte (35).

### 1.8.2 Amino acids.

According to Sherman *et al.* intraerythrocytic parasites can either utilize the hemoglobin or free amino acids from the host amino acid pool (35). Infection of the erythrocyte by the malaria parasite destroys most of the energy coupled transport systems. It seems that free amino acids enter

the erythrocyte via a concentration gradient with the parasite acting as a metabolic sink. Carrier-mediated transport is therefore only required for the highly charged molecules (35). The metabolized hemoglobin is stored inside the parasite as a brown pigment (haemozoin) conglomerate consisting of ferriprotoporphyrin IX coupled to a parasitic polypeptide (35).

### 1.8.3 Nucleic acids

In the early 1940's it was shown that malaria parasites contained DNA by using Feulgen stain (36). According to Kemp *et al.* the parasite has 14 chromosomes consisting of about 60 genes. The RNA content is about 5 times greater than the DNA content and is mostly localized on abundant cytoplasmic ribosomes in the intraerythrocytic parasite (37) .

Purines, in contrast to pyrimidines, cannot be synthesized *de novo* in *Plasmodium* and are therefore salvaged for nucleic acid synthesis (37). Adenosine, inosine and hypoxanthine are taken up in relation to the stage of the parasite, whereas ATP, AMP and IMP are taken up in limited amounts (34). Apparently thus, exogenous sources of purines are needed for nucleic acid synthesis. The erythrocyte purine pool, of which 80% exists in the form of ATP, is probably the main source. Indeed, according to Yamada and Sherman , up to 25 % of the purine requirements can be obtained from the erythrocyte pool with a concomitant sharp decline of the ATP level in infected erythrocytes (37).

In both the erythrocyte and the parasite, purine interconversion enzymes were identified and Yamada and Sherman proposed that adenylate nucleotide catabolism is towards the formation of hypoxanthine and that

the hypoxanthine present in the cytosol of the erythrocyte is taken up and consumed by the parasite (37).

The salvage pathway for purines are illustrated in Fig 1.5.

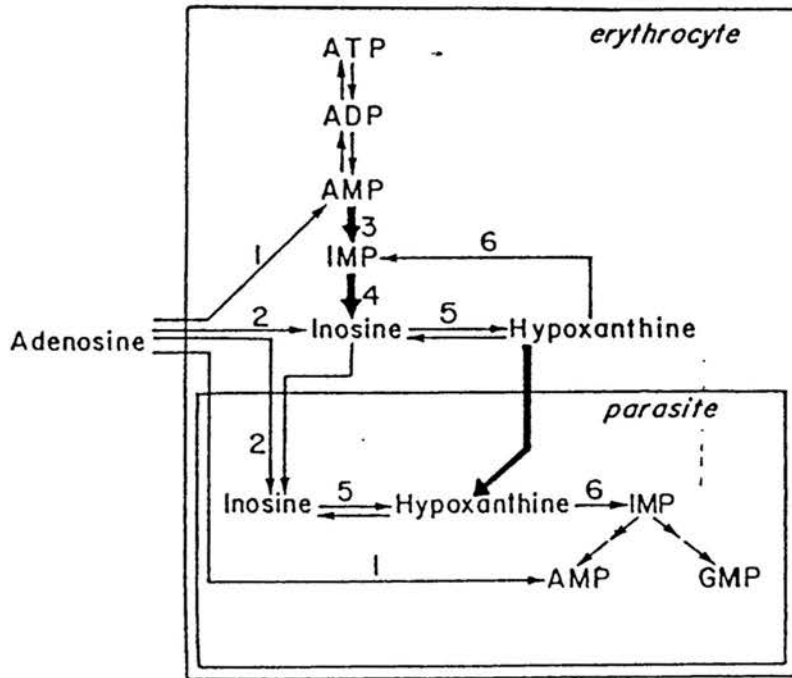



Figure 1.5 Purine salvage pathway in the erythrocyte and *Plasmodium*. 1. adenine kinase 2. adenosine deaminase 3. 5'AMP deaminase 4. 5'IMP deaminase 5. inosine nucleoside phosphorylase 6. hypoxanthine phosphoribosyl transferase (35).

