

Sensitivity to dihydrofolate reductase inhibitors and molecular karyotypes in some *Plasmodium falciparum* isolates in Kenya.

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JULIUS ODUOR NYALWIDHE

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Thesis submitted in partial fulfilment of the requirements for the degree of Master
of Science in Parasitology in the University of Nairobi

DECLARATION

This Thesis is my original work and it has not been presented for a degree in any other University.

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Julius Oduor Nyalwidhe

Date: _____

This Thesis has been submitted for examination with our approval as University supervisors.

Signed: _____

Prof. G.K. Kinoti

Date: _____

Signed: _____

Dr. B. Khan

Date: _____

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ABSTRACT

Ten Kenyan isolates of *Plasmodium falciparum* were characterized by their molecular karyotype and antimalarial drug sensitivity. For chromosomal analysis pulse field gradient gel electrophoresis was used. *Plasmodium falciparum* isolates from malarious areas in Nyanza, Rift Valley and Coast provinces were cultivated *in vitro* and used for the karyotype study. The sensitivities of cultured isolates to three antifolate malarial drugs (pyrimethamine, cycloguanil and chlorcycloguanil) and sulphadoxine which is a sulphonamide were examined by the *in vitro* radioisotopic methods of Desjardins *et al.* (1979) with minor modifications.

Considerable variation was found in the antimalarial drug response of the 10 parasite isolates. Among the four antimalarial drugs examined, chlorcycloguanil and cycloguanil had similar potencies. They were more potent than pyrimethamine and sulphadoxine. Sulphadoxine and pyrimethamine had similar potencies. Cycloguanil and chlorcycloguanil, which are closely related biguanides were highly correlated in their antimalarial activities. There was no correlation between the activity of the three antifolates and sulphadoxine.

The isolates from the Rift Valley province showed significantly lower susceptibility to the four antimalarial drugs tested than those from the Coast and Nyanza provinces. The isolates from Coast province showed the highest susceptibility.

The chromosomal bands were found to range in number from 14 to 16 and in size from 600 to 4300 kilobase pairs, and there was extensive variation in chromosome size. Two isolates had more than the constant number of chromosomes, suggesting a mixed population of parasite strains in natural malarial infections. There were inter and intra-regional differences in the sizes of the chromosomes. However, there was no correlation between band size and drug sensitivity. The degree of polymorphism shown by the

chromosomes was such that each isolate could be individually characterised by its chromosomal pattern.

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DEDICATION

This work is dedicated to my dear family for they have made me whom I am.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Malaria is the most important parasitic disease in the tropics in terms of morbidity and mortality (WHO, 1990). The causative agents are single celled protozoan parasites belonging to the order Haemosporidia, family Plasmodiidae and genus *Plasmodium*. Four species infect man, namely *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. *Plasmodium falciparum* occurs throughout tropical Africa, Asia and Latin America and it is responsible for 80% of malaria worldwide. The disease due to this species is usually severe and frequently fatal.

Malaria is prevalent in Kenya and all the four species of malaria parasite occur. *Plasmodium falciparum* is by far the most common (80-85%), followed by *P. malariae* (10-15%); *P. ovale* is only occasionally seen and *P. vivax* is reported infrequently (Roberts, 1974).

There are three clearly defined epidemiological situations found in Kenya depending upon ecological conditions (altitude, rainfall and humidity). These are: 1) endemic malaria, 2) epidemic or seasonal malaria, 3) no malaria transmission (Roberts, 1974). The World Health Organisation (WHO, 1963) recommended the use of the spleen rate in children aged 2-9 years to define the degree of endemicity. The spleen rate is the percentage of children aged 2-9 years who have a palpable spleen in an area where malaria is endemic.

Table 1 shows the endemicity of malaria in various parts of Kenya. Figure 1 shows the duration (annual) of transmission in different parts of the country.

Table 1. Malaria epidemiology by type and area.

Classification/degree	Spleen rate age 2-9	Area
(1) Endemic (a) holoendemic (b) hyperendemic (c) mesoendemic (d) hypoendemic	> 75% 50-74% 10-49% <10%	Coast Province, coastal area; Tana River, Kano Plains, Taveta. North Nyanza, Bungoma, Busia, Shimba Hills (Coast). Machakos, Kitui, Thika; parts of North Nyanza, Murang'a and Embu below 1,300 m. Meru, Pokot, Samburu, Isiolo, Baringo.
(2) Epidemic	Variable	Highland over 1,600 m with high rainfall and dry areas with exceptional rainfall: Masailand, Nandi, Kericho, Kisii, North Eastern Province, Eastern Kitui, Londiani, Elgeyo.
(3) No transmission (sometimes anophelism without malaria)	None	At altitude over 2,000 m: Aberdares, Mt. Kenya, Mt. Elgon (forest, moorland, plateaux).

Source: Roberts, 1974.

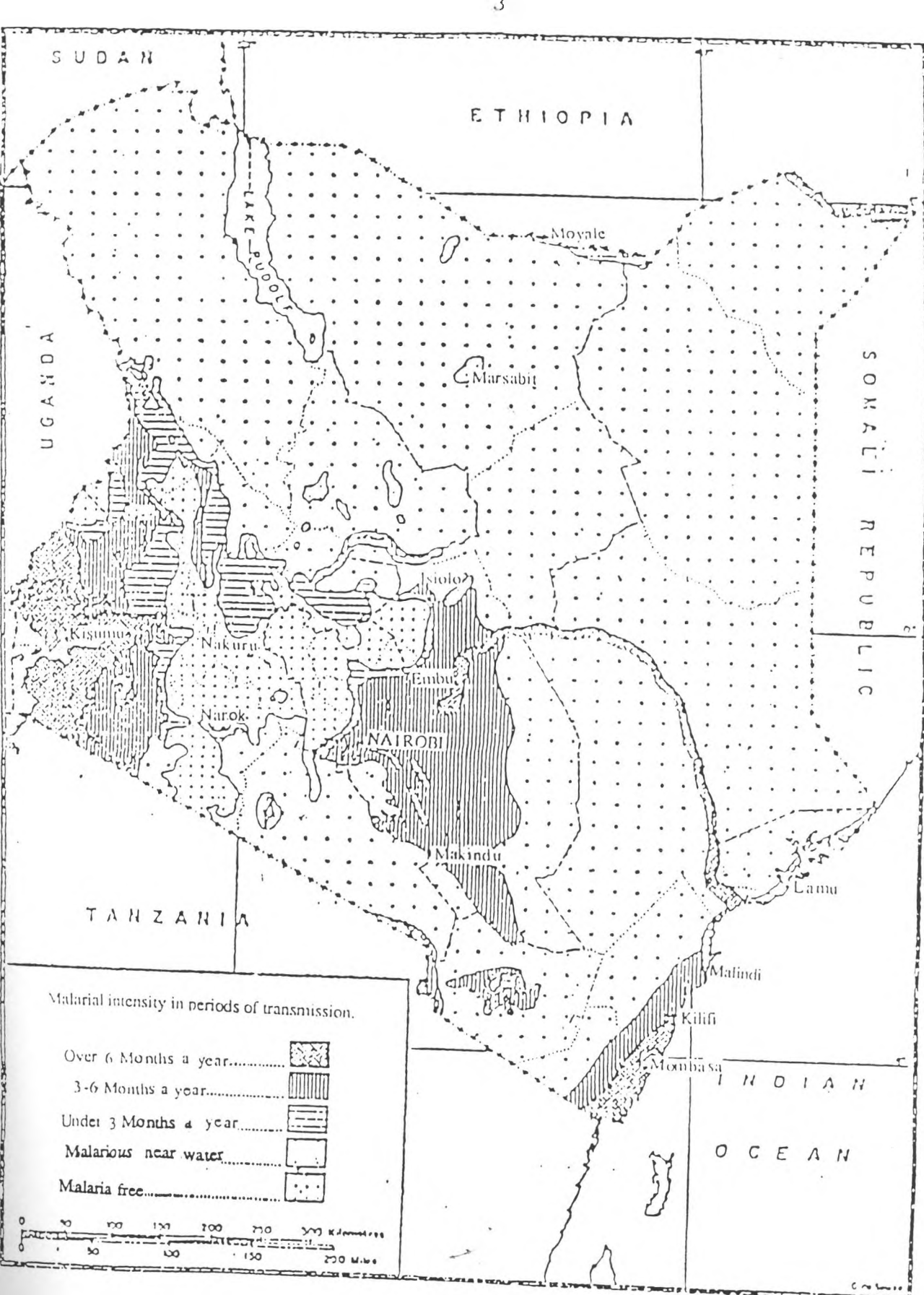


Figure 1. Duration of malaria transmission periods in Kenya. Source: Atlas of Kenya (Survey of Kenya, 1970).

Malaria parasites are inoculated into the human host by the bite of infected female mosquitoes belonging to the genus *Anopheles*. The stage of the parasite introduced into the host's blood stream by infected mosquitoes is the sporozoite. The sporozoites invade hepatocytes where they undergo asexual division (schizogony) to release merozoites. The merozoites invade red blood cells and grow into trophozoites, the earliest stage being the ring form. As the trophozoites grow, they undergo multiple nuclear divisions to form schizonts. This is followed by cytoplasmic division, resulting in mature schizonts (segmenters) which contain merozoites. Infected red blood cells rupture and release merozoites into the blood stream where they infect fresh red blood cells. Here some merozoites develop into schizonts while others undergo differentiation into sexual stages, immature macrogametocytes or microgametocytes. Gametocytes then mature but do not undergo further development until they are taken up by a feeding mosquito. In the mosquito gut, microgametocytes undergo rapid deoxyribonucleic acid (DNA) replication and cell division to form eight flagellated male gametes. These are released and one fertilizes a macrogametocyte to form a diploid zygote. The zygote undergoes meiosis and each develops into an elongated form called an 'ookinete'. The ookinetes penetrate the epithelial lining of the stomach of the mosquito and develop into oocysts which undergo sporogony to produce large numbers of haploid sporozoites. These migrate to the salivary glands of the mosquito from where they are injected into a human host when the infected female *Anopheles* bites (Garnham, 1966).

1.2 Literature Review

1.2.1 Chemotherapy of Malaria.

In Kenya, malaria is one of the most serious health problems in terms of morbidity and mortality (Bonte, 1974). Currently, there are no effective vaccines for malaria and other important protozoal diseases, and the mainstay of control is

chemotherapy (Geary and Jensen, 1986). The clinical features of falciparum malaria are highly variable and depend upon the level of immunity of the host, the duration and level of parasitaemia, and the occurrence of complications. Treatment will therefore be governed by these factors and in addition by the susceptibility of the particular strain of parasite to antimalarial drugs (WHO, 1973).

Antimalarial drugs can be divided into four different groups, based on the parasite stage they affect. Tissue schizontocides act on pre-erythrocytic stages of the parasite and thus prevent invasion of the erythrocytes. Blood schizontocides act on the erythrocytic stages of the parasite. Gametocytocides destroy the sexual forms, while sporontocides prevent or inhibit the formation of oocysts and sporozoites in the anopheline vectors (WHO, 1986).

Antimalarial drugs can also be divided into different groups based on chemical structure and biological activity. These include cinchona alkaloids, e.g. quinine; 8-aminoquinolines, e.g. primaquine; 9-aminoacridines, e.g. mepacrine; 4-aminoquinolines, e.g. chloroquine; biguanides, e.g. proguanil and chlorproguanil; diaminopyrimidines, e.g. pyrimethamine; sulfanes and sulphonamides, e.g. sulphadoxine and also certain antibiotics (WHO, 1986).

Antimalarial chemotherapy is based on the ability of drugs to be toxic to the parasite while affecting the host to a minimal extent (Knox, 1977). Each drug in use has been selected because it has one or more specific actions against the malaria parasite when administered to the patient in the appropriate dosage. If this recognised action does not occur when the drug or its active metabolites has reached the parasite, the parasite is adapted to the altered chemical environment and it is resistant to the drug. Drug resistance can be defined as the ability of a parasite strain to multiply or to survive in the presence of a concentration of a drug that normally destroys parasites of the same species or prevents their multiplication (WHO, 1965). Resistance may be relative, yielding to increased doses of the drug tolerated by the host or it may be complete, when the parasite withstands the maximum doses tolerated by the host (WHO, 1965).

The emergence and spread of resistance are the result of interactions between the parasite, the human host, the drug and the vector, enhanced by particular ecological features (Wernsdorfer, 1991). As chemotherapy plays an important role in the control and treatment of malaria, the development of drug resistance by *P. falciparum*, the most common and dangerous parasite, has become a matter of serious concern in many parts of the world. Chloroquine resistant *P. falciparum* (CRPF) was first reported over 30 years ago from South America (Young and More, 1961) and shortly afterwards from Thailand (Harinasuta *et al.*, 1962). In 1979 chloroquine resistant infections were reported in non-immune visitors to Kenya and Tanzania (Campbell *et al.*, 1979; Fogh *et al.*, 1979), although it was not until 1982 that CRPF was found in a Kenyan infant in Western Kenya (Spencer *et al.*, 1983). Since then, susceptibility to chloroquine *in vivo* has continued to decrease in all locations studied. The emergence of *P. falciparum* resistant to chloroquine and other 4-aminoquinolines, used as first line drugs in malaria control programmes, led to the development of alternative drugs or drug combinations which are effective.

However, cases of resistance are now being detected for such drugs as pyrimethamine and proguanil, and even drug combinations such as pyrimethamine / sulphadoxine (Fansidar) and pyrimethamine/sulfalene (Metakelfin), which had become valuable alternative treatments for chloroquine-resistant falciparum malaria (Bruce-Chwatt, 1982). In Kenya, pyrimethamine / sulphadoxine is an effective treatment for most *P. falciparum* malaria infections, despite resistance to pyrimethamine when used alone for treatment (Nguyen-Dinh *et al.*, 1982; Spencer *et al.*, 1984). The mechanisms of drug resistance include a decrease in the affinity of the target, an increase in the quantity of the target, a decrease in the physiological importance of the target and a decrease in the uptake of the drug (Hitchings, 1978).

The antimalarial activities of antifolates, such as pyrimethamine and proguanil, depend on the ability of the drug to inhibit an enzyme of the "thymidylate cycle", dihydrofolate reductase (DHFR; 5, 6, 7, 8-tetrahydrofolate: NADP⁺ oxidoreductase,

EC 1.5.1.3) which exists as a bifunctional enzyme with thymidylate synthetase (TS) in *P. falciparum* and other protozoa (Garret *et al.*, 1984; Peterson *et al.*, 1990).

Pteroylglutamic acid (folic acid) is a water soluble vitamin that plays a crucial role in cellular metabolism. The vitamin is a 2-amino-4-oxopteridine with a side chain incorporating both p-aminobenzoic acid and glutamic acid. Folic acid must be reduced to the tetrahydro form, which is the active acceptor of single carbon units that are subsequently transferred enzymatically from an appropriate co-factor to precursor molecules that lead to the synthesis of purine and pyrimidine (e.g thymine) components of nucleic acids, and of the important amino acid, methionine (Krungskrai *et al.*, 1990). In contrast to their mammalian hosts, the malaria parasites are able to synthesize folates *de novo*. The parasites also synthesize their pyrimidines *de novo* in the thymidylate cycle but they are dependent on preformed purines salvaged from the host.

