ASSESSMENT OF SENSITIVITY OF *P. FALCIPARUM* TO ANTIFOLATE ANTIMALARIALS IN SOUTHERN GHANA BY POLYMERASE CHAIN REACTION (PCR) ASSAY SYSTEMS.

A THESIS SUBMITTED

BY

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DECLARATION

The experimental work described in this thesis was carried out by me, at the DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF GHANA, LEGON under the supervision of Prof. F.N. Gyang. I declare that this work has not been and is not being submitted for any other degree.

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DEDICATION

To my parents MR & MRS FRANCIS K. MAK-MENSAH for their love, care and financial support and especially to my sister AKPENE for her visits.

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ABBREVIATION

A (A260)	-	absorbance (absorbance at 260nm)
bp	-	base pair
Cyc-R	-	cycloguanil-resistant parasites
Cyc-S	-	cycloguanil-sensitive parasites
dATP	-	deoxyadenosine tri-phosphate
dCTP	-	deoxycytidine tri-phosphate
dGTP	-	deoxyguanosine triphosphate
dTTP	-	deoxythymidine tri-phosphate
dTMP	-	deoxythymidine mono-phosphate
dNTPs	-	mixed deoxy nucleotide triphosphate (dATP, dCTP, dGTP, and dTTP)
dUMP	-	deoxyuridine mono-phosphate
DNA	-	Deoxyribonucleic acid
DHFR	-	Dihydrofolate-reductase
Fig.	-	Figure
M	-	molar =mol/litre = mol/dm^3
Min	-	minute
mM	-	millimolar
mg	-	milligramme
ml	-	millilitre
PCR	-	Polymerase Chain Reaction
PNS	-	PCR negative samples

PPS - PCR positive samples

Pyr-R - Pyrimethamine-resistant parasites

Pyr-S - Pyrimethamine-sensitive parasites

s - Seconds

TDR - Tropical Diseases Research

ug - microgramme

ul - microliter

uv - ultraviolet

ABSTRACT

Drug treatment of malaria is being hampered by the emergence of drug resistant strains of malaria parasites. Rapid methods for monitoring patients' response to treatment, applicable to field conditions, would aid management and control of drug resistant *P.falciparum* infections.

In this study, mutation specific polymerase chain reaction assays using 3'-mismatched oligonucleotide primers which annealed to the wild or mutated parasites gene encoding dihydrofolate reductase-thymidylate synthase (DHFR-TS) were used to survey *P.falciparum* strains in two Regions of Southern Ghana (Volta and Greater Accra Regions). Mutations were identified directly from blood samples obtained from patients attending Out Patients Departments in Hospitals. Amplified *P.falciparum* DHFR-TS nucleotide sequences of 337bps were obtained when reaction products were analyzed by electrophoresis.

Of 151 smear positive samples analysed, 13 (8.6%) contained the Asn-108 codon AAC that confers pyrimethamine resistance, 133 (88%) samples contained only the wild type Ser-108 codon AGC and none contained the Val-16 codon GTA found in cycloguanil-resistant pyrimethamine sensitive parasites. Out of the 13 pyrimethamine resistant cases, 10 were found in samples obtained from the Volta Region while the rest (3) were from Korle-Bu Teaching Hospital in the Greater Accra Region. No PCR product was found in 5 (3.3%) of the 151 samples. After second (nested) PCR, only one sample showed amplified product: contamination was negligible.

PCR amplification of the DHFR-TS presents a rapid alternative to *in vitro* drug testing for monitoring the resistance of *P. falciparum* to antifolate antimalarials.

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Malaria continues to be the infectious disease with the greatest impact on mankind. It is responsible for many health problems such as fever, headache and muscular pains among others. Malaria is often described as a great imitator of other diseases, sharing various characteristics with other childhood illnesses such as influenza, tuberculosis, typhoid, brucellosis and urinary tract infection. Even more confusing, it may coexist with other diseases (Commey, 1989). Besides, it is the leading cause of morbidity and mortality in Ghana (Ahmed, 1989 & 1992).

Chemotherapy is essential in malaria management for as much as it reduces mortality and morbidity by eliminating malaria parasites from human hosts. But rapid spread of multidrug resistant strains of the parasite has brought about changes in the demand for antimalarials.

Antifolate antimalarials consist of sulfonamide, sulfones and dihydrofolate reductase (DHFR) inhibitors (Zolg et al, 1990).

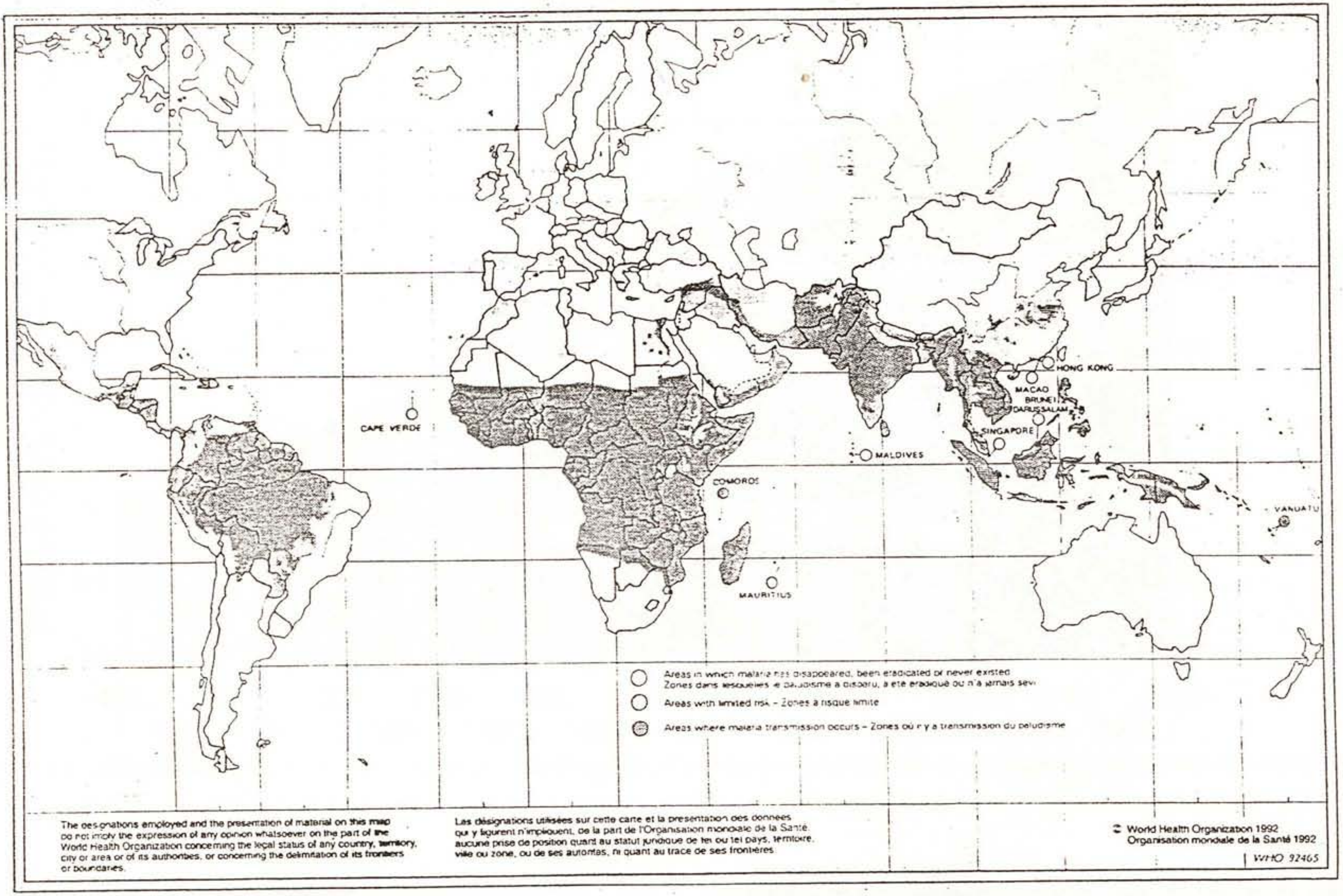
DHFR inhibitors include pyrimethamine, proguanil and chlorproguanil. They are primary tissue schizontocides,effective against the pre-erythrocytic stages of the malaria parasites hence eliminate infection before the merozoites are liberated into the blood stream. They,therefore,have causal prophylactic action which may be effective even when blood schizonts are resistant to these agents.

Pyrimethamine and cycloguanil (active metabolite of proguanil) are structural analogs (Fig 7). Their antiplasmodial activity is due to the inhibition of the parasites' DHFR which exists as a bifunctional enzyme with thymidylate synthase (TS). DHFR catalyses the reduction of dihydrofolate to tetrahydrofolate which is convertible to folate coenzymes involved in purine, pyrimidine, amino acid metabolism and in the initiation of protein synthesis. Therefore, inhibition of DHFR results in inhibition of cell division and schizogony leading to cell death.

Various combinations of sulfones or sulfonamide with DHFR inhibitors are being promoted and increasingly used for the prevention and treatment of chloroquine resistant *falciparum* malaria. However, recent observations indicate that strains of *P.falciparum* resistant to these combinations have appeared in South East Asia, South America (Peterson *et al.*, 1991) and some parts of Africa (Schapira, 1984).

Sequence analyses of DHFR from pyrimethamine-resistant and-sensitive *P.falciparum* show that amino acids 16, and 108 play important roles in the development of resistance to antifolates. Parasites with a point mutation from Ser-108 to Asn-108 are resistant to pyrimethamine with only a slight decrease in susceptibility to cycloguanil. Mutations causing change from Ala-16 to Val-16 and from Ser-108 to Thr-108 are responsible for parasites' resistance to cycloguanil (Peters *et al.*, 1973 and Inselburg *et al.*, 1987).