### 1.9 Continuous culture of *P. falciparum*.

The first continuous culture of the blood stages of *P. falciparum* was achieved in 1976 by Trager and Jensen, who thereby revolutionized research on malaria (38). The relative slowness with which the techniques for the cultivation have been developed is mainly due to the inadequate knowledge of the biochemistry of the parasite, the host erythrocytes and their constituents (35). The parasites are dependent on the hosts erythrocyte which is demonstrated by their ability to invade and multiply only inside the erythrocyte. The integrity of the erythrocyte seen in relation to the supporting medium is of primary importance. The culture

system provided large amount  for DNA and RNA isolation and metabolic experiments for research on the parasite for lethal malaria in man (7). The continuous *in vitro* culture of the sporogonic stages has not yet been achieved, although it seems that all the stages of sporogony are capable of being supported *in vitro* (39, 40).

The failure to eradicate malaria during the 1950 world campaign and the upsurge in malaria over the last few decades led to a revised strategy for malaria control. The inadequacy of the prevalent methods to contain the disease, highlights the need for a malaria vaccine (8). Vaccines, which are being tested at present, show some problems but the knowledge gained on the diversity and escape routes of the malaria parasite, should eventually lead to a method to control the disease.

### *1.10 Objectives*

The first and foremost objective of the study was to establish long-term *in vitro* cultures of *Plasmodium falciparum* as a source of antigenic material for immunochemical studies. Wild isolates were obtained from malaria-infected patients during a national survey aimed at recording the distribution of resistance to chloroquine and its potential substitute, mefloquine (Chapter 2). Due to the initial difficulties experienced in establishing wild isolates in culture, investigations were undertaken to identify factors which may limit parasite growth. The influence of the gas composition of the medium and changes in the concentrations of metabolites and byproducts on parasite growth, received particular attention (Chapter 3). In order to determine antibodies specifically bound to membrane surface antigens or receptors, appropriate quantitative methods are needed. An existing micro-immuno-assay method was modified

and tested for its ability to detect the inverse relationship between thrombocyte count and thrombocyte-associated immunoglobulins of malaria-infected patients (Chapter 4). Lastly, different conditions for the fixation of malaria-infected erythrocytes were compared in order to select the most appropriate procedure. The aim was to investigate the ultrastructural detail of the parasite and provide a method to localize specific antigens *in situ* (Chapter 5).



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# *DRUG SUSCEPTIBILITY AND SHORT-TERM CULTURE OF Plasmodium falciparum.*

## 2.1 INTRODUCTION

Drug therapy provides a second defense front against malaria parasites after vector control (1). Eradication of the *Anopheles* mosquito is a sure way of controlling malaria infection. Campaigns undertaken in 1955 by the WHO using DDT had early success in Europe, North America and urban areas of tropical countries, but Africa, which is the central "hard core" of malaria, remained neglected (1). The eventual failure of the eradication campaigns were due to over-optimism of the potential of insecticides to eradicate the vector and inadequate understanding of the epidemiology (2). Safe and effective prevention and treatment of the victims of malaria are therefore contemporary priorities requiring constant research to assess and improve chemotherapy.

The continued long term use of a particular prophylactic such as chloroquine may eventually lead to complete resistance against this drug, by selection for resistant parasites. Chloroquine is the preferred drug for chemoprophylaxis even in areas where resistance towards the drug is becoming significant (3). The drug gave the best clinical results with the least complications. Quinine is also effective and safe but has a relatively short half-life in the body (10 h). In areas where resistance against chloroquine is pronounced proguanil is prescribed simultaneously

(4). The combination drug Fansidar (pyrimethamine and sulphadoxine) is also used in chloroquine resistant areas. This drug is taken once with the first signs of an infection because of its high toxicity. Mefloquine, a new drug which proved useful for prophylaxis is rather restricted to treatment of highly resistant malaria cases only (4). As drug resistance snowballs however, an effective vaccine against malaria is a growing need in the near future.