The enzymes critical to the synthesis of thymidylate have been demonstrated in several species of protozoans. The enzyme DHFR catalyses the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF), the precursor of folate cofactors required for one carbon transfer reactions (Blakey, 1984). The DHFR is metabolically linked to thymidylate synthetase (TS) which catalyses the conversion of uridylate and the folate cofactor 5, 10 - methylenetetrahydrofolate to thymidylate (dTMP) and dihydrofolate (Ferone, 1977). For each mole of dTMP produced, 1 mole of THF is destroyed, and continued reduction of DHF by DHFR is necessary for continued thymidylate synthesis (Figures 2 and 3).

Therefore, inhibition of either DHFR or TS by the antifolates causes a functional dTMP deficiency and consequently, in the absence of folate cofactors, pyrimidine biosynthesis cannot occur. This disrupts replication of DNA and merozoite development during schizogony is curtailed (Cowman and Lew, 1989).

The effect of the antifolates is selective because the DHFR of the *Plasmodium* species is different from that of the mammalian host (Garret *et al.*, 1984), allowing for

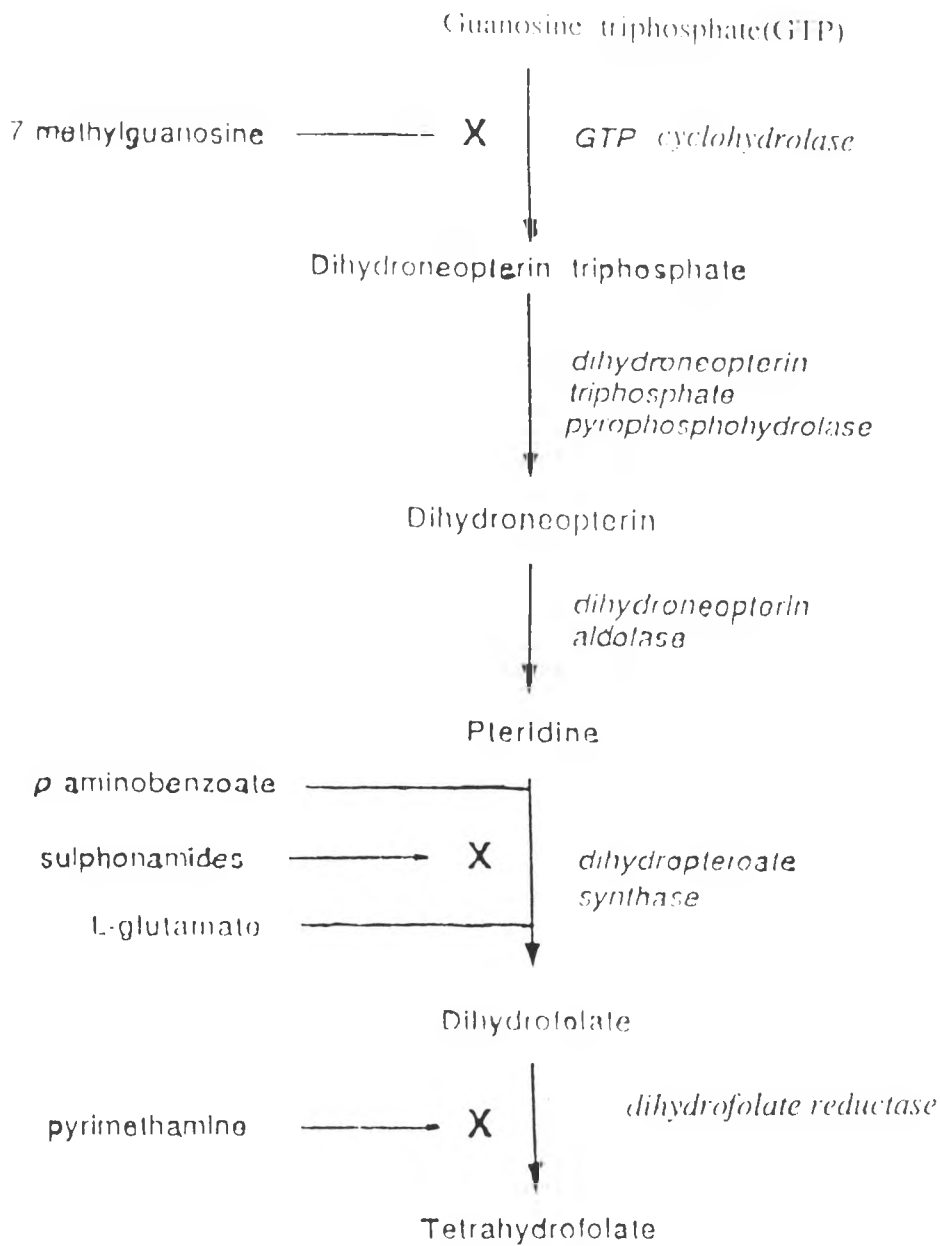
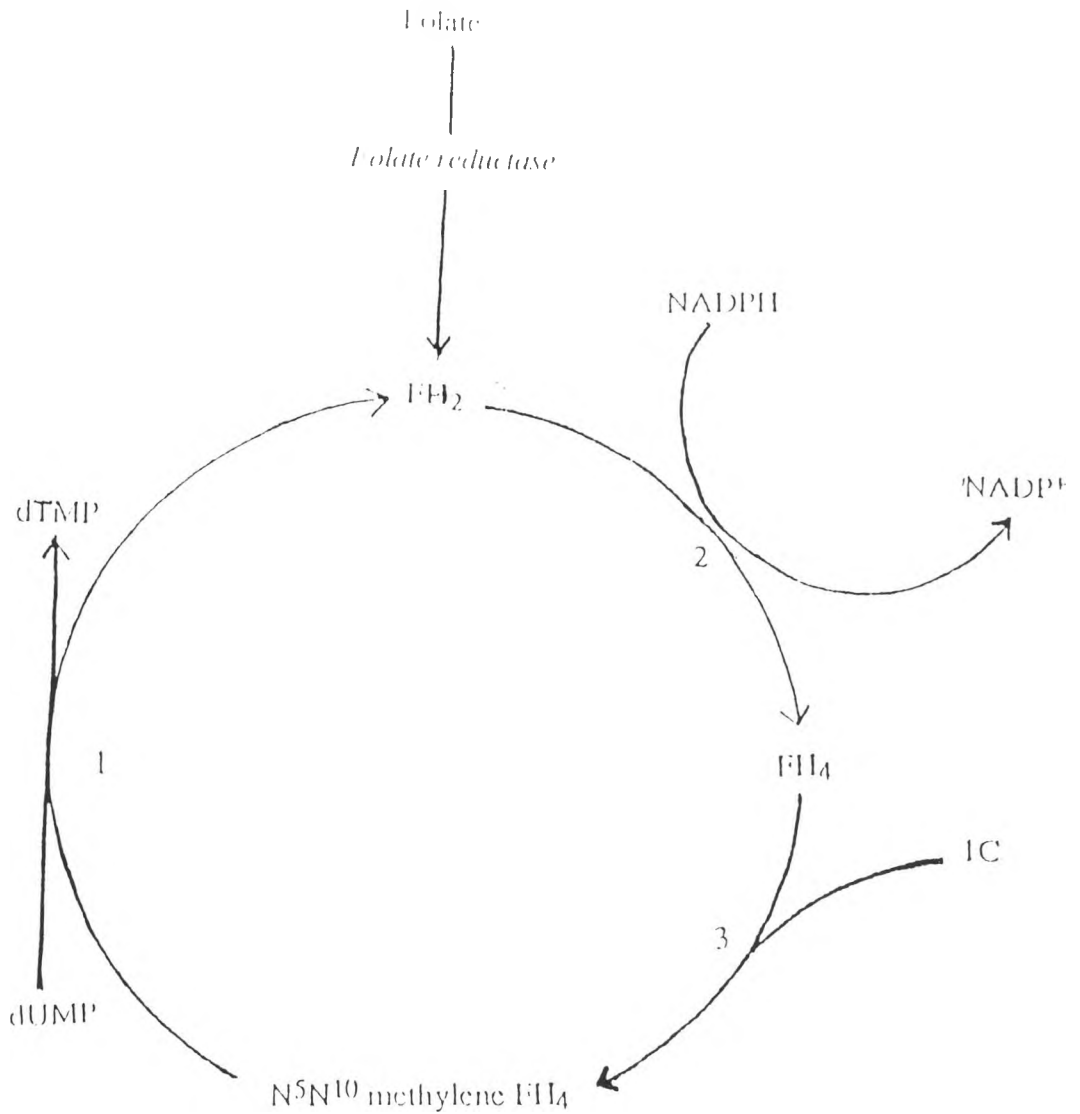


Figure 2 The *de novo* synthesis of tetrahydrofolate in *P. falciparum*. The crosses mark potential sites of inhibition (Krungskrai *et al.*, 1990).



1. *Thymidylate synthase*
 2. *Dihydrofolate reductase*
 3. *Methyltransferase*
- FH₂ dihydrofolate
 FH₄ tetrahydrofolate
 dUMP deoxyuridine monophosphate
 dTMP deoxythymidine monophosphate
 IC 1 carbon unit

Figure 3 Thymidylate synthase cycle (Gutteridge and Coombs, 1977)

the specific inhibition of the protozoan enzyme (Hitchings, 1978). The *P. falciparum* enzyme exists as a bifunctional protein consisting of DHFR and TS with a mass 200,000 daltons. The mammalian enzyme consists of separate DHFR and TS enzymes with the DHFR having a mass of 20,000 daltons. The DHFR enzyme of *P. falciparum* has a higher binding affinity for the antifolate drugs than the mammalian enzyme (Hitchings, 1978). These differences provide the basis for successful chemotherapy of malaria using the right dosages of the antifolate to inhibit the activity of DHFR of the parasite without affecting the corresponding host enzyme.

The biguanide metabolites cycloguanil and chlorcycloguanil are more potent than pyrimethamine against *P. falciparum* isolates *in vitro* (Watkins *et al.*, 1987). Cycloguanil, the active metabolite of proguanil, has been used sparingly because of the dual problem of rapid excretion (Robertson, 1957) and development of resistance (Rollo, 1980). Proguanil is activated *in vivo* by the mixed function oxidase system of the hepatic microsomes to form the active compound cycloguanil (Armstrong and Smith, 1974). However, it is not known whether pyrimethamine and cycloguanil share the same binding site on the DHFR enzyme, nor is it clear whether the acquisition of resistance to one of the drugs causes concomitant resistance to the other (Foote *et al.*, 1990).

There are several factors which may bring about the development of pyrimethamine resistance in clones of *P. falciparum*. Walter (1986) suggested that the development of resistance could arise from a mutation which results in the production of a DHFR enzyme with reduced affinity for the drug and its normal substrate dihydrofolate. Subsequent studies on the enzyme and gene in *P. falciparum* have indicated that the predominant mechanism of resistance to the dihydrofolate reductase inhibitors consists of mutations within the DHFR-TS gene that alter the binding affinity of the drug to the enzyme (McCutchan *et al.*, 1984; Chen *et al.*, 1987; Cowman *et al.*, 1988; Peterson *et al.*, 1988).

Kan and Siddiqui (1979) reported increased amounts of DHFR produced by resistant *P. falciparum*, suggesting that resistance was associated with gene amplification. Walter (1986) reported similar amounts of the enzyme in sensitive and resistant strains but found differences in the isoelectric points. Another example of overexpression of the enzyme is the 5 to 10-fold increase in expression of the enzyme in a pyrimethamine-resistant clone of *P. falciparum* selected under *in vitro* drug pressure (Inselburg *et al.*, 1987). However, there were structural changes in the DHFR enzyme that also altered its activity. There might have been several combined events to produce the observed drug resistance since the clone line was obtained by mutagenesis and selection after sequential increases in drug concentration.

Increased production of the enzyme suggests that gene amplification may be a mechanism of resistance in some protozoans. Gene amplification may result in the production of a greater amount of the enzyme which effectively counteracts the drug. This has been shown to occur in *Leishmania* spp resistant to methotrexate, a DHFR inhibitor. Three independently derived *Leishmania* promastigote cell lines resistant to the antifolate have been shown to overproduce the bifunctional protein DHFR-TS by amplification of a region of DNA from chromosome 4 that contains the gene for DHFR-TS (Washteyn *et al.*, 1985).

In *P. chabaudi*, pyrimethamine drug pressure selected for the duplication of the DHFR-TS gene (Cowman and Lew, 1989). This was not achieved by tandem duplication but by the duplication and rearrangement of a portion of chromosome 7 which contains the DHFR-TS gene. This chromosomal duplication increased the chromosome number from 14 to 15 (Cowman and Lew, 1989). The two derived chromosomes (450 kilobases and 1.1 megabases) were smaller than the original chromosome 7 (1.3 megabases) so that essentially only a 200-kilobase region was duplicated. As a result of the duplication of the DHFR-TS gene there was at least a double overproduction of mRNA and presumably the enzyme. This may explain the ability of the pyrimethamine-resistant lines to grow in increased amounts of the drug.

Therefore gene amplification may be involved in some types of pyrimethamine resistance in some *Plasmodium* species. In *P. falciparum* the dihydrofolate reductase /thymidylate synthetase gene is located in chromosome 4 (Gu *et al.*, 1990).

Chloroquine resistance of *P. falciparum* is similar to the multiple drug resistance phenotype (MDR) of mammalian tumour cells, as both involve expulsion of drug from the cell and both are reversed by calcium channel antagonists (Krogstad *et al.*, 1987). A homologue of the mammalian multidrug resistance gene (pfm₁) has been implicated in chloroquine resistance because it is amplified in some chloroquine resistant parasites but not in any of the sensitive isolates examined (Foote *et al.*, 1989). The pfm₁ gene is located in chromosome 5, and in one chloroquine resistant line with an amplified pfm₁ gene, chromosome 5 is greatly enlarged (Foote *et al.*, 1989) probably due to tandem duplication of the pfm₁ gene.

Therefore resistance of *Plasmodium* species to antimalarials can occur by diverse mechanisms ranging from point mutations in specific genes (McCutchan *et al.*, 1984; Chen *et al.*, 1987; Cowman *et al.*, 1988; Peterson *et al.*, 1988) to amplification of specific genes (Kan and Siddiqui, 1979; Inselberg *et al.*, 1987), and drug pressure brings about selection in strains of *Plasmodium*. A comparison of isolates from different areas of the country with different incidence patterns may provide information on the effect of drug pressure on sensitivity.

1.2.2 Genomic Organisation of *Plasmodium falciparum*.

Malaria parasites have been traditionally differentiated by characters such as morphology and host specificity. This has led to the creation of more than 80 species as well as numerous subspecies of these organisms (Garnham, 1966). In recent years genetic studies have complemented this approach, demonstrating how variant forms are generated by mechanisms such as recombination and mutation (Beale *et al.*, 1978; Beale, 1980).