Current worldwide distribution of malaria



The designations employed and the presentation of material on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

Les désignations utilisées sur cette carte et la présentation des données qui y figurent n'impliquent, de la part de l'Organisation mondiale de la Santé, aucune prise de position quant au statut juridique de tel ou tel pays, territoire, ville ou zone, ou de ses autorités, ni quant au tracé de ses frontières.

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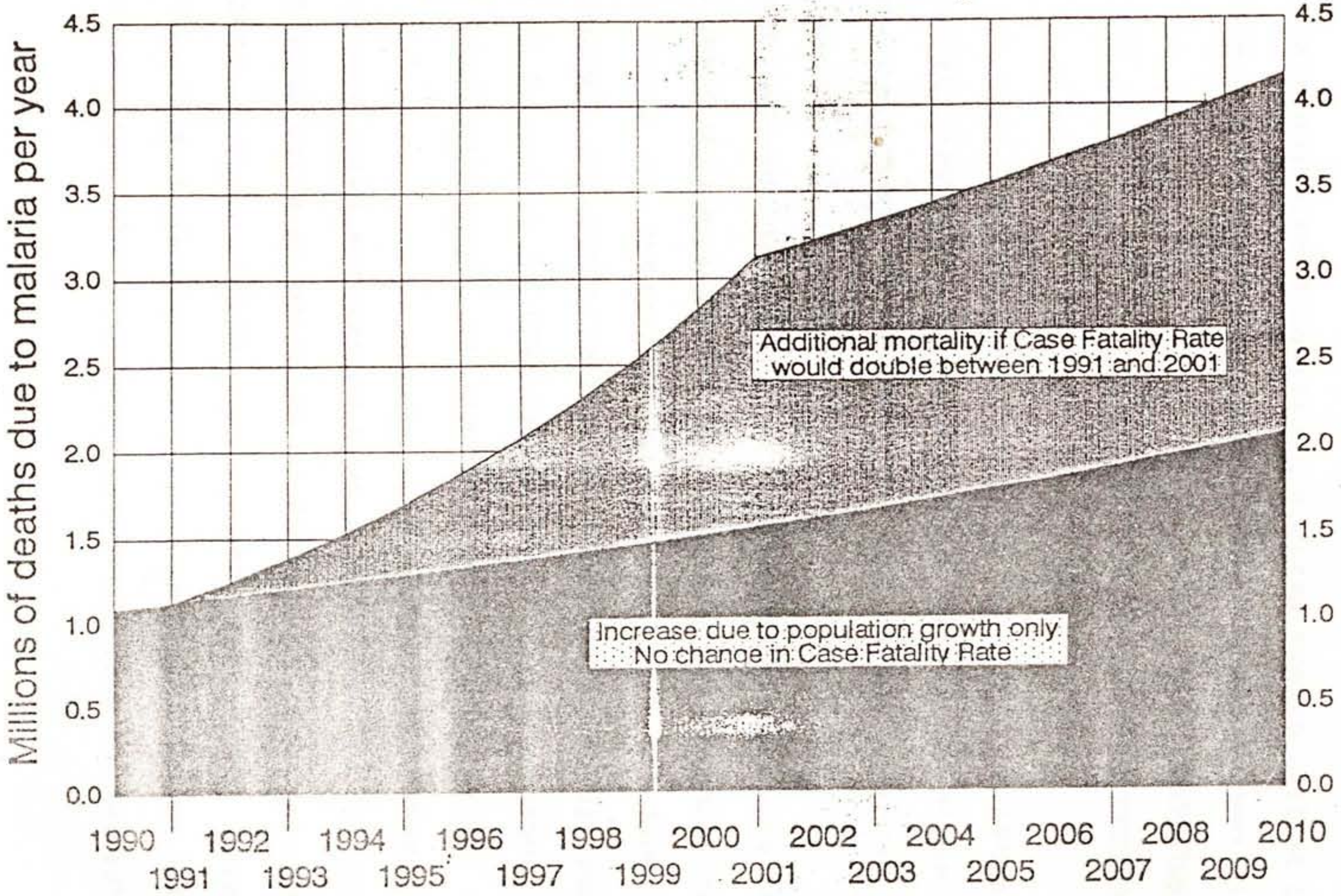
Fig.1

Fig.2
Worst case scenario for malaria mortality



The specific codon sequences of these mutations have not been found to vary among resistant *P.falciparum* isolates from South America, Africa and South East Asia (Gyang *et al.*, 1992). Resistant-parasites could therefore be detected by using mutation specific primers with 3'-mismatches in a polymerase chain reaction (PCR) assay (Gyang *et al.*, 1992).

Constant monitoring and assessment of susceptibility of *P.falciparum* to antimalarials in our environment is needed so as to obtain additional information about the effectiveness or otherwise of existing drugs. This will help clinicians to prescribe alternative but equally appropriate drug combinations in order to provide more effective prevention and control of *falciparum* infection.

The aim of this project is to assess the sensitivity of malaria parasites to antifolate antimalarials in Southern Ghana by PCR assay systems.

1.2 LITERATURE REVIEW

1.2.1 EPIDEMIOLOGY

Malaria is one of the most wide-spread diseases in the world today. About 3 billion of the World's 5 billion population live in "malarious areas" mainly in tropical Africa (Fig 1) where some 500 million people are at risk (Deming *et al.*, 1989). Some 300 million people are believed to be infected with malaria parasites with 90% of them living in tropical Africa (TDR, 1993).

Malaria claims over 1.1 million lives annually, out of which 1 million (de Raadt, 1992) including 800,000 under 5 years are from Africa (Browne, 1994). In the Volta Region of Ghana it was responsible for 59% of all reported deaths in children and 19.6% in all age groups in 1993 (Seadzi and Nyonator, 1994). With the current population growth rate, even without an increase in malaria mortality rate, the number of deaths due to malaria could double to 2 million a year worldwide by the year 2010 (Fig. 2, TDR, 1993).

Malaria accounts for 20-25% of all hospital admissions in Africa although only 8-25% of persons with malaria visit health institutions (Brinkmann and Brinkmann, 1991). In Ghana, it is the leading cause of morbidity, responsible for 40% of all out patients' visits (Ahmed, 1992), 43% in Volta Region (Seadzi and Nyonator, 1994) and 38% in Greater Accra Region (Adadey, 1993).

The spread of resistance to antimalarial drugs among parasites has compounded the seriousness of the malaria menace. Development of virgin territories and civil strife are additional aggravating factors (Browne, 1994).

1.2.2 **AETIOLOGY**

Malaria is a mosquito-transmitted blood infection caused by parasitic *plasmodia*. Human malaria parasites belong to the zoological order of *Haemosporidia* family: *plasmodiidae*, genus: *Plasmodium* (Bruce-Chwatt, 1982) with a characteristic of undergoing one cycle of asexual division in the tissues (exoerythrocytic schizogony) and another cycle of pigment producing asexual division in the red blood cells (erythrocytic schizogony) of the vertebrate host. Besides, they undergo a sporogonic development (sporogony) in the body of the mosquito (malaria vector).

The *Plasmodia* species are *P.falciparum, P.malariae, P.vivax* and *P.ovale*. Two make relatively minor contributions to the sum of human suffering caused by malaria. *P.malariae* undergoes a prolonged period of development in the mosquito vector. This greatly limits its transmissibility. *P.ovale* has a limited geographic distribution while *P.vivax*, and *P.falciparum* on the other hand are pathogens of great importance (McGregor, 1982).

P.falciparum and *P.vivax* are wide-spread throughout the World and have the ability, given suitable circumstances, to spread rapidly and cause severe, incapacitating illnesses. Of these 2 parasites, *P.falciparum* is more dangerous because untreated infections frequently terminate in death, while untreated infections of *P.vivax* are rarely lethal.

The commonest malaria parasite in Ghana is *P.falciparum*. Records of out patient attendances in some rural health centres indicate that between 1981 and 1987, 97.9% of the malaria parasites identified were *P.falciparum* and 0.8% *P.malariae* (Ahmed, 1989). *P.falciparum* rates in various parts of Ghana were found to be between 90 and 98% (Coulburne and Wright,1995) *P.ovale* which is rare was identified only once. However, mixed infections of *falciparum* and *malariae* accounted for 1.2% (Ahmed, 1989).

The biological characteristics of *P.falciparum* are significantly different from those of the other human malaria parasites. These account for the peculiar, often bizarre clinical features and pathology of its malaria (Commey, 1989).

The differences include, i) internal sporulation with *P.falciparum* completing the latter part of its asexual cycle in the capillaries where the parasitized red cells adhere to each other (Eling and Souerwein, 1995) and to the endothelial lining (Commey, 1989) of small vessels resulting in varying degrees of obstruction, ii) marked invasiveness from the rapid rate of parasite multiplication where parasite density is only limited by the number of red blood cells and iii) "asynchronicity" of parasite multiplication accounting for the unpredictable and frequently irregular temperature presentation (Commey, 1989).

1.2.3. **ENTOMOLOGY**

Human malaria is only transmitted by the female anopheline mosquito. Surveys in Accra have demonstrated the importance of *An. gambiae* as an urban malaria vector (Chinery, 1969). Chinery (1984) documented the adaptation of *An.gambiae* to breed in water-filled domestic containers, an important factor in the maintenance of urban malaria transmission.

In the southern part of the Volta Region high rates of malaria prevalence were associated with high densities of *An.gambiae* and *An.funestus* resting in houses and feeding predominantly on man. *An.melas* which breeds in lagoons was also present. Vector infection rates ranged from 0 to 21% (Ahmed, 1992).