Continuous *in vitro* cultivation of *Plasmodium falciparum* opened up a new era in the development and evaluation of antimalarial chemotherapy (5). Large varieties of isolates can now be tested in a much shorter time. The amount of antimalarial drug used can be quantified and resistance to a variety of drugs can easily be determined. Using the principles of *in vitro* continuous culture for *P. falciparum*, a microtest was developed to test drug sensitivity (6). Standardized kits are now available for a large variety of drugs, e.g. chloroquine, amodiaquine, mefloquine and quinine. These are now widely used for the assessment of drug susceptibility of the parasites, and have replaced the use of the old macrotest. The major advantages of the microtest is that it is performed in microtiter plates which have been predosed, thus requiring less drug and allowing a large range of concentrations to be tested at the same time. Only 100 ul of blood is required which can be easily drawn from the finger (6).

As with most biological systems the *in vitro* technique also has some limitations or disadvantages. The most pronounced problem is the artificial time-course to drug exposure. The *in vitro* system does not allow for the fact that the chemotherapeutic drug is metabolised and/or changed and excreted. Another disadvantage of *in vitro* testing is the fact that some



of the effective antimalarial drugs are not selective and are toxic to the host. The *in vitro* system is also subject to artefacts such as drug adherence to the glass or polystyrene supporting medium (6). Although the test has been successfully used to test chloroquine and mefloquine resistance, it is not capable of determining susceptibility to combinations of sulpha-drugs and pyrimethamine, due to the high concentration of 4-aminobenzoic acid and folic acid in RPMI 1640 medium. Other tests have also been developed to test drug susceptibility. A 48 hour test, which is similar to the microtest, uses four times more blood and ten times more culture medium than the microtest and the medium must be supplemented with 10 % human serum. Although the test can evaluate chloroquine susceptibility it is mostly used for dihydrofolate reductase inhibitors like pyrimethamine using a special medium (6).

The first requirement for testing human antimalarial chemotherapeutic drugs is a growing malaria culture. Long term continuous culture will be discussed in Chapter 3, but the *in vitro* microtest requiring only short-term culture, will now be described.

### *2.1.1 Antimalarial drug classification and mechanism of action.*

Antimalarial drugs can be categorized according to the stage of the parasite they affect. Specific antimalarial drugs can affect more than one stage.

#### **i) Schizontocides.**

##### **(a) Tissue schizontocides used for causal prophylaxis.**

These drugs eliminate the parasite in the pre-erythrocytic stage.

Proguanil, pyrimethamine and antifol combinations and certain antibiotics are included in this group. From these compounds only proguanil and pyrimethamine are prescribed primarily for prophylactic use (8).

As typical examples of "antifols", this group of compounds inhibits the activity of dihydrofolate reductase. They are not plasmodicidal but plasmodistatic and are only used as prophylactic antimalarial drugs. Blocking of the asexual cycle leads to the accumulation of late stage parasites in infected cells which eventually disintegrate. Resistance to proguanil and pyrimethamine have been reported between one and six years respectively since first used in therapy in 1952 (9). Pyrimethamine is absorbed slowly and has a prolonged biological activity. High doses lead to folic acid deficiency which inhibits hematopoiesis (8).

**(b) Tissue schizontocides used to prevent relapses.**

These drugs eliminate the hypnozoites or dormant liver forms of *P. vivax* and *P. ovale*. In this group only 8-aminoquinoline derivatives such as primaquine are presently in use. Little is known on the mode of action of 8-aminoquinolines, especially why they are more effective against tissue stages than blood forms of *Plasmodia* (10).

**(c) Blood schizontocides used for clinical or suppressive cure.**

Antimalarial drugs such as quinine, chloroquine and mefloquine belong to the blood schizontocide group. Intra-erythrocytic stages of *Plasmodium* are susceptible to these compounds. Schizontocides are also used in suppressive therapy to merely reduce the parasite load. A lowering of the parasitemia may result in a clinical cure as was demonstrated with

*P. vivax* infections. These drugs are also used to eliminate *P. falciparum* and *P. malaria* infections.

Although chloroquine causes a number of effects that singly or in combination may indicate to its primary mechanism, the detailed process is not yet understood. From early work it was hypothesized that chloroquine interacts with the DNA and inhibits DNA and RNA polymerase (10). Another theory holds that blood schizonticidal antimalarials compete with the parasite protein(s) which form complexes with the lytic ferriprotoporphyrin IX (FPIX), an oxidation product of hemoglobin digestion, to prevent its sequestration as inert haemozoin crystals. These FP IX drug complexes are membranolytic and cause parasite death by abolishing internal membrane permeability (11). The increasing amount of resistance against chloroquine as antimalarial has led to the use of tetracycline and quinine as the choice antimalarial drug combination.