The study of parasite chromosome content and structure, in addition to other biochemical studies may contribute to the understanding of the biochemistry of the malaria parasites, pathogenesis and epidemiology, and eventually to its control (Gu *et al.*, 1990). Genetic evidence suggests that the intraerythrocytic parasite is haploid (Sinden and Hartley, 1985); Walliker *et al.*, 1987) and the chromosomes are structurally similar to those of lower eukaryotes.

The pulse field gradient gel electrophoresis (PFGE) technique has proved remarkably useful for studying the chromosomes of *P. falciparum*. Pulse field gradient gel electrophoresis separates chromosome-size DNA molecules in agarose matrices by subjecting them to electric fields that alternate in two directions (Schwartz and Cantor, 1984). The larger the molecule, the larger the fraction of each cycle needed before net translation can occur, and thus the smaller the net translational motion. DNA molecules that have been resolved by PFGE range in size from ordinary restriction fragments, less than 10 kilobases (Kb), to intact chromosomal DNAs up to more than 5 million base pairs (mb) (Schwartz and Cantor, 1984; Van der Ploeg *et al.*, 1985; Smith *et al.*, 1986; Carle and Olson, 1987; Volrath and Davis, 1987; Orbach *et al.*, 1988).

Using PFGE, workers have reported fourteen chromosome-size DNA molecules in the intraerythrocytic asexual stages of *P. falciparum* (Kemp *et al.*, 1987; Langsley *et al.*, 1987 Wellems *et al.*, 1987), but the clear resolution of the different chromosomes was not achieved (Weber, 1988). Separation of chromosome-size DNA by PFGE has been shown to be sensitive to many experimental variables such as temperature, pulse time, voltage and concentration of the gel (Cantor *et al.*, 1988a, b).

Gu *et al.* (1990) established electrophoresis conditions that allow an unambiguous separation of 13 chromosomal bands from *P. falciparum* strain FCR3 using the contour clamped homogeneous electric field (CHEF) apparatus (Chu *et al.*, 1986). The chromosomes range in size from 720 kilobase pairs to 3.65 megabase pairs. All the species of *Plasmodium* studied to date (*P. falciparum*, *P. vivax*, *P. chabaudi*, *P. berghei* and *P. vinckei*) have 14 chromosomes (Langsley *et al.*, 1987; Sharkey *et al.*, 1988; Sheppard *et al.*, 1989). This compares to an electron microscopic

study of serially sectioned FCR3 strain of *P. falciparum* which identified fourteen pairs of kinetochores in the intraerythrocytic asexual stages (Prensier and Slomianny, 1986).

The PFGE separated chromosomes can be analysed using DNA hybridization techniques to determine differences in the size and contents of the chromosomes from the different parasite samples. The principle of DNA hybridization is that a single stranded DNA fragment containing a specific *P. falciparum* DNA sequence is identified, purified, labelled with a tracer (often a radioisotope) and used to probe the chromosomal profiles. When the probe is applied to Southern blotted chromosome profiles, the sequences in the probe hybridize with complementary DNA sequences in the separated chromosomes. The bound DNA can then be revealed by autoradiography if the probe was labelled with a radioisotope (Southern, 1975; Maniatis *et al.*, 1982).

No previous study of the molecular karyotypes of Kenyan isolates has been reported. This study, using *P. falciparum* isolates from different parts of the country with different infection patterns may elucidate intra- and inter-regional variations in the molecular karyotypes of the parasite.

1.3 Justification

The dihydrofolate reductase inhibitors pyrimethamine, proguanil and chlorproguanil have played a major role in the prophylaxis and treatment of *P. falciparum* infection. They are often used in conjunction with a sulphonamide drug such as sulphadoxine, and along with chloroquine they have been the most extensively used antimalarial drugs in Kenya. However, there are increasing reports of resistance to the antifolates when used alone or in combination with sulphonamides. Drug resistance has become a serious problem confronting public health workers in the affected endemic regions. Consequently it is necessary to monitor sensitivity to predict or detect resistance and if so indicated to modify therapeutic approaches.

Basic knowledge of the chromosomes of *Plasmodium falciparum* is of importance in understanding the mechanisms responsible for the genetic diversity of the parasite. Since the chromosomes of *P. falciparum* do not condense during division, it was not possible to study them until the recent development of pulse field gradient gel electrophoresis which separates intact chromosome-sized DNA molecules. The study of the genomic organisation of malaria parasites is important in trying to elucidate the mechanisms of resistance to antimalarial drugs. No previous study of the molecular karyotypes of malaria parasites in Kenya has been done. This study involves isolates of *P. falciparum* from three different parts of the country (Figure 4) with different epidemiological patterns.

1.4 Objectives

The specific objectives of this study were:

1. To assess the sensitivity of isolates of *P. falciparum* from three malarious areas of Kenya to pyrimethamine, cycloguanil, chlorcycloguanil and sulphadoxine.
2. To compare and to find out if cross-resistance occurs among the different antimalarial drugs used against the Kenyan isolates of *P. falciparum*.
3. To compare the genomes of pyrimethamine-sensitive and pyrimethamine-resistant isolates using pulse field gradient gel electrophoresis and oligo dihydrofolate reductase gene probe hybridization.
4. To compare inter- and intra- geographical region variations in the genome of *P. falciparum* isolates from different regions of Kenya.

5. To find out if distinct strains of *P. falciparum* occur within and among endemic areas in Kenya.

1.5 Definitions

The present study uses the terms “isolate”, “strain”, and “clone” as defined by Carter and Walliker (1978), viz:

Isolate: a single sample of parasite material derived from a naturally infected host specimen on a unique occasion and preserved in the laboratory either by continuous *in vitro* cultivation or by cryopreservation in liquid nitrogen. The parasite cells present in an isolate are not necessarily genetically homogeneous and may indeed contain representatives of several distinct strains or clones.

Strain: the progeny of parasites obtained from a particular isolate and having special properties distinguishing them from other members of the same species.

Clone: the progeny of parasites descended from a single cell by asexual reproduction and therefore genetically identical to each other and to the parent cell.

CHAPTER 2

MATERIALS AND METHODS

2.1. Source of *Plasmodium falciparum* isolates.

The present study used venous blood samples previously collected from malaria patients by KEMRI staff and cryopreserved in ampoules in liquid nitrogen. The infected blood samples were collected in 1986 from malarious regions of Kenya (Fig 4), namely, Nyanza Province, Rift Valley Province and Coast Province (Table 2). The parasites used in this study were randomly selected from the KEMRI *P. falciparum* bank where they were cryopreserved in Rowes cryosolution (2.5% glycerol, 3% sorbitol, 0.35% sodium chloride) (WHO, 1981).

2.2 Methods**2.2.1 Culturing of *Plasmodium falciparum*.**

Parasites already adapted to continuous *in vitro* culture for ongoing KEMRI studies were cultured using the methods of Haynes *et al.* (1976) and Trager and Jensen (1976) with minor modification. The parasites were grown in modified RPMI medium 1640 (Gibco Ltd, Paisley, Scotland) containing no added para-aminobenzoic acid or folic acid, which antagonise the antimalarial activity of dihydrofolate reductase inhibitors (Watkins *et al.*, 1985). The medium was supplemented with 10% heat inactivated normal human serum, 25mM HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) and 25mM sodium bicarbonate (Sigma Chemical Company). The parasites were grown in 0⁺ve erythrocytes maintained at a concentration of 6%

Table 2. Origin of *Plasmodium falciparum* isolates

Isolate	Province	Locality
JP62	Coast	Jilore
JP119	Coast	Jilore
M24	Coast	Malindi
KIL9	Coast	Kilifi
K67	Nyanza	Kisumu
K39	Nyanza	Kisumu
S158	Nyanza	Saradidi
ENT30	Rift Valley	Entasopia
ENT36	Rift Valley	Entasopia
ENT41	Rift Valley	Entasopia



Figure 4. Map of Republic of Kenya showing localities from which isolates were obtained.

haematocrit in a 5 millilitre culture volume. The cultures were incubated at 37°C in a gas environment of 3% CO₂, 5% O₂ and 92% N₂ in 50ml screw-cap disposable tissue culture flasks (Corning Glass Works, Corning NY).

Culture medium was changed daily and fresh uninfected erythrocytes were added at least twice a week depending on the culture parasitaemia, red cell count and any increase in the number of abnormal parasites. Growth rates were monitored every two days by making thin smears of the cultures. The smears were fixed using methanol and then stained in Giemsa. Parasite counts were made from inspection of 10,000 red blood cells per smear. The number of infected erythrocytes was used to determine the percentage parasitaemia.

$$\text{Percentage parasitaemia} = \frac{\text{number of infected RBCs}}{\text{total number of RBCs}} \times 100$$

To obtain high yields of parasites for DNA studies, parasites were grown in suspension cultures, where the culture flasks were placed in a shaking incubator. The shaking action probably resulted into a higher reinvasion rate of the erythrocytes by merozoites leading to the high parasitaemias obtained. High yields of parasites were also obtained by changing the culture medium twice per day and using 15% (v/v) heat inactivated serum in the culture medium.

Only cultures containing normal parasites as seen under microscope (Plate 2) were used in drug sensitivity and karyotype studies.

2.2.2 Drug Sensitivity Testing

The activity of pyrimethamine (Burroughs Wellcome Co., Research Triangle Park, NC, USA), cycloguanil (ICI, Macclesfield, UK), chlorcycloguanil and sulphadoxine (Hoffman-La Roche, Nutley, N-J., USA) were tested using the

radioisotopic technique of Desjardins *et al.* (1979). The RPMI 1640 medium used contained no p-aminobenzoic and no folic acid which antagonise the antimalarial activity of the dihydrofolate reductase inhibitors and sulphadoxine (Watkins *et al.*, 1985).

Parasites were incubated each drug for 48 hours (Nguyen-Dinh and Payne, 1980; Spencer *et al.*, 1983) before the addition of ^3H radiolabelled hypoxanthine. The 48 hour incubation exposes the full cycle of asexual reproduction to the test drug, thus permitting the detection of any inhibitory effects on the all developmental stages of the parasite. It allows the determination of the susceptibility of *P. falciparum* to slow-acting antifolates (Spencer *et al.*, 1984), and also makes it possible to test the drug sensitivity of asynchronous parasites from *in vitro* cultures (Nguyen-Dinh *et al.*, 1982; Spencer *et al.*, 1983).

The radioisotopic technique of Desjardins *et al.* (1979) provides quantitative measurements of the antimalarial activity of the drugs on the basis of inhibition of uptake of the radiolabelled nucleic acid precursor hypoxanthine by the parasites during short term (18 hours) cultures.

Pyrimethamine, cycloguanil and chlorcycloguanil were dissolved to required stock concentrations in sterile distilled water and ethanol. Each compound was first dissolved in a measured volume of either water or ethanol, which was then diluted with its counterpart to yield a 70% ethanol-30% water mixture containing a known concentration of the drug. Sulphadoxine was first dissolved in distilled water and then 1 M sodium hydroxide was added dropwise to complete solubilisation. The stock solutions had the following concentrations: pyrimethamine, 16.72 mM; cycloguanil, 10.05 mM; chlorcycloguanil, 4.00 mM; and sulphadoxine, 229.72 mM. The stock solutions were diluted in the microtitre plates to obtain concentrations such that the expected drug concentration required for 50% inhibition of uptake of radiolabelled hypoxanthine into parasite nucleic acid (ID_{50}), would fall in the middle of the range of dilutions (Webster *et al.*, 1985). This was done after several preliminary

drug tests which gave an indication of the estimated 50% inhibitory dose value for each drug using the ten isolates.

Microculture techniques were used to assess the sensitivity of the *P. falciparum* isolates. Microtitre plates containing 96 flat-bottomed wells were used (Fig 5).

A repeating dispenser (Hamilton Company, Reno Nevada, USA) was used to place 25 μ l of the complete culture medium in each well of the microtitre plate. For each test drug, the diluted drug solution, prepared as described above, was added to two wells of row 2, and an automatic microdilutor (Titertek Microtitration Equipment, USA) was used to make twofold serial dilutions down the plate in each column. On completion, row 1 remained free of any drug and each drug was present in duplicate columns at eleven concentrations over a 1024-fold range in rows 2 through 12.

A suspension of the *P. falciparum* parasites was then placed in the wells. The suspension consisted of fast growing *P. falciparum* stock cultures (growth rate at least three fold per 48 hours) diluted in culture medium which contained sufficient non-infected type O^{+ve} human erythrocytes to yield a final haematocrit of 1.5% and parasitaemia of 0.4%. A constant volume of 200 μ l of the suspension was placed in each well of the microtitre plate, except wells F, G and H of the 1st row each of which received 200 μ l of a suspension of unparasitised type O^{+ve} human erythrocytes in culture medium at a concentration of 1.5%. The total volume in each well was then 225 μ l. Thus the row 1 wells served as controls, with wells A, B, C, D and E, containing parasitised erythrocytes but no drugs, and wells F, G and H containing unparasitised erythrocytes and no drugs.

The plates were placed in a humidified airtight box (Belco Glass Inc., Vineland, NJ, USA) which was then flushed with a gas mixture of 3% CO₂, 5% O₂, and 92% N₂ and sealed. The box was then placed in an incubator at 37°C for 48 hours.

Uptake of ³H radiolabelled hypoxanthine (Amersham, USA) was used as an index of growth of parasites. The isotope was supplied as a lyophilate (6.2 ci/mMol) in ampoules containing 5mci. The contents of a single ampoule were dissolved in 5ml of sterile distilled water to make a stock solution which was stored at 4°C.