Malaria transmission requires that an infected human and a susceptible one be accessible to the mosquito. The mosquito is normally confined to a flight range of up to 2km (Agyepong *et al.*, 1992). The closer humans are to the breeding places, the higher the contact between them and the vector.

Transmission in the Volta Region is year-round, with seasonal peaks, associated with rainy seasons from April to June and September to October. The situation is similar in the Greater Accra Region. Other factors affecting malaria transmission are relative humidity and temperature.

Seasonal variations in relative humidity disturb sporogony (development of parasites in the mosquito from gametocyte in the gut to sporozoite in the salivary gland) even at required temperatures. Sporogony of *P.vivax* ceases below 16°C and that of *P.falciparum* below 20°C (Agyepong *et al.*, 1992). The required time for sporogony is longer where the temperature is lower. For example, *P.vivax* requires 55 days for sporogony at 16°C as compared to 7 days at 28°C.

The incidence of malaria is highest in the early and later stages of the rainy seasons when there are collections of water in empty tins as well as pools and puddles (Osei and Anteson, 1989). Rainfall or surface water creates breeding places for mosquitoes. However, heavy rainfall may flush the breeding places of mosquitoes and prevent breeding.

1.2.4 LIFE CYCLE OF *PLASMODIUM*.

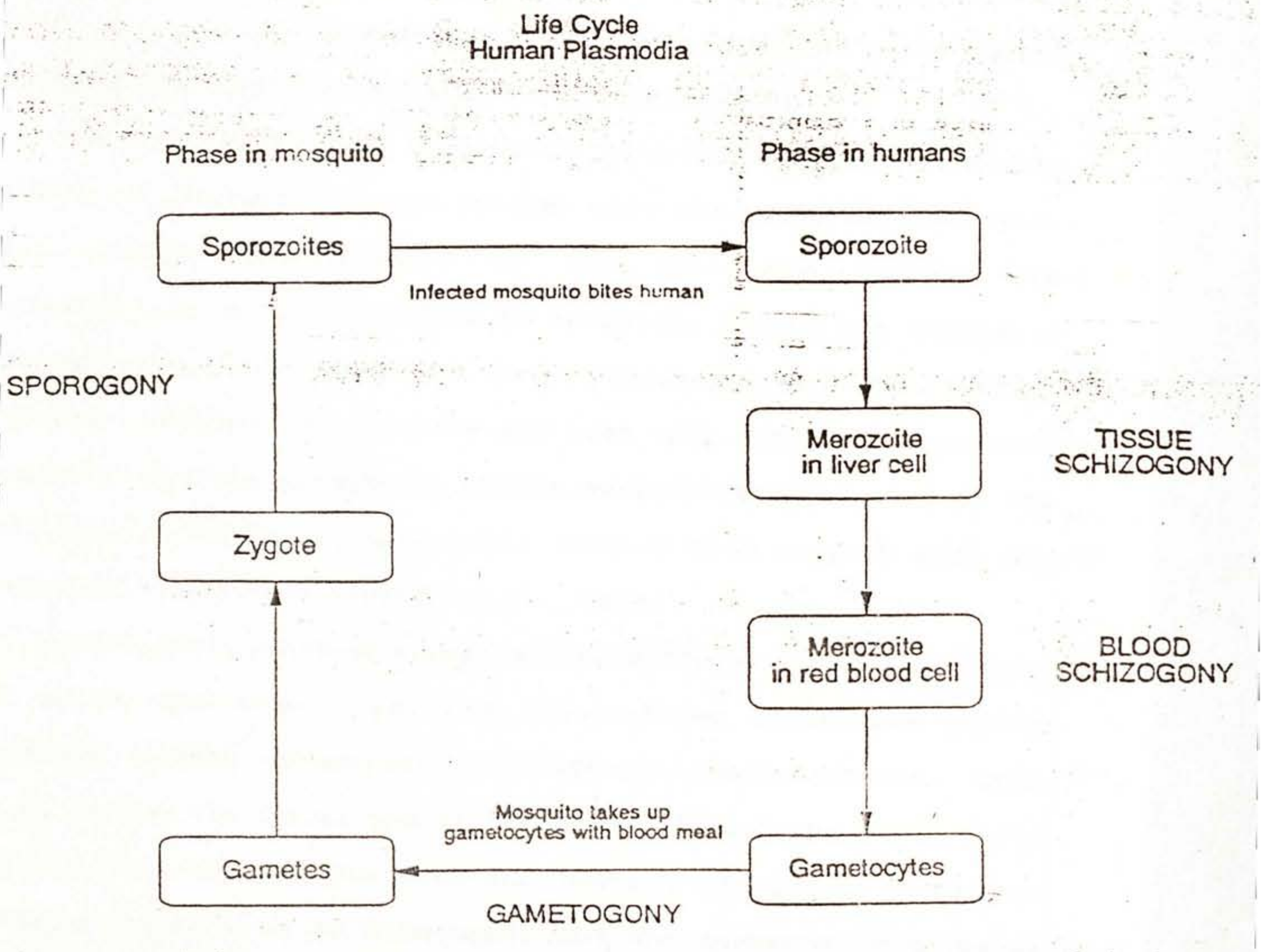
A simple sexual and extracorpuscular cycle occurs in the female *Anopheles* mosquito. It starts with the intake of gametocyte into the stomach of the mosquito when it takes a blood meal from a malaria patient. Fertilization of the female and male gametes results in formation of zygotes which subsequently develop as oocysts in the gut wall. Sporozoites released from the mature oocysts migrate to the salivary glands of the mosquito after penetrating the body cavity.

There is a pre-erythrocytic stage in man when sporozoites from infected mosquito are injected into the blood stream of the host. The sporozoites travel in circulation to the liver and infect parenchyma cells and go through non-erythrocytic schizogony to form schizonts. The red blood cells rupture discharging merozoites which enter the red cells and develop through erythrocytic schizogony to form trophozoites. The trophozoites produced in red cells are known to digest haemoglobin thereby changing the haem portion into a malaria pigment called haemozoin.

Trophozoites undergo further development producing schizonts which develop into merozoites and subsequently these merozoites may attack or re-enter red cells, some producing gametocytes. The cycle repeats when the female *Anopheles* mosquito feeds on a malaria patient (Fig 3).

2.5 INVASION OF ERYTHROCYTES BY MEROZOITES

Fig.3. Life cycle of Human Plasmodia



1.2.5 INVASION OF ERYTHROCYTES BY MEROZOITES

The merozoites are obligate intracellular forms of the parasite which can survive in extracellular milieu for only a short period of time since extracellular cultivation of the parasite has been achieved with limited success (Trager, 1950).

The interaction of merozoites with host cells is highly specific. For example, *P.vivax* invades only the young erythrocytes. Also, genetic determinants of the host may dictate the susceptibility of erythrocytes to invasion; blacks are generally resistant to *vivax* malaria. These suggest that the initial interaction between the parasite and host cell entails recognition of specific surface receptors. These surface receptors may be the Duffy coat. Duffy positive cells treated with trypsin were not invaded by *P.falciparum* (Miller *et al*., 1971).

Aikawa,(1971) provided electromicrographic evidence indicating that merozoites have a pair of flask-shaped structures called rhoptries flanked by smaller structures called microsomes. Upon contact between the apical end of the merozoite and the erythrocyte surface, the erythrocyte membrane becomes thickened forming a circumferencial site of attachment and the membrane invaginates (Aikawa *et al*., 1978).As the invagination deepens, the attachment site is no longer at the apical end, but moves along the circumference of the merozoite so that as the parasite enters, the site of attachment remains at the entry point of the invagination.

A parasitophorous vacuole created by the internalization of host membrane envelopes the merozoite. This finding was confirmed by the use of real time Normarski Interference Microscopy (Dvorak *et al.*, 1975).

1.2.6 MODIFICATION OF MEROZOITES IN ERYTHROCYTES

Once inside the parasitophorous vacuole, merozoites lose their rhoptries, microsomes and pellicular membranes and are transformed into amoeboid trophozoites (Aikawa, 1971).

The plasma membrane and the parasitophorous vacuole are also under alteration. As revealed by freeze fracture studies, intramembranous particles of the parasitophorous vacuole are reduced (McLarven *et al.*, 1977) and apparently the enzyme constituents of the membrane are rearranged (Langreth, 1977).

Erythrocytes infected with *P.falciparum* may display slit-like structures (maurer's cleft) within their cytoplasm which are believed to be extrusions of the parasitophorous vacuole membrane.

1.2.7 CLINICAL PRESENTATION OF MALARIA.

Falciparum malaria is the most lethal of the infections caused by human *Plasmodia*. It has the most complications, which can mimic several diseases including typhoid, influenza and infections of the central nervous system such as meningitis (Agyepong *et al.*, 1995).

Common signs and symptoms include chills, fever (the hallmark of clinical malaria), headache, muscle pains, vomiting, diarrhoea, abdominal pains or melaena (Commey, 1989), enlargement of the spleen and anaemia, which do not necessarily occur together. Cold sores around the mouth often occur. Enlargement of the liver, mild jaundice and some swelling of the subcutaneous tissues (oedema) may be observed.

Asymptomatic *P.falciparum* infections during pregnancy have been known to be associated with severe placenta infection, and inadequate fetal nutrition. This leads to intrauterine growth retardation. Reductions in the mean birth weight between 55 and 310gm have been reported (Antwi, 1989). Such low birth weight predisposes children to a higher infant mortality.

Classical black water fever beginning with rigors and fever followed by massive intravascular haemolysis, icterus, severe anaemia, haemoglobinuria, collapse and occasionally renal failure are considered to be more common in non-immune adults (Commey, 1989).

Fever occurs when red blood cells rupture in synchronized infections in which all the parasites are at the same stage of maturation. Thus the fever is related to the time required for schizont maturation (schizogony) which is about 48 hrs for *P.falciparum*.