Antibiotics have a weak antimalarial effect but act synergistically with quinine (12). Clindamycin, lincomycin, rifampicin and co-trimoxazole also show *in vitro* anti-schizontocidal effects against malaria and can be combined with quinine (12). All these antibiotics acts as inhibitors of prokaryotic protein synthesis. From experimental evidence it is suggested that the mechanism of protein synthesis of malaria parasites is of eukaryotic nature. Thus the inhibitory effect of antibiotics on malaria parasites probably result from the inhibition of mitochondrial protein synthesis which may also explain the relative slow clinical effect of antibiotics like tetracycline (8).

ii) *Sporotocides*. These drugs ablate transmission of the malaria parasite by inhibiting the formation of the oocysts and sporozoites in the infected vector. Primaquine is the major drug with this type of mechanism. The "antifols" also exhibit a sporontocidal action in the mosquito stages of all species.

iii) *Gametocytocides*. These antimalarial drugs will eliminate or sterilize the gametocytes at certain stages of development. Primaquine has this type of action against *P. falciparum*. Chloroquine and quinine shows gametocytocidal action against *P. vivax* and *P. malariae* but not against *P. falciparum*.

In Fig. 2.1 some of the structures of the commonly used antimalarial drugs can be seen.

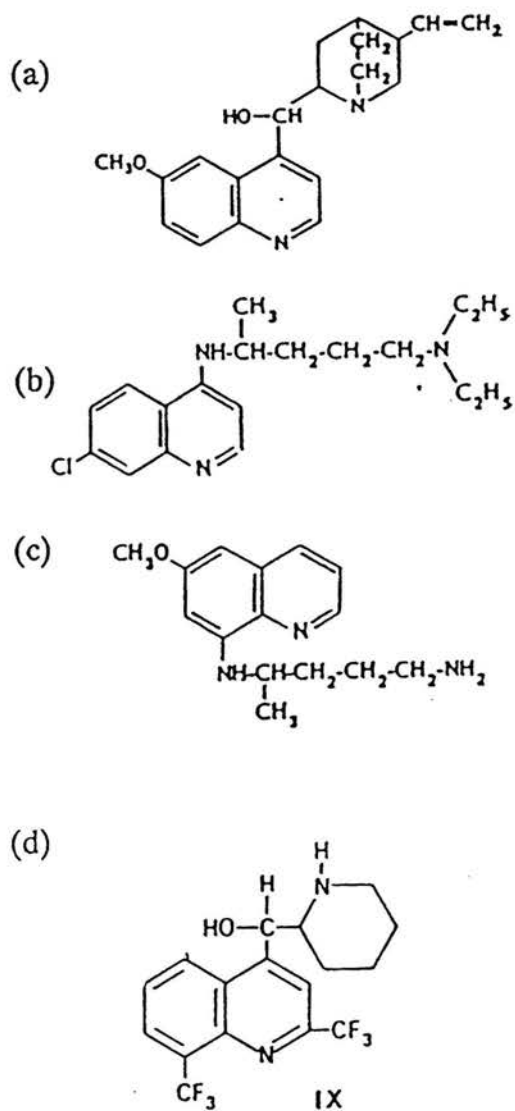


Figure 2.1 Structures of antimalarial compounds. (a) quinine (b) chloroquine (c) primaquine (d) mefloquine (WR 142490)

### 2.1.2 Chloroquine resistance.

Chloroquine accumulation in the acid vesicular compartment of the drug resistant parasites was found to be significantly less than in the susceptible parasites. Krogstad *et al.* (1987) showed that calcium channel blockers like Verapamil could reverse the resistant strain to be

chloroquine sensitive again (13). This led to the hypothesis that the mechanism of chloroquine resistance is based on a greatly increased drug efflux rate, a similar system to multi-drug resistance (mdr) in cancer cells. MDR protein, homologous to a family of ATP-driven transport proteins which act as transporters when overexpressed, expels drugs from the cell fast enough to protect against their toxic effects. Two genes (pfmdr1 and pfmdr2) were identified which coded for mdr proteins and it was found that some but not all resistant lines of parasites had amplified pfmdr1 genes and increased mdr expression. Foote *et al.* (14) found that sensitive strains can contain the mdr gene of "resistant" type sequence and concluded that other genes than pfmdr1 may be involved. A link between the mdr proteins coded for by the pfmdr1 gene and resistant parasites had been established but a more detailed understanding of chloroquine resistance requires that the other gene(s) are identified.