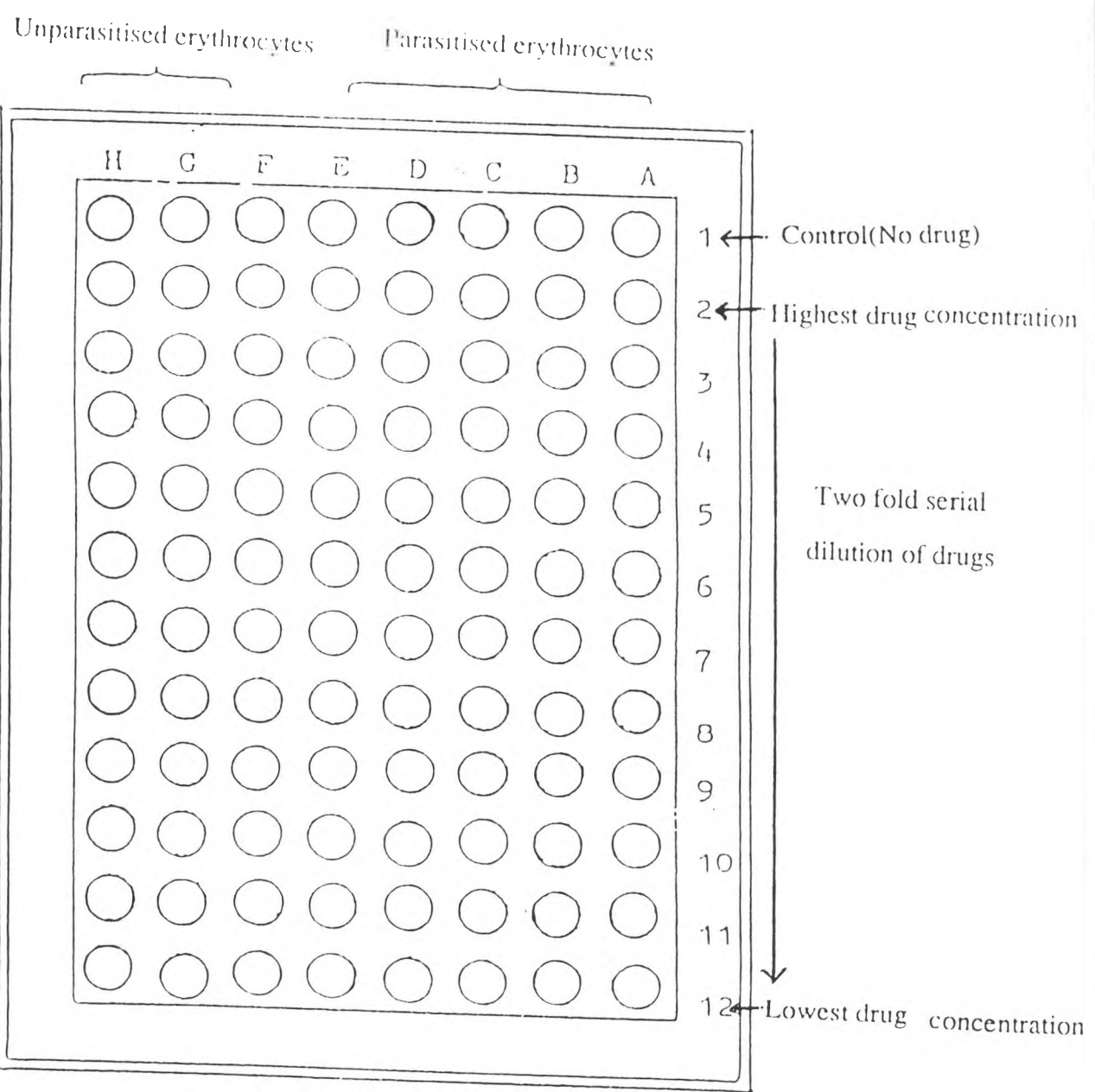


Figure 5. Design of a microtitre drug test plate.

After the 48 hours incubation period, the plates were removed from the gas box and 25µl of radiolabelled hypoxanthine solution diluted in culture medium was added to each well. The plates were then returned to the box which was again flushed with the 3% CO₂, 5% O₂ and 92% N₂ gas mixture, sealed and incubated for an additional 18 hours.

At the end of the second incubation period, each test plate was harvested on a MASH II automated cell harvester (Microbiological Associates, Bethesda, MD USA). The particulate contents of each of the wells were aspirated and deposited onto small discs of filter paper. Each disc was washed with copious amounts of distilled water, dried and then placed in a glass scintillation vial containing 1ml of scintillation fluid for beta emissions. All 96 vials, corresponding to the 96 wells of the microtitre plate, were counted in a Beckman model L81801 liquid scintillation spectrometer for a period of two minutes each.

The scintillation counter measures the incorporation of ³H-hypoxanthine into parasite nucleic acid, with the counts expressed as counts per minute (CPM). The computation of the drug concentration causing 50% inhibition of ³H-hypoxanthine uptake (ID₅₀) was modified slightly from the method of Desjardin *et al.* (1979).

The computation of the ID₅₀ values for each isolate was done in duplicate. The mean CPM values for the parasitised controls were used to estimate the mid-point (Y₅₀) as shown in the formula:-

$$\text{Mid-point (Y}_{50}\text{)} = \frac{(\text{PRBC} - \text{UPRBC})}{2} + \text{UPRBC}$$

where PRBC value is the mean of the CPM values for the parasitised red blood cells controls and UPRBC is the mean of the CPM values for the unparasitised red blood cells controls.

The ID₅₀ value for each drug was then determined by interpolation between one data point above and below the Y₅₀. Interpolated values were obtained after logarithmic transformation of both concentration and CPM values, using the formula of Sixsmith *et al.* (1982, 1984) as shown:-

$$ID_{50} = \text{antilog} \left(\log X_1 + \left[\frac{(\log Y_{50} - \log Y_1) (\log X_2 - \log X_1)}{(\log Y_2 - \log Y_1)} \right] \right)$$

where X_1 and Y_1 are the drug concentrations and X_2 and Y_2 are the corresponding CPM values for the data points. The ID_{50} gives the drug concentration required for 50% inhibition of ^3H -hypoxanthine incorporation into parasite DNA.

2.2.3 Preparation of parasite DNA for pulse field gradient gel electrophoresis

2.2.3.1 Lysis of red blood cells to release parasites.

Cultures exhibiting high parasitaemia were centrifuged at 2,500 g for 5 minutes. The packed cell volume was determined and the packed cells were resuspended in twice their volume of 0.15% saponin (BDH Ltd, Poole, England) in TSE [20 mM Tris-base (Sigma Chemical Co., St Louis, MO, USA), 100 mM NaCl (May and Baker, Essex, England) and 50 mM EDTA, pH 8.0 (Heidelberg, NY, USA)] for 5 minutes at room temperature. The cells were washed twice by centrifugation in TSE for 10 minutes at 4,500 g and then 5 minutes at 3,500 g. The saponin-treated parasite pellet was dark brown due to the presence of haemozoin, the parasite pigment (Plate 3).

The parasites were suspended in TSE and an equal volume of prewarmed (50°C) 1.5% low melting point agarose (Life Technologies Inc., Gaithersburg, USA) was added. The mixture was vortexed prior to casting gel blocks using moulds placed in ice.

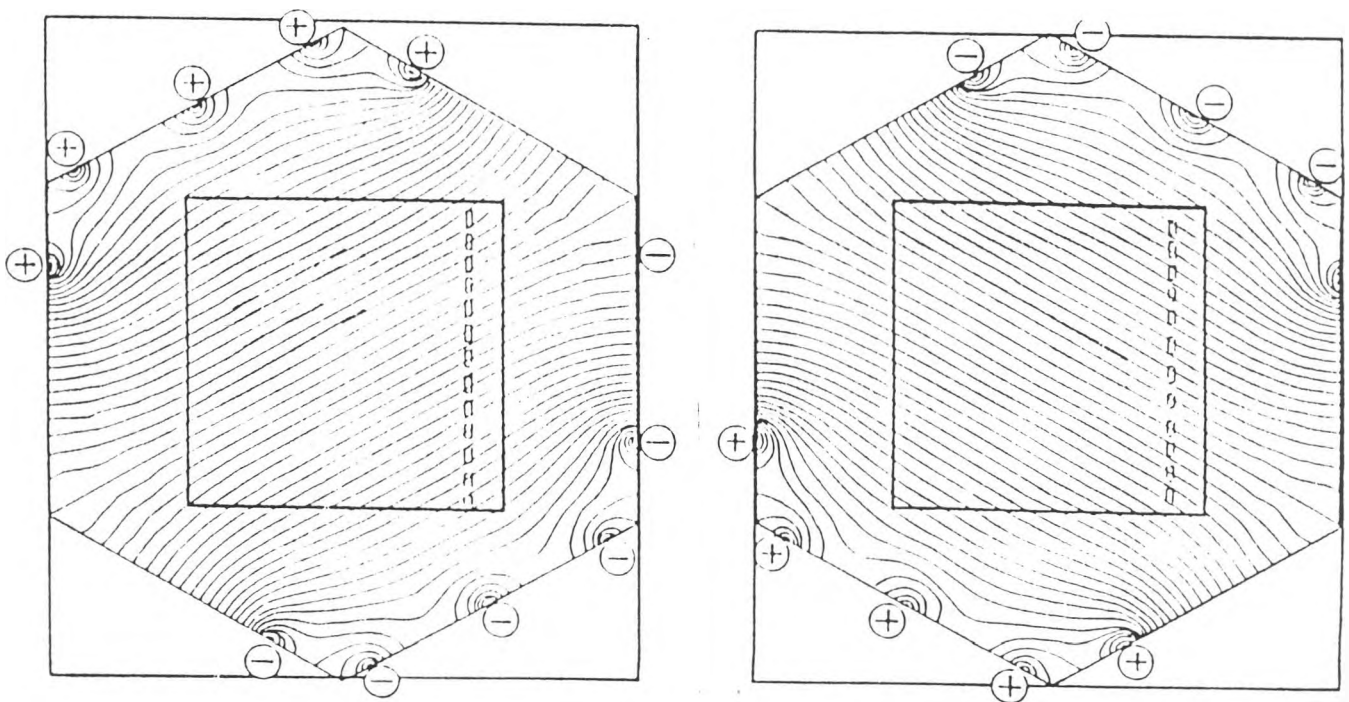
2.2.3.2 *In situ* lysis of parasites embedded in agarose.

The agarose blocks containing *P. falciparum* were incubated at 50°C for 48 hours in lysis buffer containing 5 mM EDTA (pH 8.0), 0.01% sodium dodecylsulphate

(SDS), 0.2 mM Tris-base pH 8.0 and 10 μ g/ml proteinase K (Sigma Chemical Co.) in a volume of 20 ml. After the lysis period the blocks were washed in 10 mM EDTA for 2 hours, followed by 10 mM EDTA containing 1 mM phenylmethyl sulfonyl fluoride (Sigma Chemical Company), which inactivates proteinase K, for 2 hours, followed by 10 mM EDTA six times at 15-minute intervals. They were then stored in 10 mM EDTA at 4°C (Wellems *et al.*, 1987).

2.2.4 Pulse field gradient separation of chromosome-size DNA.

Pulse field gradient electrophoresis was done using the hexagonal array electrode system (Figures 6a and 6b) of a LKB 2015 Pulsaphor electrophoresis unit (Pharmacy LKB Biotechnology, Uppsalla, Sweden) which gives a homogeneous field similar to a CHEF (contour clamped homogeneous electric field) apparatus (Chu *et al.*, 1986). Agarose blocks of size 5mm x 4mm x 1mm containing chromosome-size DNA were sealed into the wells of a 1.5% agarose gel (Life Technologies Inc., Gathersburg, USA) containing 1 x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). The chamber buffer, 1 x TBE, was recirculated at a constant temperature of 14°C at constant voltage of 120. The separations were done under the following condition: 600 seconds for 30 hours; 250 seconds for 30 hours; 180 seconds for 30 hours; and 120 seconds for 30 hours, giving a total running time of 120 hours (Plate 3). The pulse times and running hours were increased to 800 seconds for 30 hours; 600 seconds for 30 hours; 550 seconds for 30 hours; 450 seconds for 30 hours and 250 seconds for 30 hours, giving a total running time of 150 hours. The separations obtained by these are shown in Plate 4. The yeast *Saccharomyces cerevisiae* chromosomes (New England Bio Labs Inc., USA) were used as DNA size standards and their sizes are noted in the appropriate photographs.



(a) North/South Field

(b) East/West Field

Two different fields are applied, using the hexagonal array electrode. The figures show isofield lines.

Source: Pharmacia LKB Biotechnology, Uppsalla, Sweden.

Figure 6. Hexagonal array electrode system of an LKB 2015 pulsaphor electrophoresis unit.

2.2.4.1 Staining and photography of separated chromosome-size DNA.

The gels were stained with ethidium bromide (Sigma Chemical Co.) at a concentration of $1\mu\text{g/ml}$ for 20 minutes and destained for 30 minutes in distilled water. The DNA bands were photographed under illumination with ultraviolet light using a Polaroid camera and Polaroid type 667 film (Polaroid, USA).

2.2.5 Southern blot transfer.

Ethidium bromide stained gels that contained separated chromosomes were deproteinized by soaking in 0.25 N HCl for 20 minutes at room temperature. The acid was decanted and the gel rinsed several times with distilled water before adding the denaturation solution (1.5 M NaCl; 0.5 M NaOH) for 1 hour with two changes of solution. The denaturation solution was decanted and the gel was rinsed with distilled water before soaking in a neutralizing solution (3 M NaCl; 0.5 M Tris-base) for 1 hour with two changes of solution.

DNA was transferred from the gel onto a nylon filter (Hybond N, Amersham, USA) by modification of the method of Southern (1975). The filter was measured to the size of the gel, then soaked in 2x SSC (0.3 M NaCl; 0.03 M Trisodium citrate) for 5 minutes before being placed on the gel after appropriate labelling of the filter to correspond with the size of the gel. The transfer pyramid was set up as shown in Figure 7 and 10x SSC (1.5 M NaCl ; 0.15 M trisodium citrate) was used as transfer buffer. Transfer of DNA from the gel onto nylon filter, due to capillarity, continued for 48 hours.

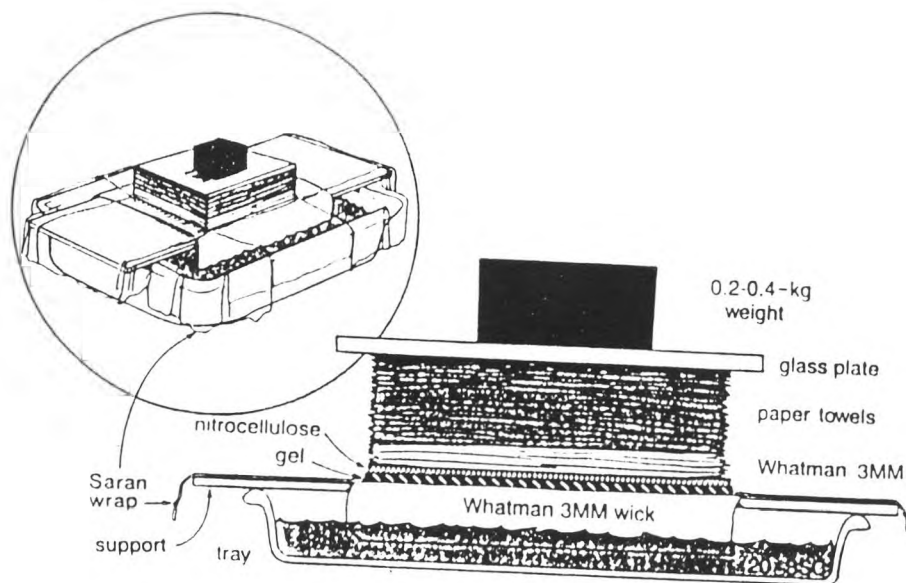


Figure 7. Southern transfer pyramid.

After 48 hours the filter was removed from the transfer pyramid, washed in 2 x SSC for 5 minutes, and dried in air. To fix and nick the DNA, each side of the filter was exposed to uv light for 2 minutes. The nylon filter was then placed in a vacuum at 80°C for 2 hours after which it was sealed in a plastic bag and stored at room temperature.

2.2.6 DNA probes for hybridization

The DNA probes used were obtained from Dr. T.E. Wellems, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, U.S.A. These probes, consisting of specific DNA segments complementary to DNA sections in various *P. falciparum* chromosomes (Table 3), were contained in plasmids.