Anaemia also occurs as a consequence of the rupture of erythrocytes during schizogony but the degree of anaemia frequently exceeds that attributable to the degree of parasitemia (Zuckerman, 1966).

The vasodilation secondary to fever results in a decrease in "effective" plasma volume and initiates homeostatic mechanisms aimed at restoring plasma volume. Secretion of antidiuretic hormone and aldosterone increases and causes a decrease in free water clearance. When the degree of renal water resorption exceeds sodium conservation, hyponatremia results.

Schizonts of *P.falciparum* become attached to the surface of endothelial cells in venules of a variety of organs, including the brain, lung, heart, intestine, kidneys, spleen and placenta. This attachment seems to be mediated by the presence of electron dense knobs on the surfaces of the infected erythrocytes. This leads to a decrease in local microcirculation (slugging of blood) resulting in hypoxemia.

The attachment of schizonts to the endothelial cells ultimately leads to transudation of fluid into the interstitial spaces. This is readily seen pathologically in acute pulmonary edema. If severe, it may lead to local microscopic haemorrhage, as seen for example, in cerebral malaria (Eling & Souerwein, 1995) or to cell death, as is observed in acute tubular necrosis and acute nephritis (Commey, 1989).

Blockage of splanchnic capillaries leads to vomiting, diarrhoea and abdominal pains. The vomiting tends to be persistent and irregular leading to the common diagnosis of acute gastritis or dyspepsia. The stools are watery and non mucous with stool microscopy often showing only few red blood and pus cells. (Commey, 1989).

Thrombocytopenia and granulocytopenia frequently accompany malaria, but spontaneous bleeding and increased susceptibility to bacteria infections are rare complications. The basis for the thrombocytopenia seems to be the sequestration of most platelets in the enlarged spleen (Skudowiz, 1973)

The basis of the granulocytopenia is less clear but might be similar to that of thrombocytopenia. Abnormalities in the concentration of humoral clotting factors also may accompany infection and clinical evidence for disseminated intravascular coagulopathy is not rare.

1.2.8 SOCIO-ECONOMIC ASPECTS OF MALARIA

Malaria contributes significantly to the loss of national productivity, absenteeism from work and school, and poor academic performance. It is the single most important contributor of the greatest number of "healthy days lost" (Adjei, 1989). From case studies in African countries including Ghana, it could be generalized that, on the average, a semi-immune person with uncomplicated malaria will be unable to work for 3 to 5 days per infection (Brinkmann and Brinkmann, 1991). It was also inferred that 2 days of adult time are likely to be lost to care for a malarious child.

In addition, it imposes an extra burden on the health service in terms of man power demands, cost of drugs (especially in the face of multidrug resistance). Also the cost of control efforts in conditions of abject poverty; costs such as transportation for health care, the cost of hospitalization and treatment, and the low level of income, take their toll on the sufferer. The result has been a vicious cycle of relationship between the disease and underdevelopment - people are sick because they are poor: they become poorer because they are sick and more sick because they are poorer.

1.2.9. THE CONTROL OF MALARIA IN GHANA.

These programmes took the form of chemoprophylaxis and vector control in order to ameliorate the precarious malaria situation in the country.

School children were given quinine tablets twice weekly. This was discontinued in 1930 because of the World Economic Depression at that time (Medical and sanitary report, 1931). From the 1930s until the outbreak of the second World war in 1939, quinine was sold at all Post Offices throughout the country so that rural dwellers could benefit. Addition of antimalarial drugs to salt was stopped as other sources of salt were available (Adjei, 1989).

In 1946, land spraying with oil, D.D.T. and drainage of breeding sites were practised to reduce the vector-human contact. By 1970, it was estimated that 727million people had been freed from the risk of malaria (Adjei, 1989). However, by 1974, the malaria situation exacerbated in several countries and that reported cases doubled. This led World Health Organisation to recommend chloroquine and pyrimethamine for malaria chemoprophylaxis.

In 1983 vector control programmes based on aerial spraying and larviciding were launched but could not be sustained because of resistance of mosquitoes to insecticides. The use of mosquito proofing materials in doors and windows has been found to be effective in preventing bites at night (Neequaye[a], 1989). Currently, Pyrethyrene impregnated mosquito nets are being used in Northern Ghana to prevent mosquito bites. Other measures such as wearing of long sleeve shirts and long trousers as well as application of repellents are also widely practised.

1.2.10 **FOLATE METABOLISM**.

Folate consists of heterocyclic pteridine, *para*-aminobenzoic acid (PABA) and L-glutamic acid (Stokstad *et al.*, 1967; Fig. 4). The pteridines (dihydroneopterin and neopterin) are synthesized by the parasites from guanosine 5-triphosphate (GTP) by the enzyme GTP cyclohydrolase. This was confirmed when labelled GTP was used (Krungkrai *et al.*, 1990).

The intracellular folate coenzyme forms are predominantly polyglutamate derivatives with peptide linkage of the *gamma*-carboxyl group and amino group of glutamyl residues. The folylpolyglutamates (or pteroylpolyglutamates) are better substrates than the monoglutamate form of folate for many folate-dependent enzymes. Folate coenzymes serve as acceptors or donors of one carbon unit in purine, pyrimidine, amino acid and in the initiation of protein syntheses.

In *Plasmodia* dihydrofolate reductase (DHFR; 5,6,7,8, tetrahydrofolate: NADP$^+$ oxidoreductase EC: 1.5.1.3) (Foote *et al.*, 1990) and thymidylate synthase (TS:5,10, methylenetetrahydrofolate: dUMP methyltransferase, EC: 2.1.2.15) exist as a bi-functional

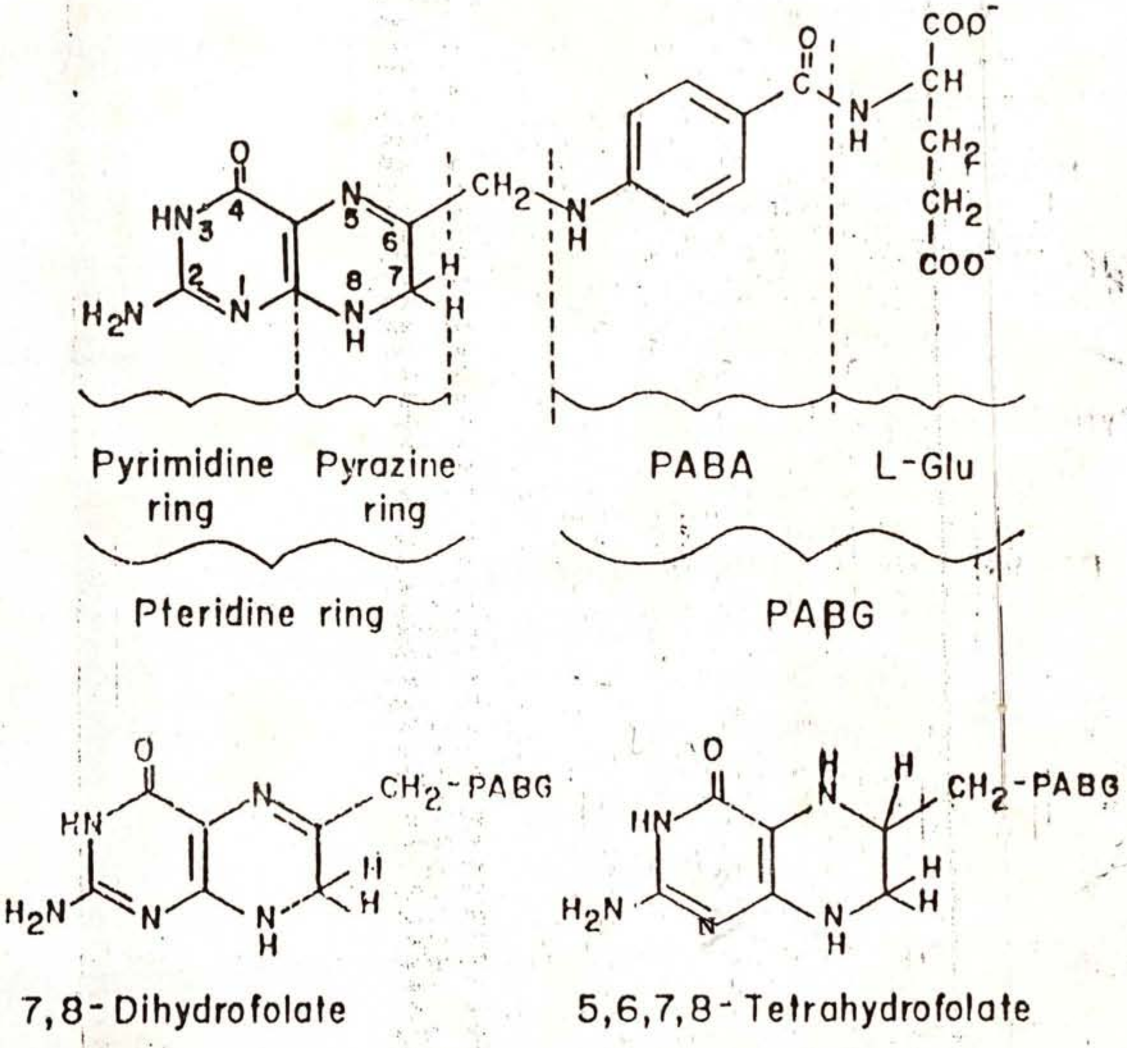


Fig. 4 Chemical structure of the folate molecule (PABA, p-aminobenzoate). Reduced forms of folate are also shown: 7,8-dihydrofolate and 5,6,7,8-tetrahydrofolate.