### 2.1.3 *New drugs.*

Research into new drugs such as the traditional Chinese remedy, qinghaosu, and mefloquine are important priorities, as new drugs are not common and malaria parasites have established resistance to most of the antimalarial drugs available (15). Mefloquine which is used in chloroquine resistant cases already proved ineffective to certain malarial species even before it was introduced. Arteether a derivative from qinghaoso seems most effective against the blood stages of the malarial parasite, but is short lived and needs to be taken several times a day (15).

#### *2.1.4 Objectives.*

The objective of the study was to test drug susceptibility, (chloroquine and mefloquine) in *Plasmodium falciparum* infected patients in the North-Eastern Transvaal area. This project was part of a national survey in 1988 to record the chloroquine resistance status and to evaluate the use of mefloquine in chloroquine resistant parasites.

## **2.2 MATERIALS AND METHODS**

### *2.2.1 Microtest.*

The microtest kit for the assessment of chloroquine and mefloquine sensitivity in *Plasmodium falciparum* growth *in vitro* (supplied by the World Health Organization) was obtained from Professor Fripp, University of Medunsa, PO Medunsa, 2024.

### *2.2.2 Samples.*

Venous blood samples were drawn in citrate/dextrose tubes from patients suffering from malaria. The blood was assayed as soon as possible, or maintained close to body temperature for a maximum of three hours, depending on the conditions of the field work. Persons that received chloroquine within the last 14 days were excluded from the test. The pre-selected patients were then subjected to a urine test for 4-aminoquinolines using Dill Glazko's solution, which indicates the use of prophylactic drugs and render the sample not useable.

### 2.2.3 *Blood smears.*

Thin and thick blood smears were made. A droplet of sample blood was placed on the microscope slide  $\pm$  1.5 cm from the frosted or marked section. The drop was smeared by using another microscope slide held at 45 ° angle. The smear slide was applied in front of the droplet blood allowing it to spread to the edges. The blood was then spread with a uniform forward stroke to cover 3/4 of the slide. After allowing time to dry, the cells were fixed with absolute methanol (E. Merck, SA Pty (Ltd), Midrand )

The thick smears were made by allowing a droplet of sample blood to dry on the microscope slide after spreading it with a matchstick.

### 2.2.4 *Giemsa staining.*

Giemsa stain was prepared fresh for staining of smears. Giemsa solution (500  $\mu$ l) was diluted in 10 ml sodium phosphate buffer (0.01 M) pH 7,2. After staining the smear for 30 to 40 minutes the slides were rinsed in distilled water. Thick smears were not fixed but stained as described for thin smears. Stained thick and thin smears were then examined for *Plasmodium falciparum* parasites using a Nikon type 104 light microscope.

### 2.2.5 *Dill Glazko's reagent (16).*

Eosin (50 mg) was transferred to a small glass-stoppered separating funnel. Chloroform (100 ml) and 1N HCl (1 ml) were added. The mixture was shaken for 3 minutes and the yellow chloroform layer was transferred to a brown glass-stoppered bottle.

Assay: To a small test tube containing 2 ml of urine, ten drops of the Dill-Glazko's reagent were added and mixed vigorously. A change from



yellow to violet red of the chloroform layer was taken as an indication of the presence of 4-aminoquinolines.

#### *2.2.6 Preparation of growth medium.*

The contents of 1 packet of RPMI 1640 powder (104 mg) were added to 10 ml sterile water and put in a Falcon tube (15 ml). To this was added 1 ml 7,2 % HEPES and 1 ml 2,4 % NaHCO<sub>3</sub> solution. The growth medium was sterilized by filtration through a 0,22 µm Millipore filter fitted onto a 20 ml syringe from which 0,9 ml aliquots were distributed in sterile 6 ml Falcon tubes for further use the same day (pH 7.4).