The SL series probes were inserted in either pC DNAlI or pUC 13 plasmids. PMBB₂ was inserted in pUC 19 plasmids (Annie Walker - Jonah, personal communication).

The probes were amplified by transformation of *Escherichia coli* and then selected on Luria-Bertani medium (Appendix) containing ampicillin. Competent JM109 *E. coli* cells were used for the amplification experiments (Maniatis *et al.*, 1982; Panyim *et al.*, 1985).

2.2.6.1 Transformation of competent cells.

The frozen competent cells were thawed out at 4°C. 25-50 nanograms of plasmid DNA were added followed by 100 mM CaCl₂; these were mixed by vortexing before incubation on ice for 30 minutes, followed by heat shock for 2 minutes in a 43°C water bath. 1ml of Luria-Bertani medium was added to the reaction tube which was then incubated for 1 hour at 37°C. After 1 hour 100 µl of the *E. coli* culture was spread on agar plates containing 40 µg/ml ampicillin. The plates were incubated inverted overnight at 37°C (Maniatis *et al.*, 1982).

Table 3. Chromosomal markers

Probes	Chromosomes marker
SL-3	4
SL-33	5
PMBB ₂	8
SL-6	10
SL-5	13
SL-1	14

2.2.6.2 Small scale isolation of plasmids.

A single colony from an agar plate was picked and inoculated into 5 ml of Luria-Bertani medium culture medium containing ampicillin (50 $\mu\text{g/ml}$) in disposable 15ml centrifuge tubes. The cultures were incubated overnight at 37°C in a shaking water bath after which the cells were pelleted by centrifugation in Eppendorf tubes at 30,000 g.

The supernatant was discarded and 100 μl of a freshly prepared lysozyme solution (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA and 20 mg/ml lysozyme) was added, the pellet was resuspended and then incubated on ice for 10 minutes. After the incubation period 200 μl of lysis solution (1% SDS, 0.2N NaOH) was added, contents were mixed gently by inverting the tube two or three times, the tube was left on ice for 10 minutes after which 150 μl of neutralizing solution (3 M sodium acetate solution, pH 4.8) was added. The contents of the tube were mixed well and then placed in a freezer at -70°C for 10 minutes, where a white precipitate formed. The suspension was centrifuged for 15 minutes at 40,000 g and the supernatant was transferred into a fresh tube. An equal volume of phenol/chloroform (1:1 v/v) was added, the mixture was vortexed and then centrifuged for 2 minutes before transferring the supernatant to a fresh tube.

To the supernatant, 0.1 volume sodium acetate solution (3 M, pH 4.8) and 2 volumes of cold ethanol were added and the mixture was placed in a freezer at -20°C for 1 hour. The DNA was pelleted by centrifugation at 30,000 g for 10 minutes. The supernatant was removed and the tube placed in an inverted position to drain excess liquid. The pellet was washed twice with 70% ethanol before being dried briefly in a vacuum dessicator. The dried pellet was then dissolved in an appropriate volume of TE (Tris-HCl 10 mM, pH 7.5; 0.1 mM EDTA).

2.2.6.3 Isolation of probes from plasmids.

The DNA samples were freed from RNA by digestion using RNAase (10mg/ml in 10 mM Tris-Hcl, pH 7.5; 15 mM NaCl). The concentration of DNA in the plasmid preparation was determined by optical density measurements using a spectrophotometer (DuPont,USA). The DNA consisted of circular transformed plasmid DNA. The purified plasmid preparations were digested using restriction endonucleases. Single digestion to linearise the plasmids was done using *Hind* III for all the different vectors. The reaction mixtures were set up as follows:

x μ l DNA (volume equivalent to 10 μ g of DNA)
 1 μ l 10x *Hind* III buffer (medium salt buffer)
 y μ l of distilled water (to add up mixture to 10 μ l)
1 μ l of *Hind* III
 10 μ l Total volume reaction mixture incubated for 3 hours at 37°C.

Double digestion to release the inserts from the plasmids was done using the restriction endonucleases *Xho*I and *Bam* HI for the SL-series under the following conditions:

x μ l DNA (volume equivalent to 10 μ g of DNA).
 2 μ l 10x High salt buffer
 y μ l of distilled water (to add up mixture to 20 μ l).
 1 μ l of *Xho*I
1 μ l of *Bam* HI
 20 μ l.

The reaction mixture was incubated for 3 hours at 37°C. Double digestion of the PMBB₂ plasmid was done under similar conditions, with the enzymes *Eco*RI and *Pst*I used in a high salt buffer.

After the double digestion, a minigel was run on a horizon 58 minigel apparatus (Bethesda Research Laboratories Inc., USA). 20 μ l of the double digests were loaded in the large wells of a 1% low melting point agarose gel. The gel was run at 40 volts for 2¹/₂ hours using 1 x TAE, pH 8.0 (40 mM Tris acetate; 1 mM EDTA), containing 0.5 μ g/ml ethidium bromide (Sigma Chemical Company). The gel was visualised

under uv and then photographed (Plate 1). A slice of agarose containing the small fragment (insert) of the double digest was cut out and put into an eppendorf tube containing 1ml of 20 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0 and warmed at 65°C to melt the agarose. After cooling to room temperature the DNA was phenol extracted and ethanol precipitated overnight at -20°C. The DNA was pelleted by centrifugation at 18,000 g for 10 minutes. The DNA pellet (*P. falciparum* probe) was dissolved in TE (0.001 mM Tris-HCl; 0.001 mM EDTA, pH 7.5) and kept at 4°C.

2.2.7 Hybridization of probes to *P. falciparum* Southern blots.

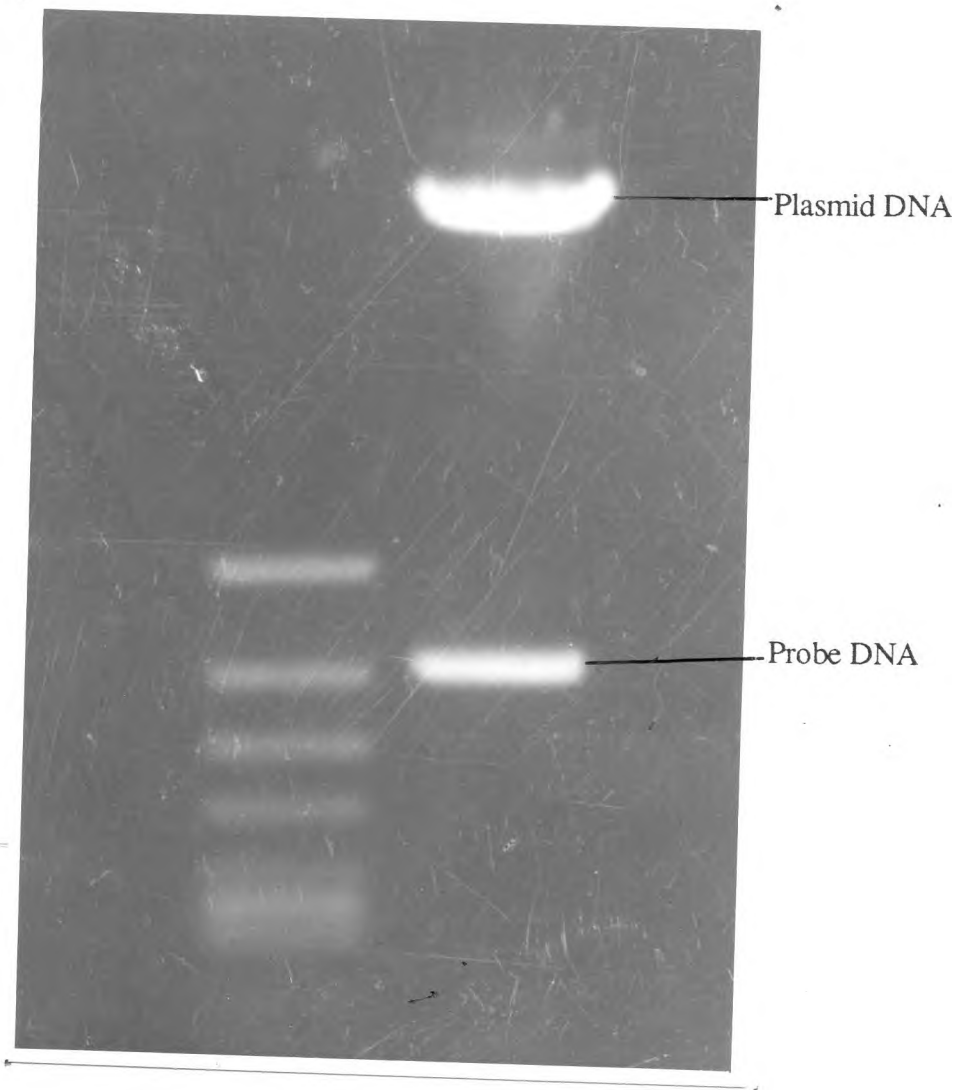
2.2.7.1 Labelling of probes for hybridization.

The labelling of the *P. falciparum* chromosomal markers and the oligo dihydrofolate reductase probe with radioactive phosphorus-32 was attempted using different methods. The random primer extension technique (Feinberg and Vogelstein 1983) and the nick translation technique (Maniatis *et al.*, 1982) were used in an attempt to label chromosomal probes. The oligonucleotide kinase technique (Maniatis *et al.*, 1982) was used to try to label the 5' end of the oligo dihydrofolate reductase gene probe. The non-radioactive enhanced chemiluminescence technique (Amersham, USA) was used to label the chromosomal probes.

2.2.7.2 Hybridization of Southern blots.

The hybridization of the chromosomal and dihydrofolate reductase gene probes was attempted using the methods of Maniatis *et al.* 1982.

Plate 1. Separation of probe from plasmid after double digestion with restriction endonucleases.



CHAPTER 3

RESULTS

3.1 *In vitro* response of 10 Kenyan *P. falciparum* isolates to four antimalarial drugs.

The activity of the four antimalarial drugs studied against 10 *P. falciparum* isolates are shown in Tables 4 and Figure 8. The ID₅₀ values were used to assign susceptibility and for the purpose of the study an arbitrary value of 100 nanomoles was used as a cut-off point between "sensitive" (<100nM) and "resistant" (>100nM) for pyrimethamine and sulphadoxine and 10nM for cycloguanil and chlorcycloguanil (Watkins, W.M. 1991, personal communication). The terms sensitive and resistant are only used relatively.

Considerable variation in antimalarial drug sensitivity was found among the 10 Kenyan isolates of *P. falciparum* studied to the four drugs. This was confirmed by Kruskal-Wallis one way non-parametric analysis of variance. The probability level was 0.01. The non-parametric multiple comparison by simultaneous test procedure (Sokal and Rohlf, 1969), an *a posteriori* test for equal sample sizes confirmed that there were significant differences in the potency of the two closely related biguanides and pyrimethamine, and between them and sulphadoxine. The two biguanides had similar potencies, while pyrimethamine and sulphadoxine had similar potencies. The isolates from the Rift Valley were found to be the least susceptible to the antimalarial drugs, followed by the isolates from Nyanza province. The isolates from Coast province were the most susceptible to the antimalarial drugs.

Using the previously described criteria to assign susceptibility, it was found that out of the ten isolates six were resistant to pyrimethamine, six to cycloguanil, four to chlorcycloguanil, and nine to sulphadoxine. (Table 4).

The isolate K67 was sensitive to all the four antimalarials and had the lowest ID₅₀ value for each of the four drugs. ENT36 was resistant to the four drugs. ENT36

was the isolate most resistant to pyrimethamine with an ID₅₀ value that is 10,000 times that of K67, the most sensitive isolate to the drug (Table 4).

In the case of cycloguanil, the most resistant isolate was ENT36 with an ID₅₀ value 100 times more than that for K67. ENT36 was the most resistant isolate to chlorcycloguanil, with an ID₅₀ value 500 times more than for K67 the most sensitive isolate. ENT41 was the isolate most resistant to sulphadoxine with an ID₅₀ value 700 times than for K67, the most sensitive isolate (Table 4).

To compare possible correlations (or cross-resistance) between the antimalarial drugs used against the 10 isolates of *P. falciparum* examined, the correlation coefficient was calculated among the drug pairs. The highest correlation was between cycloguanil and chlorcycloguanil, which are closely related biguanides (Table 5). there was also significant correlation between pyrimethamine and the two biguanides. These three inhibit the same enzyme dihydrofolate reductase. The correlation between the three dihydrofolate reductase inhibitors and sulphadoxine, which inhibits dihydropteroate synthetase, was not significant. The exact t-test for existence of correlation in small samples confirmed these results at probability level 0.05 with 8 degrees of freedom (Table 5).

Linear regression analysis was done among the dihydrofolate reductase inhibitors which had significant correlation coefficients. The regression lines which were obtained after logarithmic transformation of the ID₅₀ values are shown in figures 9, 10 and 11. The regression coefficient indicated the quality of fit achieved by the regression and values closer to 1.00 indicated a better correlation (or cross resistance) than values close to zero. Therefore, the drug pairs which generated linear regression curves with regression coefficient greater than 0.6 were considered to be correlated in their activities. The greatest regression coefficient value was between cycloguanil and chlorcycloguanil (Figure 11). The regression coefficient value between pyrimethamine and cycloguanil was 0.447 which is not significant (Figure 9) and that between pyrimethamine and chlorcycloguanil was 0.43 which is not significant (Figure 10).

Table 4. *In vitro* sensitivity of 10 Kenyan isolates of *P. falciparum* to four antimalarial drugs: Mean ID₅₀ values (nanomoles).