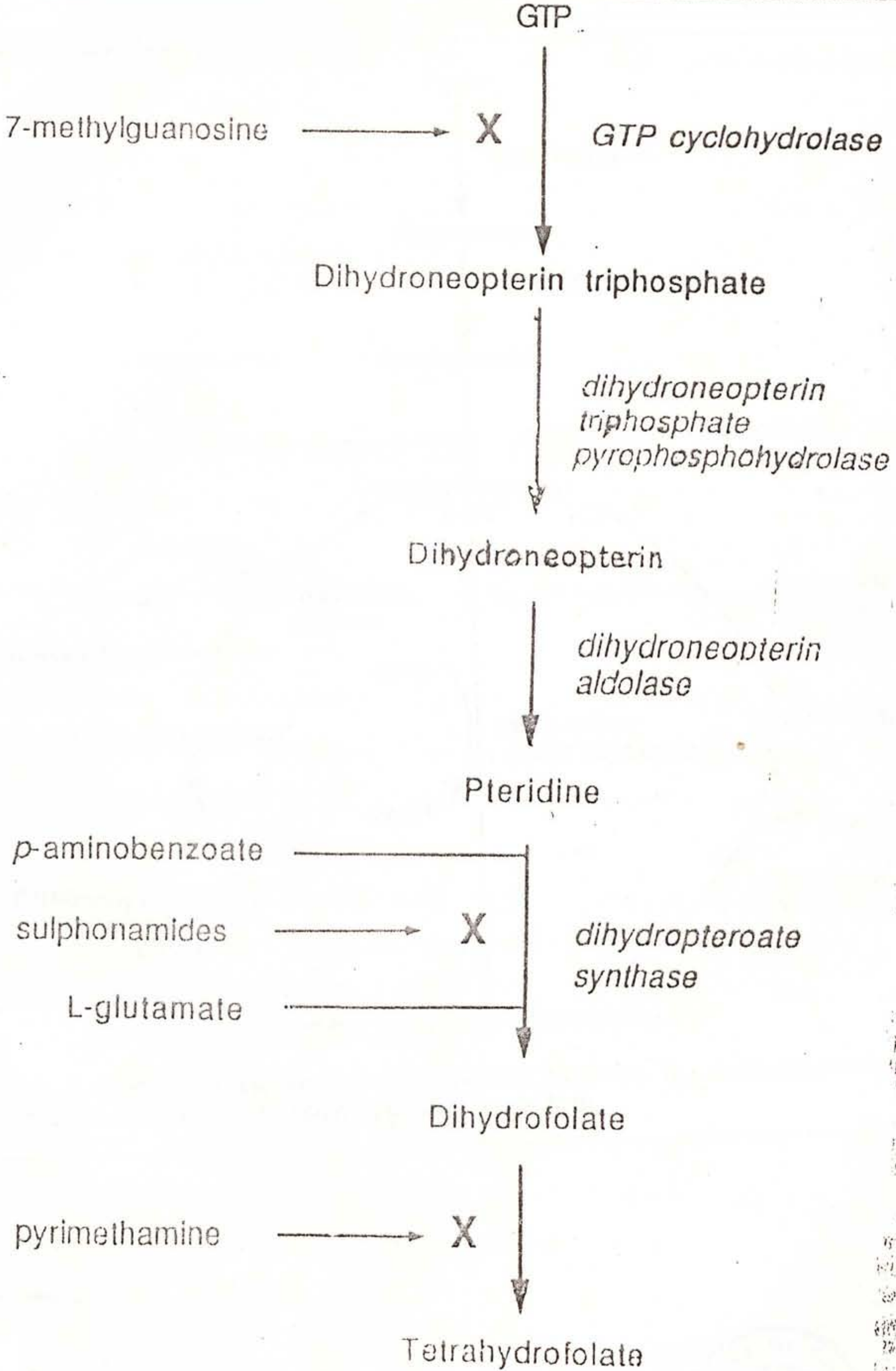


Fig. 5. The de novo synthesis of tetrahydrofolate in Plasmodium falciparum. *The crosses mark potential sites of inhibition.*

N^5 Methyltetrahydrofolate

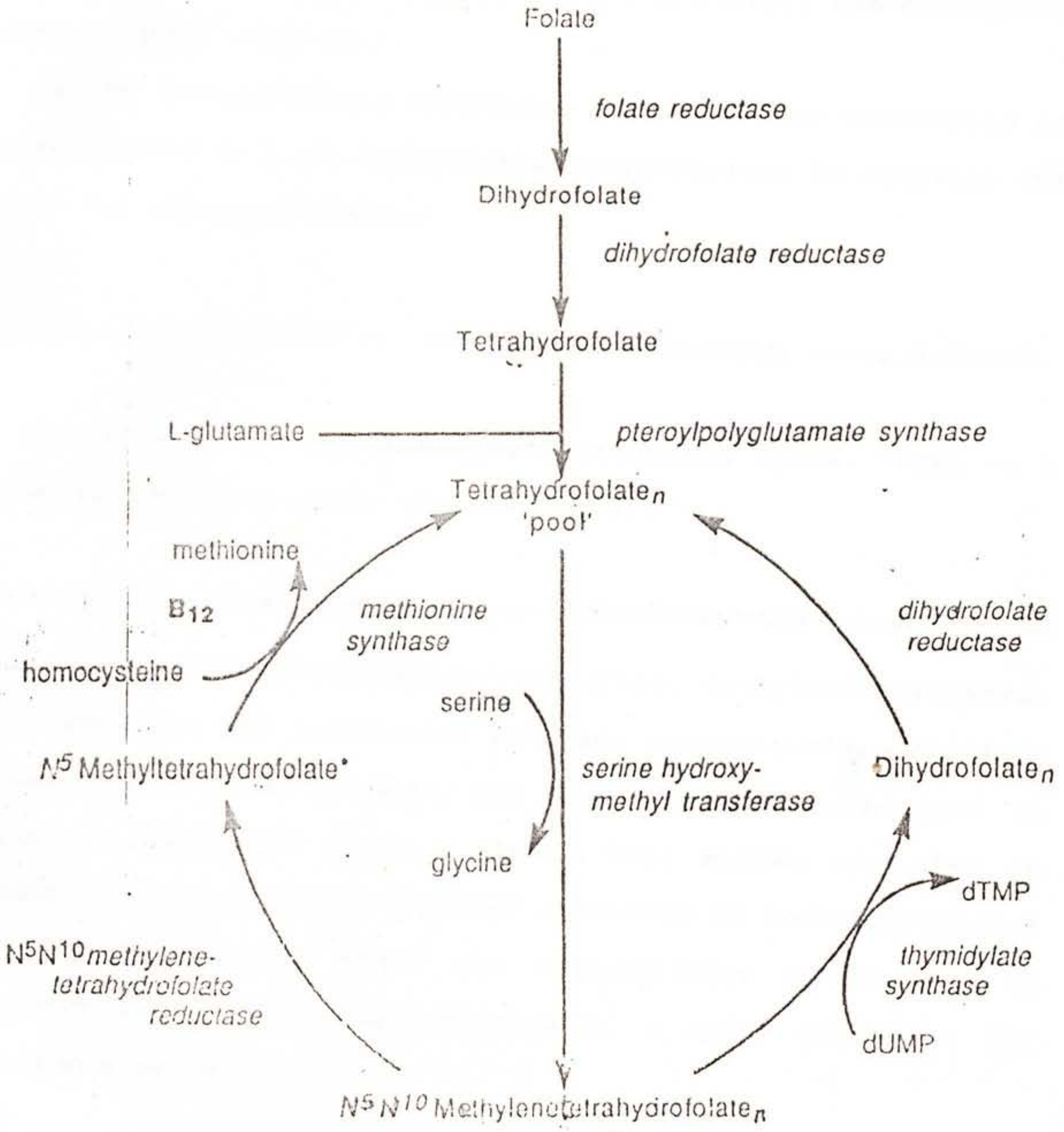


Fig. 6. Folate-dependent reactions in Plasmodium falciparum. *B_{12}, methylcobalamin; *, may be salvaged from host plasma; n, five in* Plasmodium.

protein (Bzik *et al.*, 1987). The former catalyses the conversion of dihydrofolate to tetrahydrofolate while the latter, the conversion of dUMP to dTMP (Fig. 5).

Serine hydroxymethyltransferase catalyses the conversion of tetrahydrofolate to 5,10, methylenetetrahydrofolate to complete the thymidylate synthesis cycle;

dihydrofolate → tetrahydrofolate → 5,10,methylenetetrahydrofolate → dihydrofolate.

In addition to the thymidylate synthesis cycle, there is a methionine synthesis cycle (Ferone, 1977):

tetrahydrofolate → 5,10, methylenetetrahydrofolate → 5,methyltetrahydrofolate → tetrahydrofolate,

catalyzed by serine hydroxymethyltransferase, methylenetetrahydrofolate reductase and methionine synthase respectively. Cobalamin dependent methionine synthase has recently been identified in *P.falciparum* (Krungkrai *et al.*, 1989). This enzyme may play an important role in one carbon transfer reactions of tetrahydrofolate metabolism in malaria, since the intracellular folate pool in *P.falciparum* is 5,methyltetrahydrofolate, a good substrate for methionine synthase (Fig. 6).

1.2.11 **ANTIFOLATE ANTIMALARIALS.**

Most mammalian cells use exogenous folate as the monoglutamate derivative of 5,methyltetrahydrofolate. In contrast, malaria parasites are able to synthesize folate *de novo* because they have the enzymes 6-hydroxymethyldihydropteridine, pyrophosphokinase, dihydropteroate synthase and dihydrofolate reductase (Ferone, 1977; Sherman, 1979). The last two enzymes are the targets of antifolate antimalarial drugs (Sherman, 1979; Fig. 5).

Antifolate antimalarials comprise the *para*-aminobenzoic acid competitors (sulfonamides and sulfones) and the dihydrofolate reductase (DHFR) inhibitors (Zolg *et al*., 1990).