#### *2.2.7 Performance of the microtest.*

Blood taken from the *P. falciparum* infected patient in the field was processed as follows: Infected blood (100 µl) was added to one of the 6 ml Falcon tubes containing the growth medium. In each well of the appropriate columns of the chloroquine or mefloquine presealed microtiter plate, 50 µl of the blood/medium mixture was added. The plate was closed and gently shaken to ensure that the drug deposits in the wells were completely dissolved. The cultures were then placed in a desiccator with a paraffin candle and a flask containing copper sulphate solution. After the candle was lit, the desiccator lid was partially displaced, leaving only a small opening. The lid was closed just before the flame went out. The desiccator was subsequently placed in an oven (Labotec 382) at 37 °C and incubated for 24 hours. After incubation, as much as possible of the supernatant from each well was aspirated, thick blood smears were prepared from the residual contents of each well, stained for 30 minutes with Giemsa and subsequently viewed under a microscope. If the controls

revealed 20 or more schizonts of *P. falciparum* the culture was considered to be successful and the titration values interpreted as follows:

(a) Total inhibition (100%) of growth at 4.0 pmol chloroquine or mefloquine per well: Susceptibility to standard chloroquine, mefloquine treatment.

(b) Growth at 5.7 pmol or more chloroquine, mefloquine per well: Resistance of *P. falciparum* to chloroquine and mefloquine.

(c) Growth at 4.0 pmol chloroquine, mefloquine per well, but inhibition at 5.7 pmol: partial resistance, which may be reduced by treatment of the patient with higher dosages of the drug.

### ***2.3 RESULTS***

Of thirty two patients tested, 14 assays had to be abandoned due to too low parasite counts. Results from the eighteen patients are shown in Figure 2.2.