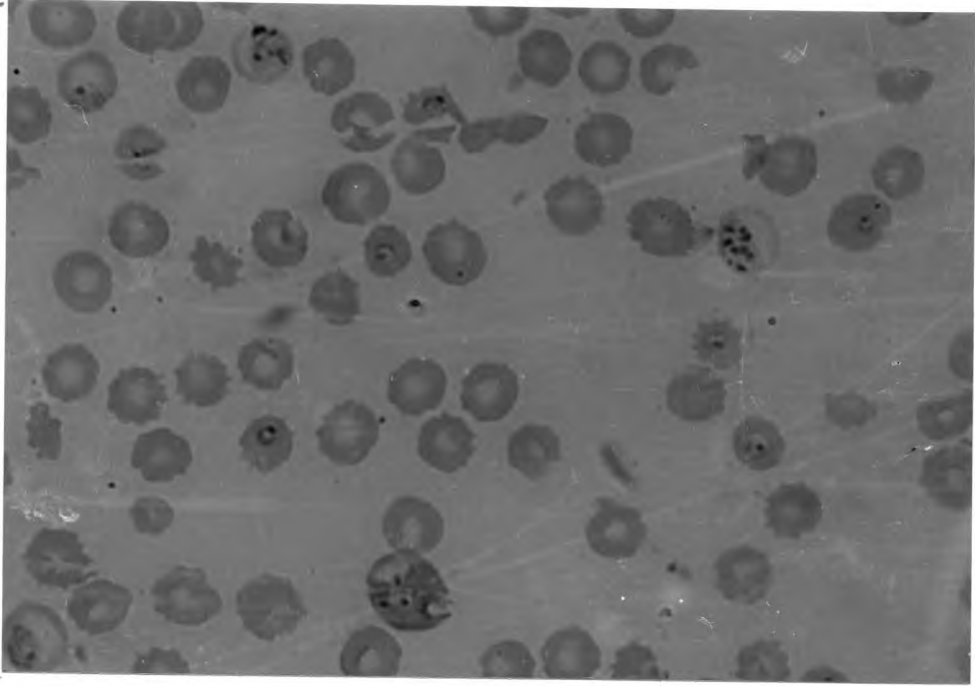
Isolate	Pyrimethamine	Cycloguanil	Chlorcycloguanil	Sulphadoxine
JP62	3.2±1.5	4.2±1.75	0.72±0.33	1439.0±0.6
JP119	236.0±8.5	14.0±1.13	4.00±1.3	187.0±0.65
M24	1.8±0.2	1.3±0.3	0.72±0.2	458.3±30.49
KIL9	352.0±1.43	3.0±1.8	5.66±0.83	173.0±5.8
K67	0.3±0.13	0.9±0.2	0.3±0.14	30.8±8.35
K39	2601.0±15.6	53.0±7.43	14.0±7.43	902.0±3.48
S158	308.0±2.3	28.1±0.61	0.62±1.1	109.6±2.6
ENT30	476.0±4.2	41.3±0.8	17.0±4.73	387.0±0.82
ENT36	3064.0±11.6	117.2±1.62	145.34±2.6	1856.0±16.0
ENT41	23.5±1.3	15.8±2.1	21.0±0.8	23161.0±24.4

± = standard deviation

Table 5. Coefficient of correlation between different drugs tested against 10 Kenyan *P. falciparum* isolates.

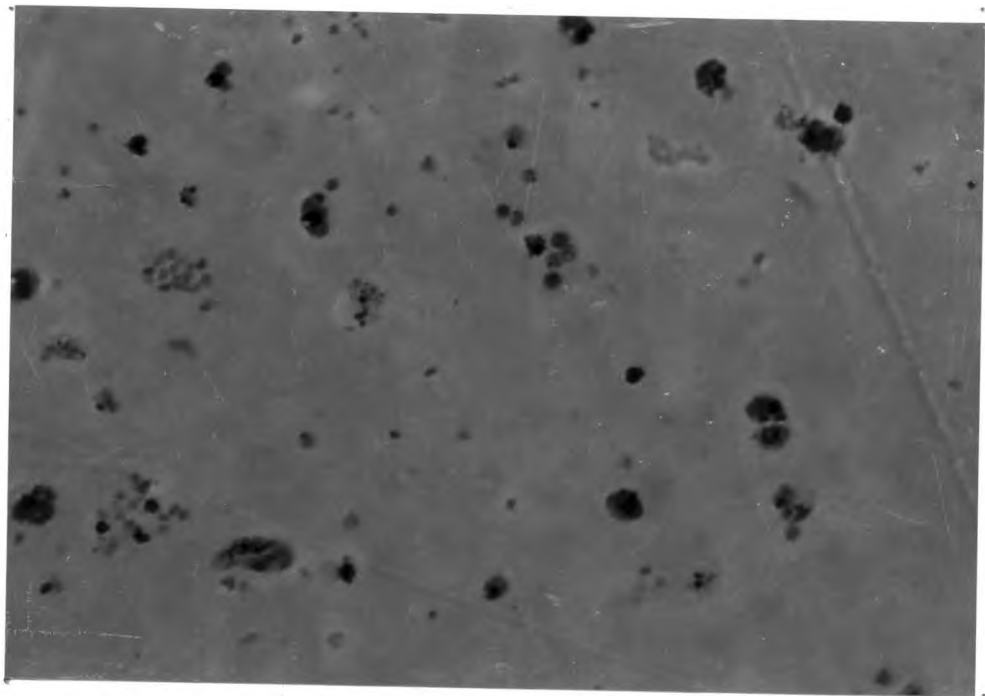
Drugs	Coefficient of correlation (r)	Significance with 8 degrees of freedom at 0.05 p
Pyrimethamine / cycloguanil	0.899	significant
Pyrimethamine / chlorcycloguanil	0.760	significant
Pyrimethamine / sulphadoxine	0.096	not significant
Cycloguanil / chlorcycloguanil	0.905	significant
Cycloguanil / sulphadoxine	-0.050	not significant
Chlorcycloguanil / sulphadoxine	0.062	not significant

Plate 1. *Plasmodium falciparum* in culture.



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Plate 2. *Plasmodium falciparum* Parasites after saponin lysis.



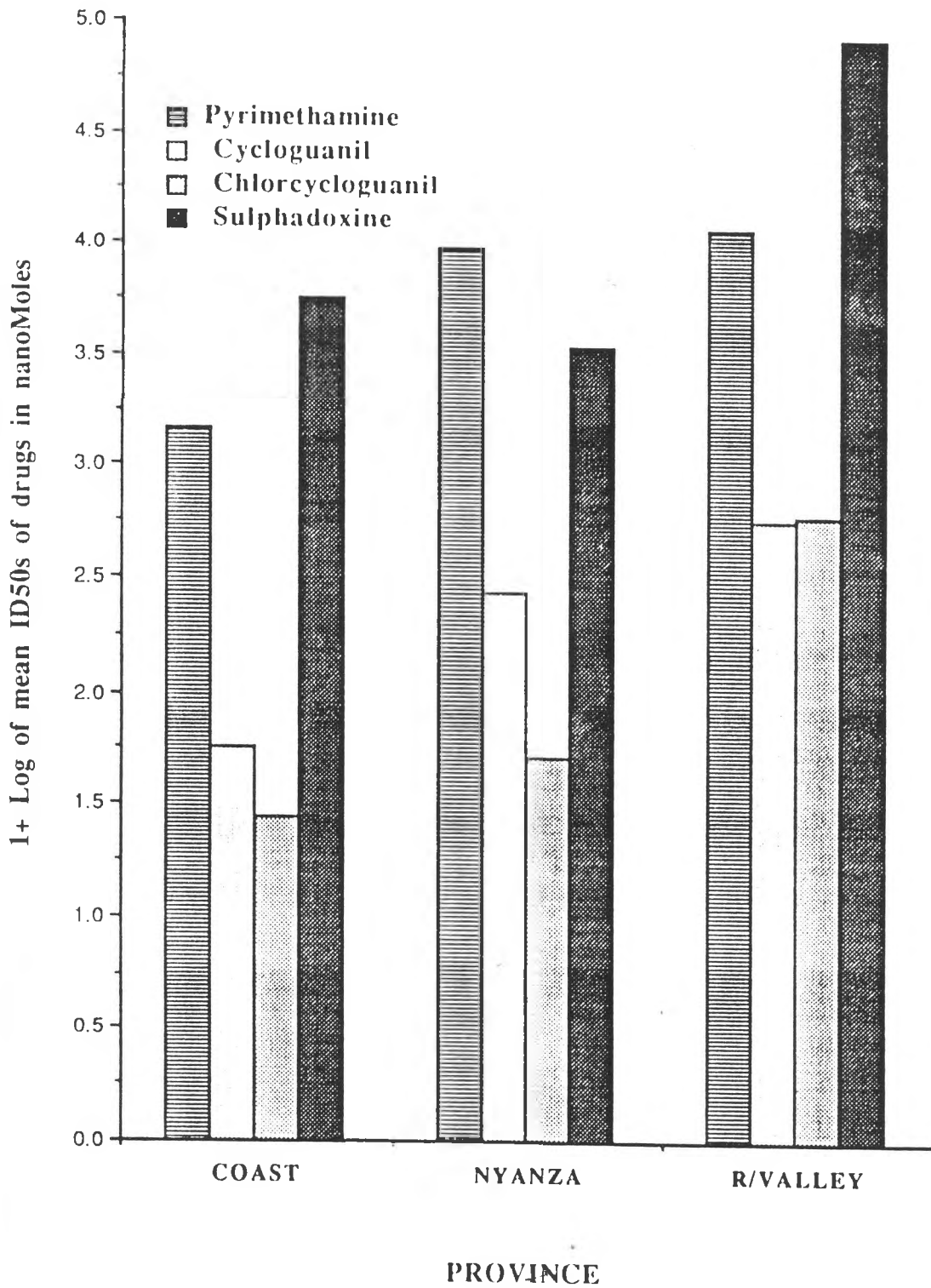


Figure 8. Mean *in vitro* sensitivities of *P. falciparum* isolates from the Coast Nyanza and Rift Valley provinces of Kenya to four antimalarial drugs.

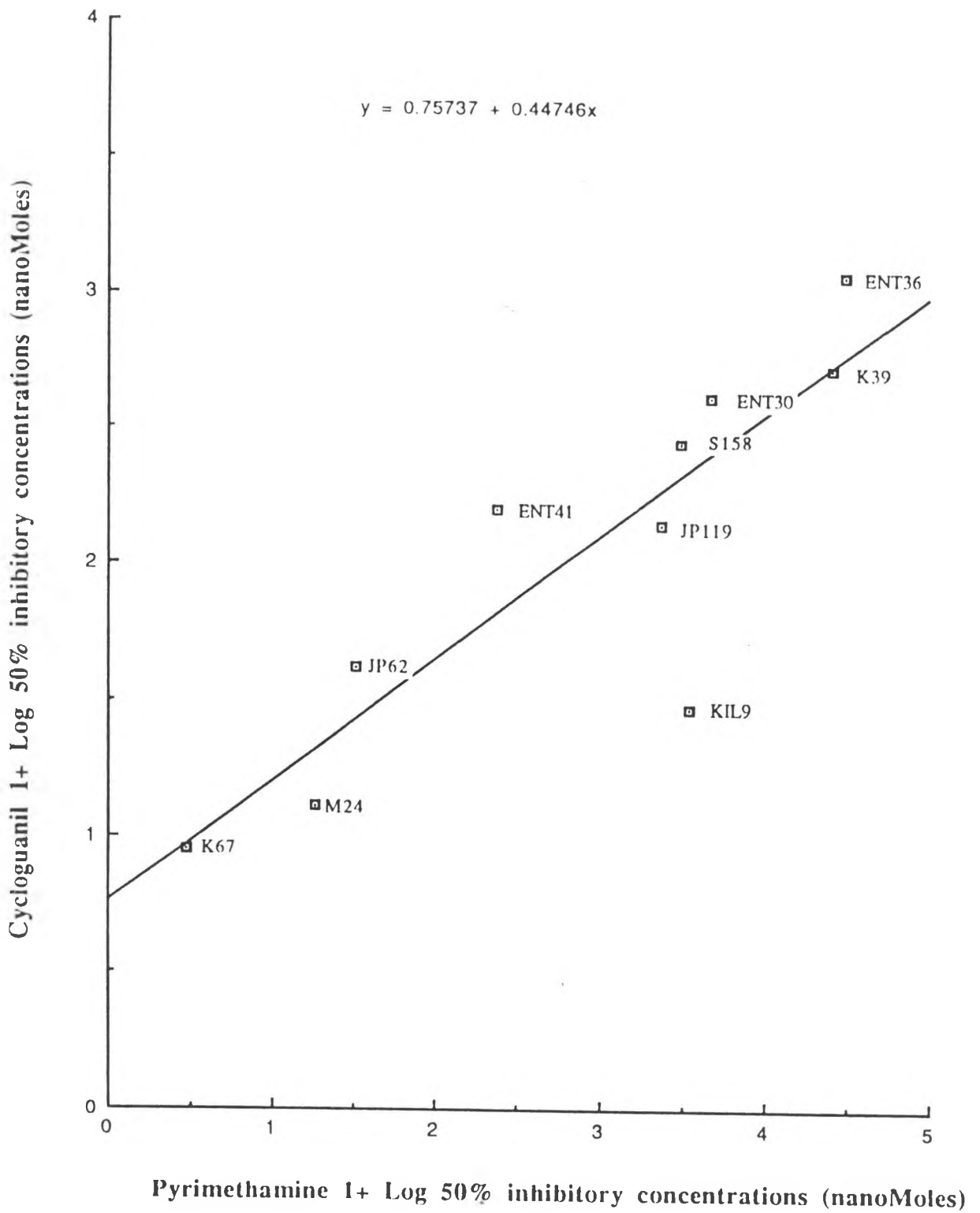


Figure 9. Regression graph obtained after logarithmic transformation of ID₅₀ values obtained in *in vitro* sensitivities of ten *P. falciparum* isolates from Kenya to pyrimethamine and cycloguanil.

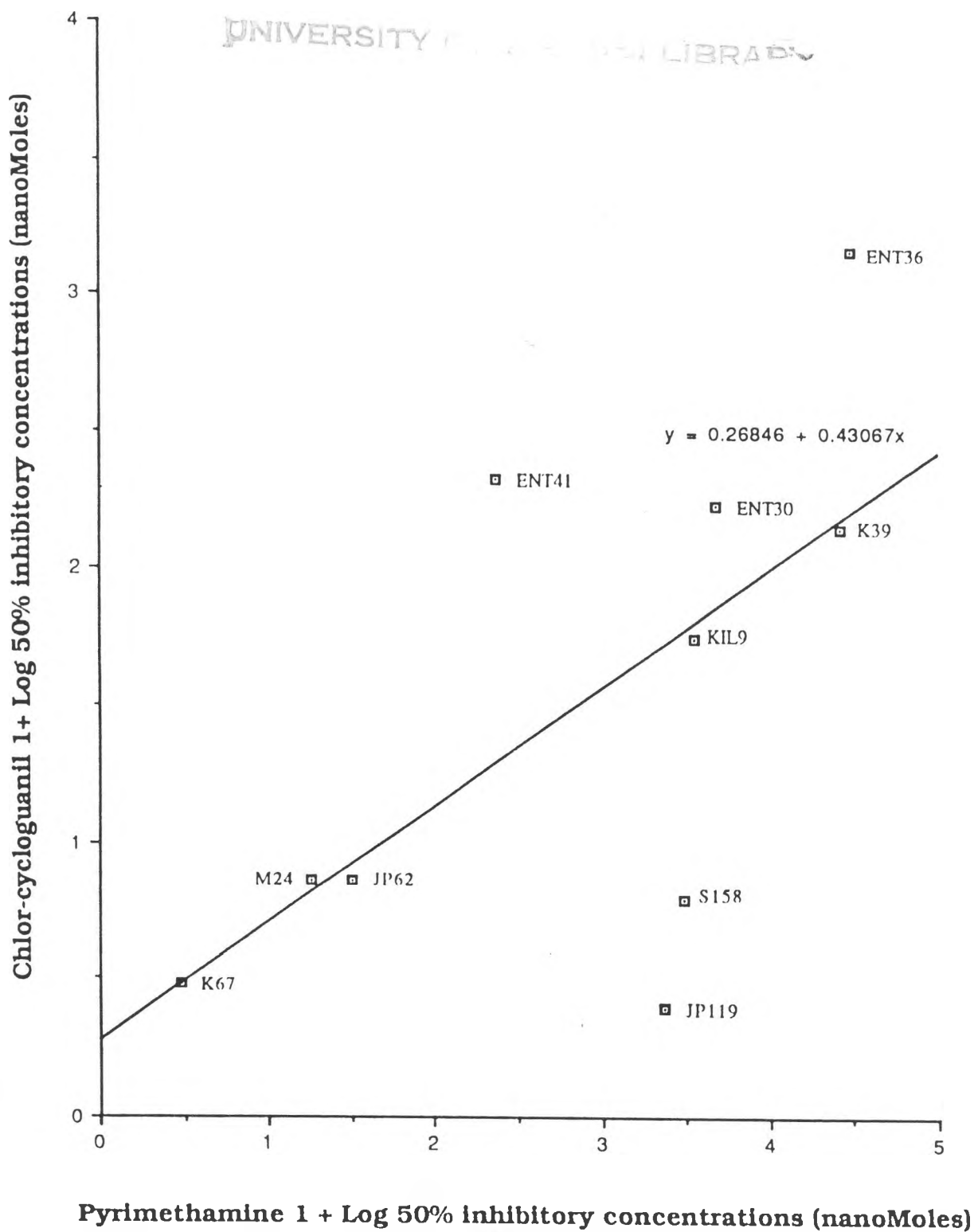


Figure 10. Regression graph obtained after logarithmic transformation of ID_{50} values obtained in *in vitro* sensitivities of ten *P. falciparum* isolates from Kenya to pyrimethamine and chlorcycloguanil.