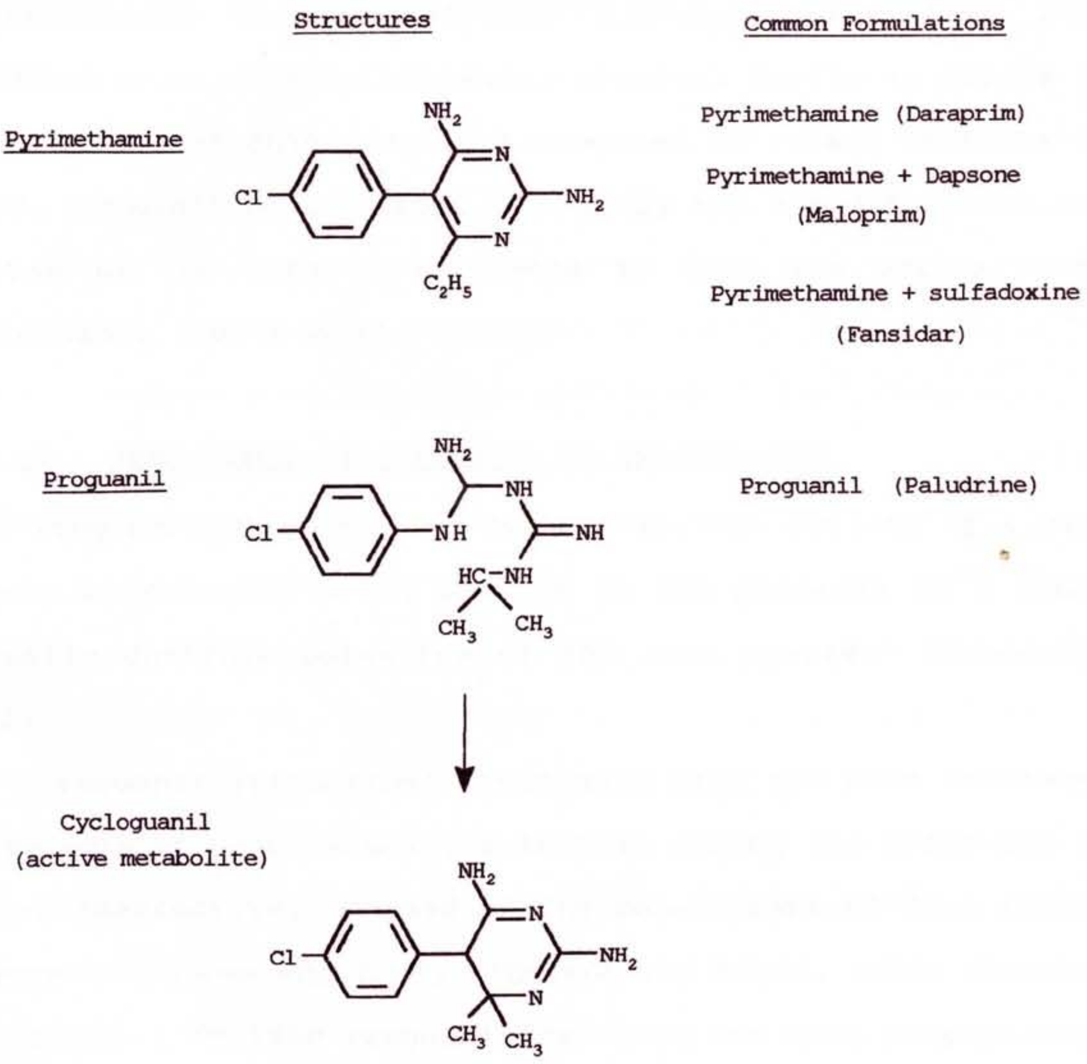
DHFR inhibitors including pyrimethamine and the biguanides (proguanil, chlorproguanil and cycloguanil) do not prevent the maturation of ring forms of the parasites to trophozoites. (lnselburg *et al*., 1987). The most conspicuous effect of DHFR inhibitors is the formation of parasites with abnormal appearance. After exposure to these drugs, the parasites' chromatins do not divide normally to form schizonts but they split into indistinct fragments of varying sizes and shapes.

1.2.11.1 Pyrimethamine

Pyrimethamine (Fig.7) affects the biosynthesis of nucleic acids and causes cell death by inhibiting DHFR (Zolg *et al*., 1989). The activity of pyrimethamine is higher than those of biguanides on parasites for a long time in the body. Pyrimethamine inhibits the development of trophozoites and schizonts.

In most countries, Pyrimethamine is only used in combination with long acting sulfonamide for the treatment of chloroquine-resistant *falciparum* malaria and in others it is used alone for prophylaxis (Peterson *et al*.,1991). However, resistance to

Fig. 7. Structures and common formulations of pyrimethamine and proguanil. Cycloguanil, the biologically active metabolite of proguanil contains a triazine ring with two substituent methyl groups instead of the pyrimidine ring and ethyl side chain of pyrithamine (Peterson *et al.*,1990)



pyrimethamine appears relatively rapidly.

1.2.11.2 Biguanides

The active metabolites of proguanil and chlorproguanil (dihydrotriazines) are responsible for the antimalarial activities of the biguanides (Schapira, 1984), and are more effective than pyrimethamine *In vitro* against *P.falciparum* resistant strains. (Eriksson *et al.*, 1989). However, clinical trails in Africa showed poor efficacy of chlorproguanil compared to others (Watkins *et al.*, 1987). Proguanil is activated *in vitro* by the mixed function oxidase system of the hepatic microsome to form the active compound, cycloguanil (Foote *et al.*, 1990).

1.2.12. RESISTANCE OF *PLASMODIUM* TO ANTIFOLATES.

Drug resistance has been defined as,"The ability of a parasite strain to multiply or to survive in the presence of a drug that normally destroys parasites of the same species" (Bruce-Chwalt, 1982).

Proguanil (paludrine), initially used for both treatment and prophylaxis of *falciparum* malaria in Asia during the 1940s and 1950s, became ineffective, because of the development of drug resistance by both *P.falciparum* and *P.vivax* (Edeson and Field, 1950; Chaudhuri *et al.*, 1952). In 1950 proguanil was used for mass prophylaxis in a region of Java. Resistance developed a year later (Neequaye[b], 1989).

Pyrimethamine (Daraprim) was introduced in 1952 as the greatest therapeutic triumph in malaria since paludrine. Again it was used extensively both for treatment and prophylaxis. After 6 months use in semi-immunes in Kenya for prophylaxis, *P.falciparum* became resistant to it (Neequaye[b], 1989). Resistance to pyrimethamine occurred more readily than resistance to proguanil. In 1962, there was a project where pyrimethamine prophylaxis was given by heads of households in the Volta Region of Ghana. There was a dramatic improvement in parasite rate for the first few weeks but after two years the parasite rates had increased to former levels (Neequaye[b], 1989).

P.falciparum resistant strains to sulfadoxine/ pyrimethamine combination (fansidar) have appeared in South East Asia, South America (Peterson *et al.*,1991) and Ghana (Akanmori *et al.*, 1989). In 1958, parasite strains resistant to both proguanil and pyrimethamine were found in Kenya (Avery-Jones, 1958).

1.2.13 MECHANISM OF RESISTANCE TO ANTIFOLATES.

Resistance to antifolates could result from a number of mechanisms including amplification of the DHFR-TS gene or increased production of DHFR enzyme, decreased permeability of cells to drug, increased activity of DHFR-TS enzyme (Inselburg *et al.*, 1987) and decreased activity for pyrimethamine by structurally altered enzyme (Zolg *et al.*, 1989). Metabolic alterations,for example, increased salvage of 5,methyltetrahydrofolate and conversion to tetrahydrofolate through methionine synthase to by-pass the DHFR step may also be one of the mechanisms. It may be possible that