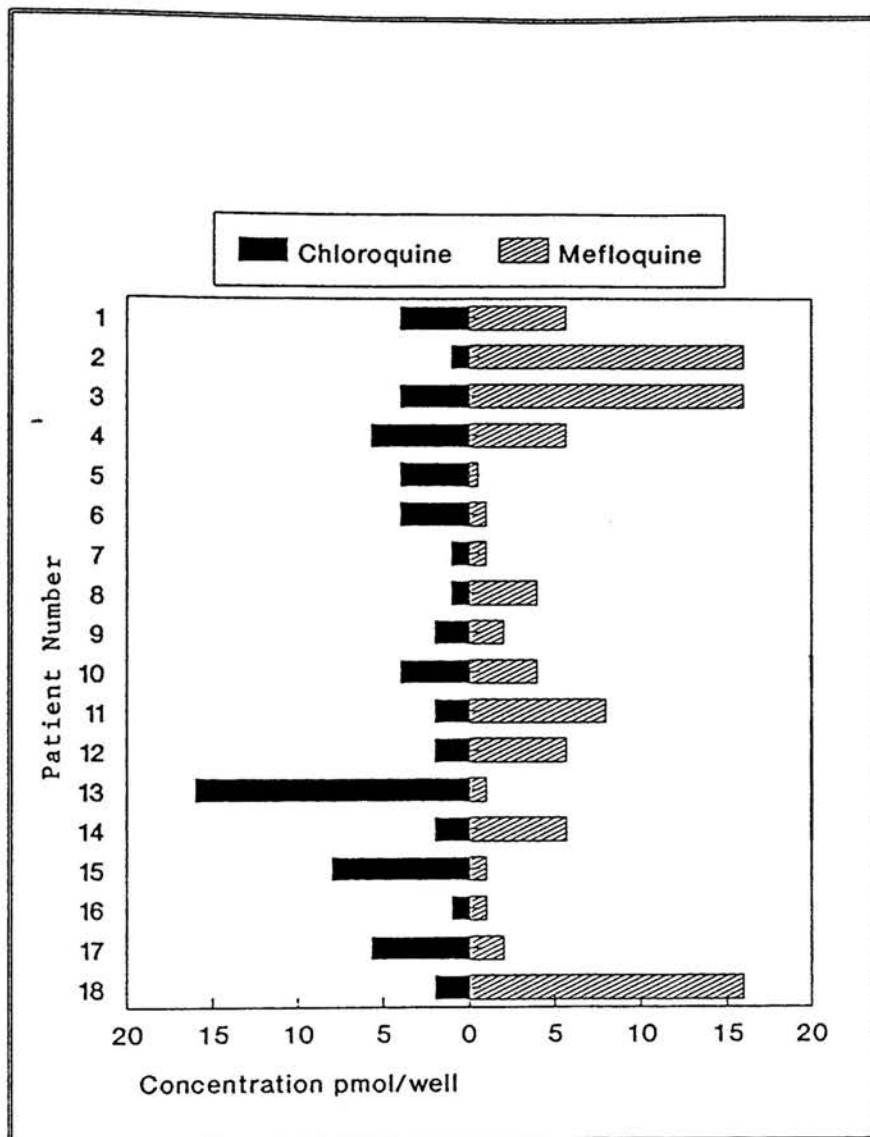


Figure 2.2 Growth inhibition from 18 patients tested for chloroquine and mefloquine resistance in North-Eastern Transvaal during 1988.

Patient 13 registered resistance to chloroquine at higher than 16 pmol/well, whereas number 15 was resistant up to 8 pmol/well. With the mefloquine test, patients 2, 3 and 18 were highly resistant and 11 only up to 8 pmol/well. It was also noticed that none of the isolates exhibited resistance to both chloroquine and mefloquine. Population pie charts of drug resistance towards chloroquine (Fig. 2.3 A) and mefloquine (Fig. 2.3 B) are shown.

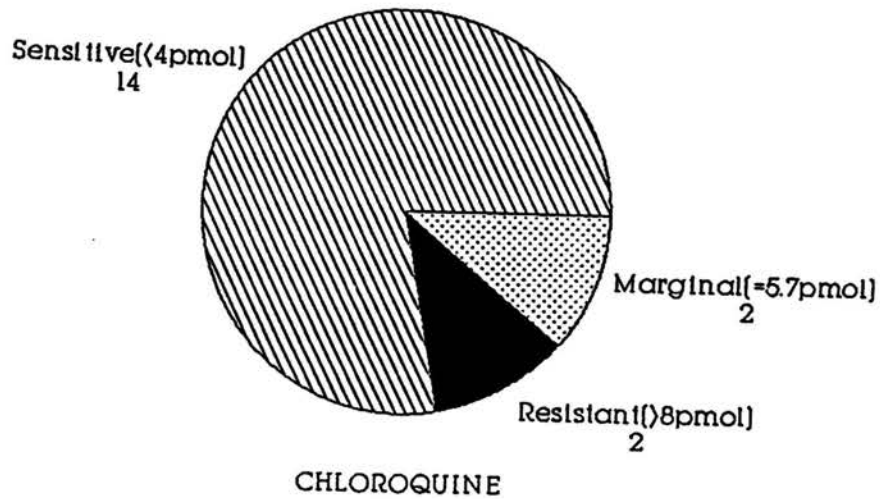


Figure 2.3(a) Population pie chart of chloroquine resistance in the North-Eastern Transvaal during 1988 (n=18).