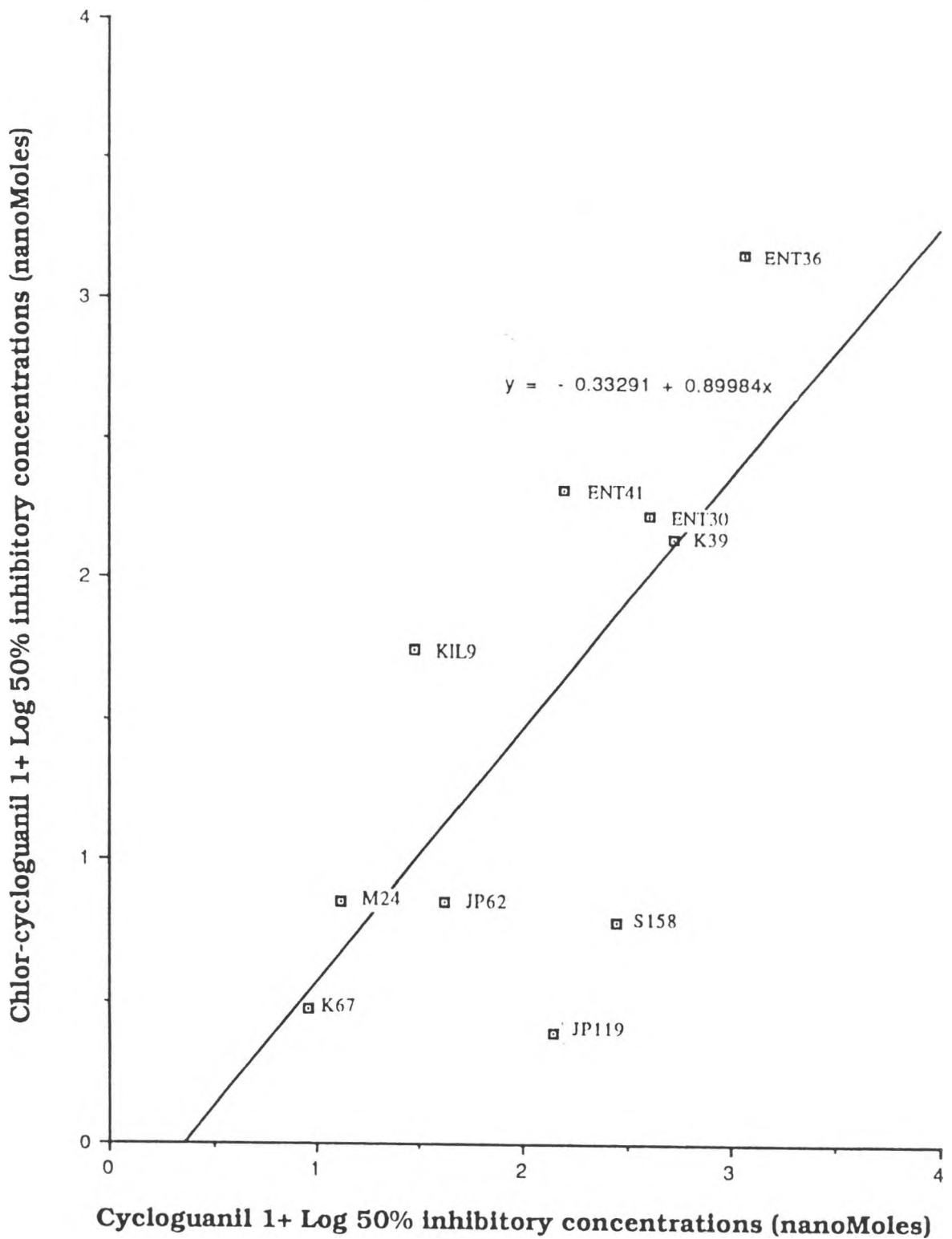


Figure 11. Regression graph obtained after logarithmic transformation of ID₅₀ values obtained in *in vitro* sensitivities of ten *P. falciparum* isolates from Kenya to chlorcycloguanil and cycloguanil.

3.2 Pulse field gel electrophoresis of *P. falciparum* chromosomes.

Results of the chromosomal analysis of the 10 Kenyan isolates of *P. falciparum* examined are shown in Plates 4 and 5. Initial attempts were made to resolve chromosomal DNA from *P. falciparum* using pulse times of 600 seconds for 30 hours, 250 seconds for 30 hours, 180 seconds for 30 hours and 120 seconds for 30 hours. Under these conditions the chromosome-size DNA separated into 9-11 bands (Plate 4) and there seem to be two compression zones, at the 2600-2900Kb and the 1600Kb regions of the chromosomal profile where chromosomes were co-migrating.

The running conditions were increased to 800 seconds for 30 hours, 600 seconds for 30 hours, 550 seconds for 30 hours, 500 seconds for 30 hour 450 seconds for 30 hours and 250 seconds for 30 hours. These conditions managed resolve the chromosomal bands up to a maximum of 16 bands for one isolate and a constant number of 14 chromosome bands for most of the isolates (Plate 5). The chromosome separations were repeated five times and the results obtained were similar under the same running conditions.

The chromosomal markers for estimating the sizes of the *P. falciparum* chromosomes were *Saccharomyces cerevisiae* chromosomes digested with different restriction endonucleases to give fragments of varying sizes. The four markers, CF13, CF14, pichia 1A and lambda concatamers were run alongside the *P. falciparum* chromosomes (Plates 4 and 5). The lambda concatamers were not separated by the running conditions used in obtaining the separations in Plate 4. In CF13 the smallest fragment had 900 kilobase pairs (kbp) and the largest fragment 2900kbp. In CF14 the smallest fragment has 295kbp and the largest fragment 2100kbp. In pichia 1A the smallest fragment has 900kbp and the largest fragment 3200kbp.

With the increase in the pulse times and running hours the resolution of the chromosomal bands was increased to 16 for one isolate, ENT36, suggesting a mixed population within the isolate, and a constant number of 14 chromosome bands for most of the other isolates (Plate 5). The multiplicity of an evenly staining bands in the ENT36 track further indicated a mixed population of parasites within the isolate.

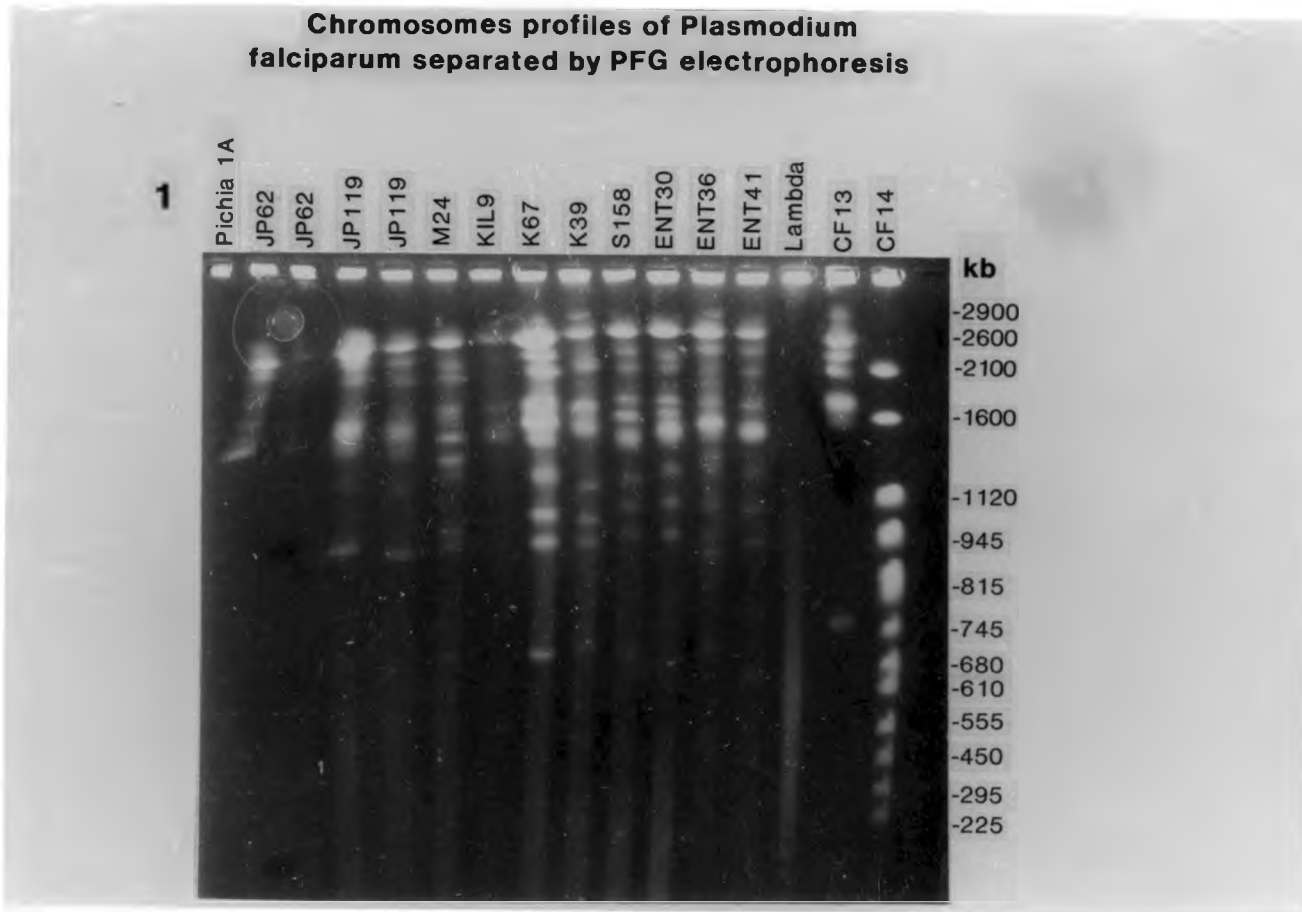
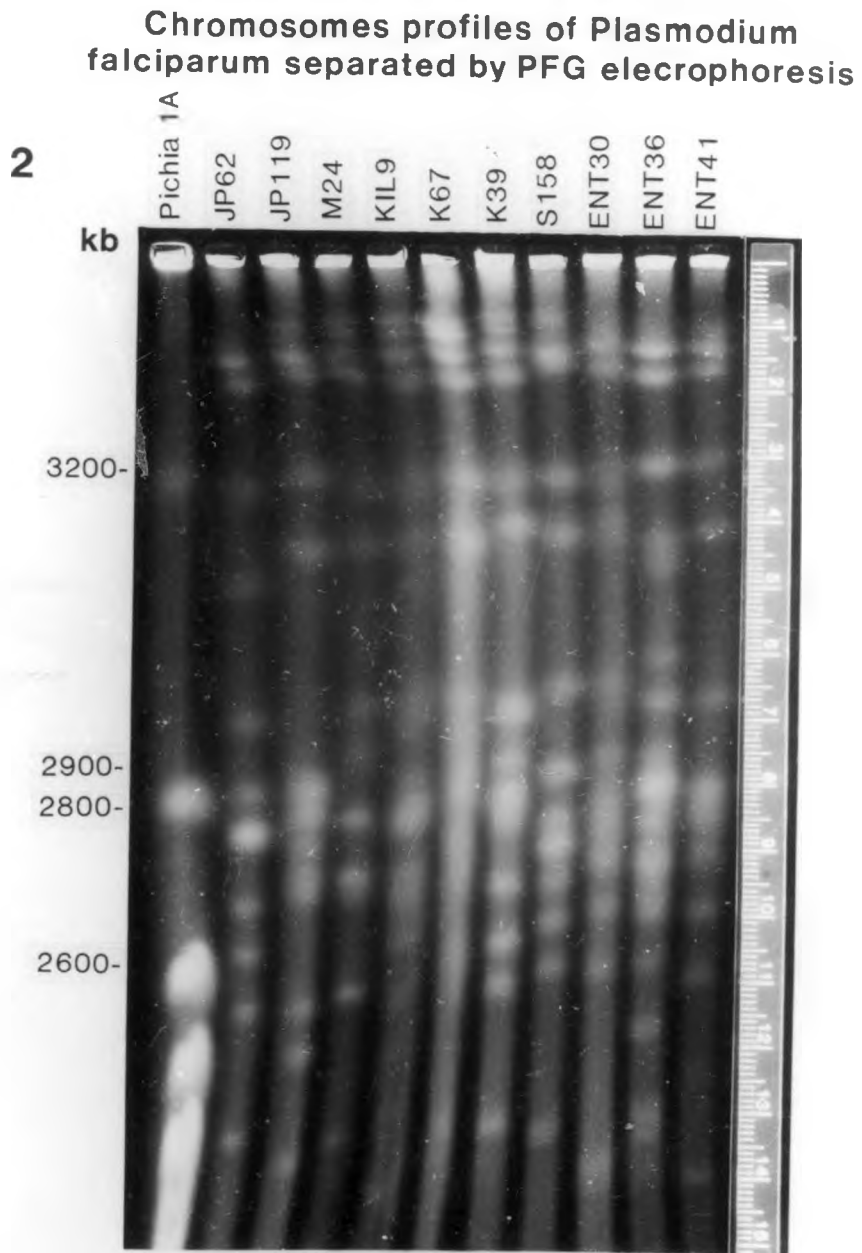
Plate 4. PFGE separation of *Plasmodium falciparum* chromosome-size DNA

Plate 5. PFGE separation of *Plasmodium falciparum* chromosome-size DNA

It was not possible to determine precisely the sizes of the chromosomes of the different isolates, although from the comparison with yeast maker chromosomes subjected to PFGE at the same time, the chromosome sizes of the isolates appeared to range from 580kb to 4450kb. The significant aspect of these results is the extent of parasite karyotype diversity in that no two isolates, not even those from the same geographical region had an identical karyotype.