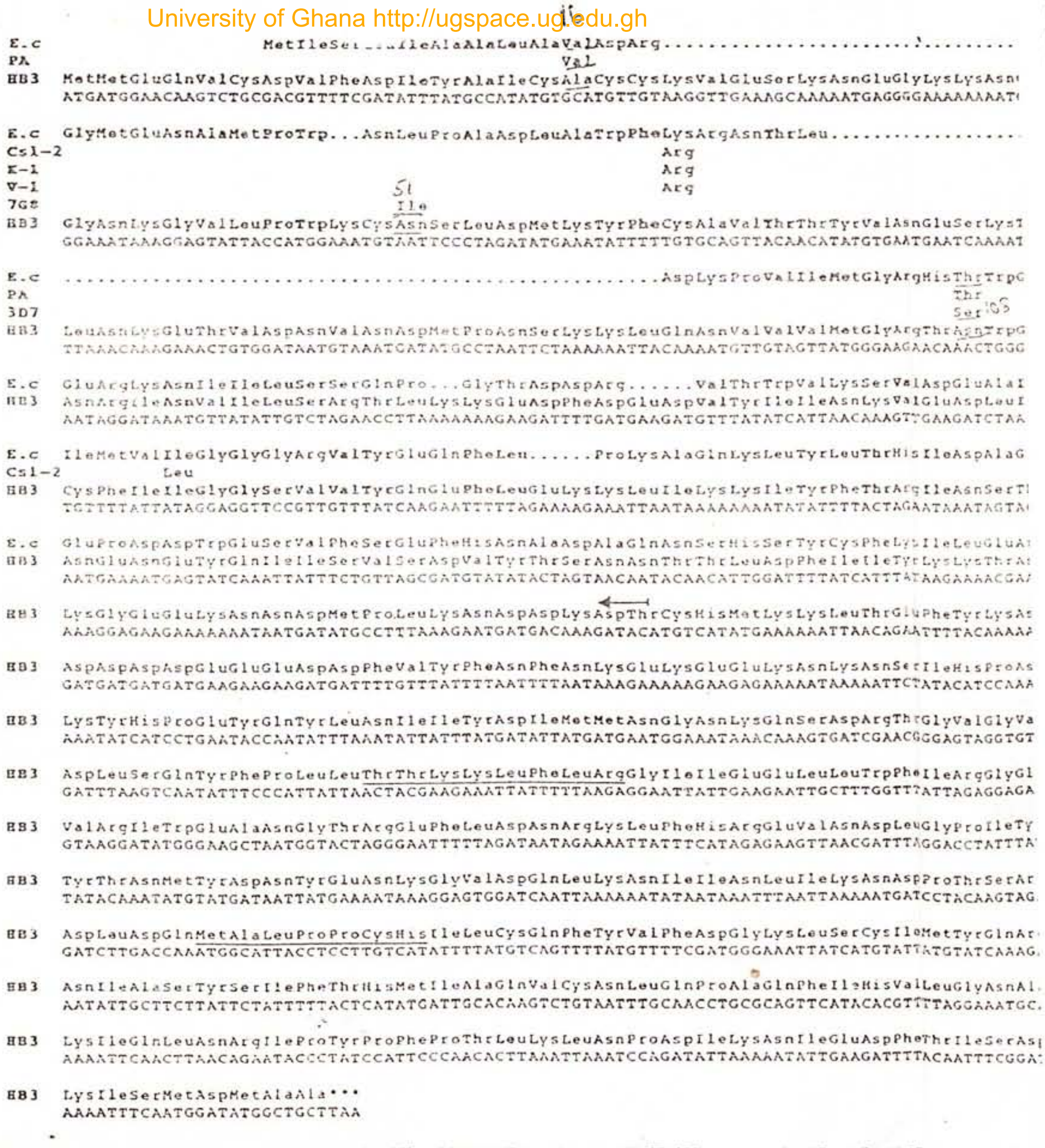


Fig. 8 27

Fig.8 DNA sequence and deduced protein sequence of the DHFR-TS gene of *E.coli* (E.C) and parasite clones, HB3, 7G8, 3D7, K-1 and V-1 (Cowman et al, 1988).

The nucleotide sequence of the pyrimethamine-resistant clone HB3 is shown in full with the deduced protein sequence. Only one amino acid change from Ser-108 (codon AGC) to Asn-108 (codon AAC) is responsible for pyrimethamine resistance of HB3.

resistant parasites have more methylcobalamin dependent methionine synthase activity than do the sensitive parasites (Krungkrai *et al.*, 1989).

Comparing the activities of DHFR isolated from pyrimethamine - resistant clones (7G8 and HB3) with that from drug sensitive clone 3D7, suggests that drug resistance is a result of structural changes in the DHFR-TS protein.

Comparison of 3D7 DHFR sequence to that of HB3 revealed that only one amino acid difference of residue 108, accounts for the parasites' resistance to pyrimethamine. It has been proven that a single mutation from Ser-108 to Asn-108 in DHFR active site (Fig.9) is responsible for decreased activity of pyrimethamine on the parasite (Gyang *et al.*, 1992; Peters *et al* .,1973; Schapira , 1984 and Inselburg *et al.*, 1987). It was also found that the initial event in the development of resistance in the parasite was duplication of chromosome 4 carrying the DHFR-TS gene (Inselburg *et al.*, 1987).

Paired mutations, Ser-108 to Thr-108 and Ala-16 to Val-16, confer *P.falciparum* resistance to cycloguanil with a slight decrease in pyrimethamine response (Gyang *et al.*, 1992).

Cross resistant parasites to pyrimethamine and cycloguanil were found to have 3 mutations in common: Ser-108 to Asn-108, lys-59 to Arg-59 and IIe-164 to leu-164.

1.2.14 <u>OTHER CAUSES OF PARASITES' RESISTANCE TO ANTIFOLATES.</u>

In the cause of malaria transmission, genetic recombination of the sexual stages of the parasites may occur if the mosquito has simultaneously injected two or more strains of gametocytes circulating in one person or has divided its feed between people carrying different strains. Hybridization between differing strains of the parasite may result in modification of the degree of drug sensitivity (Neequaye[b], 1989).

Drug pressure is the major factor responsible for selection of resistant parasites (Neequaye[b], 1989; Ofori-Adjei, 1989). This usually arises when the drug is used inappropriately in low dosages, allowing the parasites to be exposed to subcurative doses and hence survive in an otherwise hostile environment. Malaria may be caused by a mixture of parasites; some fully sensitive and others with various degrees of resistance to antimalarials. Subcurative dose of drugs then may result in the selection of the resistant parasite strains.

Gross misuse and abuse of drugs as a result of lack of control, overprescription, population movement and inadequate training of health workers are other factors contributing to the selection and dissemination of resistant *P.falciparum* strains (Riekmann, 1990).

1.2.15 **IDENTIFICATION OF *PLASMODIUM* IN BLOOD.**

Microscopy has, historically, been the mainstay of malaria diagnosis. Its use is limited by the expertise required for optimal staining and interpretation of blood films. In addition, low parasite densities are rarely detected. Another problem arises when slides are stained in bulk;parasites washed off one slide may occasionally find their way onto another (Frimpong and Kankam, 1989).

In vitro cultivation, although not meant as a substitute for *in vivo* assessment of patients' response to treatment, provides an alternative means for detecting low parasite densities. It is also convenient for the detection of drug resistant parasites (Riekmann *et al.*, 1978) and for the evaluation of efficacy of new compounds on parasites.

In spite of the above, *in vitro* cultivation gives inconsistent results because *P.falciparum* is asynchronous. That is not all parasites grow at the same rate and also not all parasites adapt to laboratory conditions. It is difficult for researchers in malaria endemic areas to obtain human sera devoid of antimalarial antibodies or chemotherapeutic drugs. In addition, the ability of the sera to support parasites' growth varies from donor to donor (Grun, 1987).

In order to obviate the above drawbacks, restriction fragment length polymorphism (RFLP), cloning and sequencing, and hybridization are being used to follow resistance to antimalarials. This is achieved by detecting mutations in the parasites' gene since all pyrimethamine -resistant and cycloguanil -resistant *P. falciparum* isolates examined had point mutations in the gene encoding DHFR-TS protein. In order to get enough materials to do these assays,cells must often be cultured to increase their numbers,a process which is time consuming and labour intensive and not always easy or successful.

Polymerase Chain Reaction (PCR) amplification of DNA presents a new alternative method for detecting these point mutations in the parasites' genome.

It offers a quick, easy and sensitive way of detecting low levels of parasite DNA that could be used to identify subpotent parasitemia (Sethabutr, 1992). PCR assay can detect low number of parasites (Foley *et al.*, 1992).

With PCR, treatment failures could be predicted on time. This early identification would permit appropriate therapy changes which ultimately could decrease the spread of drug resistance infection (Kain *et al.*, 1994).

1.2.16 PCR

The PCR, invented by Kary Mullis (Mullis and Faloona, 1987) is an *in vitro* method for the enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA.

Its principle is simple, requiring a 3-step cycling process;

1. denaturation of double stranded template DNA,
2. annealing of primers to template DNA and
3. primer extension by DNA polymerase resulting in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers.

By virtue of the exponential accumulation of billions of copies derived from a single progenitor sequence, PCR represents a form of "cell free molecular cloning" that can accomplish in an automated 3-4 hours *in vitro* reaction what might otherwise take days or weeks of biological growth or biochemical purification (Saiki *et al.*, 1988).

A cycle typically takes 3-5 minutes and is repeated 20-40 times and because the primer extension products synthesized in one cycle can serve as templates in the next, the number of target DNA copies approximately doubles at every cycle. Thus 20 cycles of PCR yield about a million fold (2^{20}) amplification (Erlich, 1992).

Mutation specific PCR (MSPCR) primers were designed with 3'-mismatches to detect point mutations in the parasites DHFR gene (Gyang *et al.*, 1992) based on the observation that oligonucleotides having a single base mismatch at their 3' ends are much less efficiently extended than are perfectly matched primers. (Peterson *et al.*, 1991).

One of the attractive features of MSPCR is the assay's independence of labelled probes. This eliminates not only the time consuming processing of 3 separate filters (each one used with one of the 3 specific probes) but also makes it possible to obtain definite results within one working day (Zolg *et al.*, 1990).

Moreover, the absence of any detectable cross priming of the mutation specific primers with incorrect target DNA on agarose gels and the high efficiency of the *Taq* polymerase with perfectly matched primers make the MSPCR typing specific and sensitive.

The initial PCR method used a Klenow fragment of *E. coli* DNA polymerase I to extend the annealed primers at 37°C. Although, a specific parasite DNA fragment could be amplified up to a million fold, what was synthesized was estimated as about 1% of the expected yield (Scharf *et al.*, 1986). Moreover, subsequent analysis with a specific hybridization probe is required to eliminate mispriming of incorrect DNA templates (Saiki *et al.*, 1986). Also the high temperature required to separate the two DNA strands at the outset of each PCR cycle inactivated the *E.coli* DNA polymerase 1 so fresh enzyme had to be added at every cycle.

The use of the thermostable DNA polymerase (*Taq* polymerase) isolated from *Thermos aquaticus* transformed the PCR into a simple reaction which could now be automated by a thermal cycling device. The *Taq* polymerase, not only simplified the PCR procedure but also significantly increased the sensitivity and the overall yield of the reaction. The higher temperature optimum (75°C) allowed the use of higher temperature for primer annealing and extensions, hence increasing the stringency of the reaction and minimizing the extension of primers that were mismatched with the template.