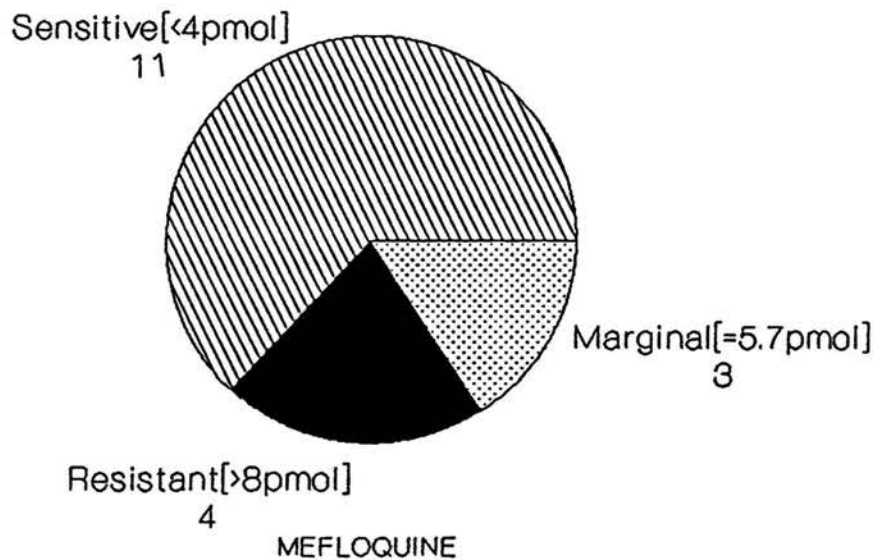


Figure 2.3(b) Population pie chart of mefloquine resistance in the North-Eastern Transvaal during 1988 (n=18).

Within the constraints of the small population tested, the results indicate a tendency towards higher resistance against mefloquine (22 %) than against chloroquine (11 %) among the patients tested.

## 2.4 DISCUSSION

The malarial parasite has the ability to adapt towards the constraints put on its propagation by chemotherapy and already shows resistance against a variety of antimalarial drugs. Chloroquine resistance has progressively increased over the last decade, especially in the Kwazulu area according to NIDTE surveys (17). In 1937, 25 % cases were found to be chloroquine resistant compared to 100% resistance measured in the Mamphene area in 1988 (17). Clinically the effectivity of chloroquine treatment (1500 mg per patient over three days) dropped from 100 % in 1983 to 85.2 % in 1987 (17). Chloroquine resistance measured for the whole Kwazulu area in 1988 was recorded at 15 % . In the North Eastern-Tranvaal, 15 to 30 clinical chloroquine resistant cases were recorded in 1988 by Hansford and Pammenter (18). This is a approximately 1 % of the total cases recorded compared to our 11 % determined in this investigation using the *in vitro* method. However, no direct extrapolation can be made between the clinical and *in vitro* determinations. Patients with malaria are not tested for the presence of prophylactic drugs before treatment is started, which may aid to cure the patient resulting in clinical resistance not being recorded. Patients also differ in their ability to metabolise drugs while in the *in vitro* system, no metabolism or excretion of the drugs takes place. Since 1988 no *in vitro* study has been undertaken to determine the chloroquine resistance in the North-Eastern Transvaal area. According to Hansford it would appear that the clinical recorded cases of resistance have declined over the last two years (Hansford, RIDTE, Box 33, Tzaneen, 0850, Personal communication, August 1991).

The use of mefloquine as a curative drug in areas where chloroquine resistance is a problem shows promise, as is shown in Fig. 2.2 where

no chloroquine resistant patients were found to be resistant against mefloquine. It was found that 4 of the 18 samples tested, showed parasite growth at mefloquine concentrations above 5.6 pmol, three of which at higher than 15 pmol per well. According to Graig Cranfield, Chairman of the WHO's committee on antimalarial drugs, some strains of parasites exhibited insensitivity against this drug even before it was introduced, which may explain our results (15). All of the samples that were chloroquine resistant in our tests were sensitive against mefloquine. This shows that chloroquine resistant malaria cases can be treated using mefloquine if the drug has the same effect *in vivo* as *in vitro*.

The possibility that antimalarials other than chloroquine could have been present in the samples using the *in vitro* assay led to the suggestion that the plasma from infected patients should be replaced with non-immune serum in the future (17). Freese *et al.* (17) found that the resistance micro-test gave better results with South African strains if the parasites were not grown in a burning candle oxygen depleted atmosphere, but in a specific gas mixture containing 3 % O<sub>2</sub>, 4 % CO<sub>2</sub> and 93 % N<sub>2</sub>. Finally Kouzretsov *et al.* (19) postulated that the parasite density was also a factor. He found that isolates with more than 100 000 parasites per ul blood needed more chloroquine for inhibition of the schizonts than the same isolates at a lower parasitemia.

It should be remembered that there is considerable variation in the infectious behaviour of malarial parasites from the same species, and field isolates generally contain genetically distinct clones of the parasite (6). The development of more sophisticated methods for the culturing and testing of parasite clones for resistance to drugs and other properties

for example by characterizing the genomic DNA by molecular biology techniques, should lead to a better understanding with beneficial implications for the chemotherapy of malaria.

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