The differences in the molecular karyotypes were not the result of preparation artifacts or modification at the DNA level such as DNA glycosylation or structural changes. The separation patterns of the isolates were reproducible and therefore degradation or aggregation of DNA molecules did not occur.

3.3. Hybridization patterns of *P. falciparum* chromosomes to specific probes.

The hybridization experiments were unsuccessful, with poor labelling of the probes when using P³². With the enhanced chemiluminescence technique (ECL), hybridization of the labelled probes to the chromosomal profiles was unsuccessful, probably due to the poor sensitivity of the technique.

3.4. Correlation of drug response and chromosomal profiles of Kenyan isolates of *P. falciparum*.

The isolates from the Rift Valley province were the least susceptible to most of the antimalarial drugs tested followed by the isolates from Nyanza province and Coast province (Table 4 and Figure 8). Correlation could not be determined between susceptibility of the isolates to the drugs and the observed chromosomal profiles, due to the failure to label probes with P³² and to hybridize the ECL labelled probes to Southern transferred chromosomes.

CHAPTER 4

DISCUSSION

4.1 *In vitro* susceptibility of 10 Kenyan *P. falciparum* isolates to pyrimethamine, cycloguanil, chlorcycloguanil and sulphadoxine.

Dihydrofolate reductase antagonists play a major role in chemotherapy and they are used against a variety of organisms ranging from tumour cells to parasitic protozoa and bacteria. Pyrimethamine and proguanil (active metabolite cycloguanil) and chlorproguanil (active metabolite of chlorcycloguanil), which are DHFR antagonists, have played a major role in the prophylaxis and treatment of *P. falciparum* infections. They are often used in combination with sulphonamide drugs such as sulphadoxine, which inhibit the enzyme dihydropteroate synthetase, and are now being reassessed as malaria prophylactics in view of the present spread of resistance of *P. falciparum* to chloroquine. There is need to study the epidemiology of resistance to these drugs because they are widely recommended for prophylaxis (WHO, 1988)

In vitro drug susceptibility assays are useful for epidemiological purposes and for evaluating new antimalarial compounds (Desjardins *et al.*, 1979). *In vitro* and *In vivo* systems for evaluation of the drug susceptibility of the same *P. falciparum* isolates have been found to produce similar results (Nguyen-Dinh *et al.*, 1982; Spencer *et al.*, 1983; Watkins *et al.*, 1988b). It is possible the susceptibility in the *in vitro* in this study reflects the *in vivo* susceptibility of the 10 Kenyan *P. falciparum* isolates studied.

The Kenyan isolates examined showed considerable variation in their sensitivities to the different antimalarial drugs studied (Table 4 and Figure 8). These results have shown that resistance to the DHFR inhibitors is multifocal and is found in all three geographical regions studied. The high frequency of resistance found is not

surprising since resistance has been reported before among some isolates from these areas (Nguyen-Dinh *et al.*, 1982) It is probable that DHFR resistant genotypes are continuing to spread at the expense of the sensitive ones in Kenya, although this process can be only be confirmed by continuing monitoring.

Watkins *et al.*(1988a) reported that the Malindi isolate M24 was sensitive to pyrimethamine and that the Kisumu isolate K39 was resistant. The two reference isolates K39 and M24 were retested during this study. The results confirmed the earlier report, thus indicating the reliability and validity of antimalarial drug test systems used during the study. The results of the drug sensitivity tests also confirmed that the phenotype is not lost during long term storage in liquid nitrogen or *in vitro* continuous culture.

The coefficients of correlation between the pyrimethamine / cycloguanil, pyrimethamine / chlorcycloguanil and cycloguanil / chlorcycloguanil were high and significant. However it is not clear whether correlation represents cross resistance or whether an accumulation of several independent mechanisms conferring resistance have occurred, even though the molecular basis of resistance to pyrimethamine and cycloguanil in *P. falciparum* has been demonstrated to involve different mutations in the dihydrofolate reductase - thymidylate synthetase genes (Peterson *et al.*, 1990). The low insignificant regression coefficient between pyrimethamine and the two biguanides, shown in figures 9 and 10, compared to the highly significant regression coefficient between the two biguanides would therefore conform to this.

Malaria resistant to pyrimethamine is common in Kenya (Nguyen-Dinh *et al.*, 1982; Spencer *et al.*, 1984) and the present study has shown that 6 out of the 10 isolates had low susceptibility to the drug as indicated by ID₅₀ values greater than 100 nM (Table 4). Among the six isolates, only ENT 36 was resistant to sulphadoxine and such double resistance may occur when they are use in combination, as Fansidar. However Fansidar is an effective treatment for most *P. falciparum* malaria attacks in Kenya at present (Watkins *et al.*, 1988a).

The marked variation in response *in vitro* to pyrimethamine, cycloguanil and chlorcycloguanil and the high level of unsusceptibility to sulphadoxine suggests that in natural populations resistance to each drug arises independently. There is good evidence demonstrating that pyrimethamine resistance is due to point mutations in the *P. falciparum* dihydrofolate reductase gene (Cowman *et al.*, 1988; Peterson *et al.*, 1988; Zolg *et al.*, 1990) and also that there are different point mutations that confer differential resistance to cycloguanil and pyrimethamine in *P. falciparum* malaria (Peterson *et al.*, 1990). It therefore seems unlikely that multidrug resistance to a variety of different drugs, as has been demonstrated for the mammalian P-glycoprotein drug efflux pump by Endicott and Ling (1989), accounts for the various types of antifolate and sulphonamide resistances seen in Kenyan isolates.

Most forms of drug resistance have a genetic basis (Walliker, 1980, 1982; Molineaux, 1986) and antimalarial drug sensitivity test data can probably be used for *P. falciparum* strain characterization. The genetic basis and stability of these characteristics can only be confirmed after transmission of the parasites through mosquitoes (Rosario and Thaithong, 1986).

4.2. Genomic organisation of 10 Kenyan *P. falciparum* isolates.

The occurrence of different karyotype forms in Kenyan *P. falciparum* isolates demonstrated by the present study indicates that different strains of the parasites occur in the different endemic areas studied. This study has shown that the chromosomal bands range in number from 10 to 16. Previous work had shown that the genome of *P. falciparum* could be resolved into 14 chromosomal bands by pulse field gel electrophoresis (Langsley and Ponnudurai, 1988). In the case of isolates having less than 14 chromosomal bands, it is possible that some chromosomes are very similar in size and they co-electrophorese. Those isolates having 15 or 16 chromosomal bands (KIL9 and ENT36, respectively) are likely to be mixed populations; that is isolates

containing more than one parasite strain. The multiplicity of unevenly staining bands on the ENT36 track further indicated a mixed population of parasites within the isolate.

The molecular karyotypes of *P. falciparum* are known to be highly polymorphic (Kemp *et al.*, 1987) with genetically homologous chromosomes having considerable size range. It has been shown further that in some instances deletion might be responsible for changes in size, and this could involve either repetitive DNA (Van der Ploeg *et al.*, 1985) or coding sequences and sub-telomeric regions (Corcoran *et al.*, 1986; Pologe and Ravetch, 1986).

A significant aspect of the results of the present study is the extent of parasite diversity. Most chromosomes, particularly the smaller ones, exhibited extensive size polymorphism and no two isolates, not even those from the same geographical region shared an identical chromosomal karyotype (Plates 4 and 5). The smaller chromosomes (chromosomes 1 to 7) varied very markedly in size. They may do so because they contain a larger than normal amount of repetitive DNA and significant deletions can be tolerated. This situation has already been described for the human Y chromosome (Schmid, 1985). Frequent recombinations could account for the diversity of the genotypes within a region.

Further studies were carried out to look for specific amplification of the DHFR gene but they were unsuccessful due to the non-labelling of the chromosome-specific and DHFR gene probes with P^{32} and non-hybridization of the ECL-labelled chromosomal probes to the Southern transferred chromosomes. Therefore it was not possible to distinguish between sensitive and resistant isolates on the basis of their chromosomal profiles alone.

The extensive polymorphism amongst homologous chromosomes coupled with the large numbers of parasites in a single infected person may explain the adaptability of the parasite when subjected to selective pressure such as that imposed by the immune system of the host or antimalarial drugs. The basic events leading to the development of drug-resistant *Plasmodium* are probably mainly due to mutations. Mutations not only probably account for the occurrence of considerable variation among strains of a

species in sensitivity to various types of drugs, but they also provide a degree of heterogeneity in populations of parasites and thus set the stage for resistant strains to emerge either spontaneously, which subsequently are selected by drug pressure.

The likelihood of drug-resistant strains of *Plasmodium falciparum* developing depends on several factors, including the pharmacological properties of the chemotherapeutic agent. Peters (1967) suggested that the slope of the dose response curve, rate of action and types of susceptible life cycle stages could be influential. If a drug has a relatively flat dose-response curve larger amounts are required to eradicate all parasites than are necessary for almost complete suppression of the parasitaemia, and may exceed the safe dose. Therefore conventional doses may fail to eradicate parasites at the extreme end of the spectrum of drug insensitivity. If a drug acts slowly or only against certain stages of asexual development the possibility of arrested forms, which may or may not be destroyed by the host's immune system, is increased. The drugs sharing the features referred to above include pyrimethamine, cycloguanil, chlorcycloguanil and sulphadoxine and they are prone to permit the emergence of resistant forms. It is noteworthy that these drugs act specifically by inhibiting nucleic acid synthesis; and the emergence of resistance to pyrimethamine in *P. falciparum* was associated with the use of the drug.

The differences in the endemicity patterns in the various malarious regions could also account for the variation in drug susceptibility. It has been reported that drug resistance tends to emerge in situations of relative intense transmission (epidemics) but not as much as in endemic areas with immunity in the human population (Molineaux, 1986). Entasopia is an area of seasonal malaria, with higher transmission during the rainy season (Watkins *et al.*, 1988b). Nyanza and Coast provinces are regions of hyper to holoendemic malaria with transmission all the year round. It is possible that most of the residents of the localities in Nyanza and Coast provinces are immune to malaria compared to the residents of Entasopia who are not immune. Therefore, in immune populations, drugs act in concert with the immune system killing most of the parasites. The overall effect is a smaller gene pool of

resistant parasites in endemic areas. The different patterns of transmission and levels of immunity could explain the differences in susceptibility to the drugs especially between Entasopia and the other two localities.

The extensive chromosome size polymorphism even within a single endemic area may be important to the efforts aimed at controlling *P. falciparum* infection. Depending on the frequency with which a change in the karyotype of the parasite is actually accompanied by a change in its phenotype, the fluidity of the *P. falciparum* genome could complicate efforts to control the disease by drugs or molecular vaccines.

4.3. CONCLUSIONS

Five conclusions can be drawn from the results of the present study:

1. There is marked variation in the sensitivity of the 10 Kenyan isolates of *P. falciparum* studied to the antimalarial drugs assayed. The isolates from the Rift Valley province were the least susceptible to the four antimalarial drugs studied, followed by the isolates from Nyanza province. The isolates from Coast province were the most susceptible.
2. Among the dihydrofolate reductase inhibitors studied cycloguanil and chlorcycloguanil had the same potencies but they were more potent than pyrimethamine and sulphadoxine. Pyrimethamine had the same potency as sulphadoxine which inhibits dihydropteroate synthetase but their correlation coefficient was not significant.. There was a high and significant correlation coefficient between cycloguanil and chlorcycloguanil, and also between them and pyrimethamine.
3. Pyrimethamine-sensitive and pyrimethamine-resistant isolates of *P. falciparum* cannot be differentiated on the basis of their molecular karyotypes alone.

4. Genetic diversity of the Kenyan *P. falciparum* isolates occurs both within and between the three provinces of Kenya. No two isolates, even within the same geographical region, had identical molecular karyotypes.
5. Genetically diverse strains are present in Kenyan isolates of *P. falciparum*, as shown by pulse field gradient gel electrophoresis and drug susceptibility results.

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Appendix

Composition of solutions used in the study.

Denaturation solution	1.5 M sodium chloride 0.5 M sodium hydroxide made up in distilled water
Elution buffer	10 mM Tris-HCl pH 7.4 10 mM EDTA made up in distilled water
Ethidium bromide	10 mg/ml in distilled water
Giemsa stain	Giemsa powder 3 g Glycerol 250 ml Absolute methanol 250 ml
High salt buffer 10x	500 mM Tris-HCl, pH 7.4 100 mM Magnesium Chloride 10 mM Dithiothreitol made up in distilled water
Loading buffer	0.04% Bromophenol 20% Glycerol made up in distilled water.
Low salt buffer 10x	100 mM Tris-HCl, pH 7.5 100 mM Magnesium Chloride 100 mM Dithiothreitol made up in distilled water

Luria-Bertani (LB) medium	1% Tryptone 0.5% Yeast extract 10 mM Sodium Chloride 1.5% Bactoagar 50 µg/ml ampicillin made in distilled water
Lysis buffer	5 mM EDTA, pH 8.0 0.01% SDS 0.2 mM Tris-HCl, pH 8.0 10 µg/ml Proteinase K made up in distilled water
Lysozyme solution	50 mM glucose 25 mM Tris-HCl, pH 8.0 10 mM EDTA 20 mg/ml, lysozyme made up in distilled water
Medium salt buffer 10x	50 mM Sodium Chloride 10 mM Tris-HCl, pH 7.5 10 mM Magnesium Chloride 1 mM Dithiothreitol made up in distilled water
Neutralization solution	3 M Sodium Chloride 0.5 M Tris-HCl, pH 7.5 made up in distilled water
RNAse solution	10 mg/ml RNAse 10 mM Tris-HCl, pH 7.5 made up in distilled water

Rowes cryosolution	2.5% Glycerol 3% Sorbitol 0.35% Sodium Chloride
RPMI 1640 medium	10.4 g RPMI powder 5.94 g HEPES buffer solid 960 mls autoclaved distilled water
SDS 1% solution	1 g Sodium lauryl sulfate 100 ml distilled water
Sodium bicarbonate solution (5%)	5 g anhydrous sodium bicarbonate 100 ml distilled water
Standard saline citrate 20x	3 M Sodium Chloride 0.3 M Tri-Sodium citrate made up in distilled water
TAE (Tris-Acetate) 1x	40 mM Tris-acetate 1 mM EDTA made up in distilled water
TBE (Tris-HCl; Boric acid; EDTA)	1.0 M Tris-HCl, pH 7.5 1.0 M Boric acid 2.0 mM EDTA made up in distilled water
TE (Tris-HCl ; EDTA)	10 mM Tris-HCl, pH 7.5 0.1 mM EDTA made up in distilled water

TSE (Tris-HCl; Sodium Chloride; EDTA) 20 mM Tris-HCl, pH 7.5
100 mM Sodium Chloride
50 mM EDTA
made up in distilled water