At optimum temperature, a catalysis constant of *Taq* polymerase approaches 150 nucleotides/sec/ enzyme molecule (Erlich, 1992). At lower temperatures, *Taq* polymerase has extension activities of about 0.25 and 1.5nucleotides/sec at 22°C and 37 C respectively. Very little DNA synthesis is seen at very high temperatures (>90°C). Although *Taq* DNA polymerase has very limited ability to synthesize DNA above 90°C, the enzyme is relatively stable to and is not denatured irreversibly by exposure to high temperatures. *Taq* polymerase retains about 50% of its activity after 130min, 40min and 5-6 min at 92.5°C, 95°C and 97.5°C respectively (Erlich, 1992). Purified 94 kilodalton *Taq* DNA polymerase does not contain an inherent 3-5 exonuclease activity (Tindall and Kunkell, 1988).

At 37°C many of the mismatched primers are stable enough to be extended by the Klenow enzyme resulting in non-specific amplification products. The use of *Taq* polymerase reduces the competition by non-target products for enzyme and primers because the *Taq* polymerase is more specific than the Klenow enzyme.

In the later cycles, the amount of enzyme is no longer sufficient to extend all the annealed primer/template complexes in a single cycle leading to a reduced efficiency and a "plateau" in the amplification reaction. The reassociation of the template strands at high product concentration may also contribute to the plateau effect. In addition, exhaustion of primers or deoxynucleotide tri-phosphates (dNTPS) or inactivation of polymerase, substrate excess and competition by non-specific products may lead to the plateau effect.

Primer dimer is an amplification artifact often observed in the PCR when many cycles of amplification are performed on a sample containing very few initial copies of template. It is a double stranded fragment whose length is very close to the sum of the two primers, one over the other primer. Primer dimers if formed at an early cycle, easily overwhelm a reaction and become the predominant products. It can be avoided if low concentrations of enzyme and primers are used.

The composition of the PCR buffer usually affects the efficiency and specificity of the reaction. In particular, excess Mg^{2+} will result in the accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce yield. Innis *et al.*, (1988) reported that reduction or elimination of KCl and gelatin from the PCR buffer could improve PCR yield.

Higher concentrations of dNTPs may promote misincorporation by the polymerase; as dNTPs appear to quantitatively bind Mg^{2+}, the amount of dNTPs present in a reaction will determine the amount of free Mg^{2+} available.

PCR false-positive and -negative results are not uncommon but are avoidable. False-positive results may occur when DNA fragments from previous PCR assays serve as substrates (contaminants) for the generation of more products. False -negative results may be caused by inhibitors or low concentration of DNA templates. These false PCR reactions could be avoided if precautions are taken. Some of these precautions are preparing DNA templates, carrying out PCR assays and analyzing PCR products in different rooms. Others are wearing of gloves, using sterilized double distilled water and different sets of pipetting devices in the rooms.

1.2.17 *P. FALCIPARUM* DNA ISOLATION.

Epidemiological studies of parasitic diseases are often hampered by the need to isolate parasite DNA before performing PCR. The presence in blood of compounds that inhibit PCR amplification has been a major problem associated with direct amplification of

DNA in haemolysates.By testing various blood components, haemoglobin has been found to inhibit the *Taq* polymerase. Heparin and chelating agents like EDTA which are used to prevent degradation of target DNA are also found to be inhibitory (Barker, 1992).

Therefore, most PCR methods have used organic extraction and ethanol precipitation to purify template DNA prior to amplification (Kadokami and Lewis, 1990). However, this procedure is cumbersome, requiring many manipulation steps and has the risk of potential exposure to hazardous chemicals. It also presents greater opportunity for sample contamination with exogenous template DNA.

A simple and rapid procedure for isolating parasite DNA from a 20 ul of blood sample (Foley *et al*., 1992) is modified and used. It makes use of the capacity of PCR to amplify a target sequence from crude DNA preparations (Saiki *et al*., 1986, Sjobring *et al*., 1990). The DNA in a sample need not be chemically pure to serve as a template provided that the sample does not contain inhibitors of *Taq* polymerase. This protocol has been used to detect 77 parasites per ul of whole blood (Foley *et al*., 1992).

It involves freezing of blood for lysis of red cells after thawing. The haemoglobin released from the lysed erythrocytes is washed with 5 mM Sodium phosphate buffer pH 8 (SP8) several times until a clear supernatant is observed. Boiling then denatures nucleases and proteases and releases the DNA.

The whole procedure takes only 50 min (Foley *et al*., 1992) and is carried out in a single centrifuge tube, reducing the chances of contamination. The use of small volumes of blood makes this method exceptionally valuable for field studies where low parasitemias are common and also where venous bleeding of children is inconvenient and requires authorized expertise.

1.2.18 GEL ELECTROPHORESIS OF PCR PRODUCTS.

Many biological molecules such as DNA carry electric charges, the magnitude of which depends on the pH and the composition of the suspending medium (buffer). These charged DNA molecules migrate in solution to the electrode of opposite polarity when an electric field is applied (Plumer,1987).

The technique is simple, rapid to perform, and capable of resolving mixtures of DNA fragments that cannot be separated adequately by other procedures like density gradient centrifugation Maniatis *et al.*, 1989).

The electrophoresis is usually carried out on a supporting medium. A composite gel consisting of 1% agarose and 2% nusieve garose is used in this study as the support.Nusieve agarose makes he gel more compact and affords one easier detection of low olecular weight *P.falciparum* DHFR-TS gene. The electrophoretic obility depends on the gel concentration, electric field applied, ize and conformation of DNA and electrophoresis buffer.

In general, better separation is obtained with gels of high concentration. But large fragments can take several days or even eeks to elute from such gels, so from a practical stand point, it s better to dilute gels for large DNAs. Gel concentrations below

0.8% are not self supporting and tend to collapse. Experience with analytical gels shows that small DNA fragments separate better on concentrated gels.

Also, when a higher electric field is applied, there is a heating effect such that an excessive amount of water is lost from the buffer by evaporation. If the heating is excessive, the DNA molecules may be denatured.

Closed circular, nicked circular and linear DNA of the same molecular weight migrate through the gels at different rates (Maniatis *et al.*, 1989). More ethidium bromide binds to the linear DNA molecule than to the nicked circular or to the closed circular DNA molecules. In effect, mobility of linear DNA molecules decreases while that of the closed circular DNA molecules increases with the mobility of the nicked circular DNA being intermediate.

Buffer solutions also influence the electrophoretic mobility. The buffer solution is carefully selected so that it will not react with the DNA molecules under investigation. Phosphate buffers support growth of bacteria and also precipitate with ethanol (Southern[a], 1979). Tris-borate buffer gives good resolution of DNA fragments and has significantly higher buffering capacity than Tris-acetate which becomes exhausted during extended electrophoresis, the anode becomes alkaline and the cathode acidic (Maniatis *et al.*, 1989). At high ionic strength, sharp bands are obtained but much heat is produced which affects resolution of DNA fragments.

1.2.19 STAINING DNA IN AGAROSE GEL.

The most convenient method of visualizing DNA in gels is by use of the fluorescent dye, ethidium bromide. Ethidium bromide can be used to detect both single and double stranded nucleic acids. But the affinity of the dye for single stranded nucleic acids is relatively low and the fluorescent yield is poor.

Although the electrophoretic mobility of linear duplex DNA is reduced in the presence of the dye by approximately 15% (Sharp, *et al.,* 1973), the ability to examine the gel directly under UV-illumination during or at the end of the run is a distinct advantage. Photographs of gels may be made by polaroid camera using incident UV-light.

1.2.20 MEASUREMENT OF DNA SIZE BY ELECTROPHORESIS.

Estimation of molecular size of nucleic acids in gel electrophoresis is important for the identification and for studies of native and denatured DNA (Oerter *et al.*, 1990).

The positions of the fragments are detected by staining with ethidium bromide and their lengths or molecular weights are determined by comparison with the mobility of nucleotide standards of known sizes. Usually this is done manually by interpolation from plot of size against mobility (Fisher and Dingman, 1971). It gives a straight-line over a low molecular weight range.

The standard curves obtained from such plots often show pronounced curvature in the high molecular weights and unless nucleotide standards are used with molecular weights close to the molecule to be measured, errors in the measurement can be caused by drawing a curve through the standard plots which may introduce significant subjectivity into the interpolation process (Duggleby *et al.*, 1981).

When electrophoresis is carried out at low voltage gradients, a plot of size (L) versus reciprocal of mobility (1/m) shows a linear relationship (Southern[b], 1979). At a high voltage gradient the curvature in the plot of L against 1/m is exaggerated. However, the data can be made to fit a straight-line, by plotting L against [1/(m-mo)], where mo, is a factor calculated to give the best fit to a straight-line:

$L = k_1/(m-mo) + k_2$ (Southern[b], 1979).

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1. MATERIALS

Agarose.	FMC Bio Products, Rockland, USA.
Boric acid.	BDH Chemical Ltd, Poole, England.
Bromophenol Blue.	Fluka Chemie, Switzerland.
Citric acid.	Eastman Kodak Corporation Rochester, N.Y.
Disodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4.12H_2O$, 358.15).	Hopkins & Williams, Essex, England
DNA molecular weight marker (ϕx174 RF1 Hinf I digest).	Sigma Chemical Co. St. Louis, MO. USA
Ethidium bromide	Fluka Chemie, Switzerland.
Ethylene-Diamine-Tetra Acetic acid (EDTA)	Fluka Chemie, Switzerland.
Glucose	Hopkins & William, Essex England.
Malaria parasitized blood.	Korle-Bu Teaching Hospital, Korle-Bu. E.P. Church Hospital, Adidome. University Hospital, Legon. Keta Government Hospital, Keta. St. Anthony's Hospital, Dzodze. Catholic Hospital, Bator. Sogakofe Health Centre, Sogakofe.

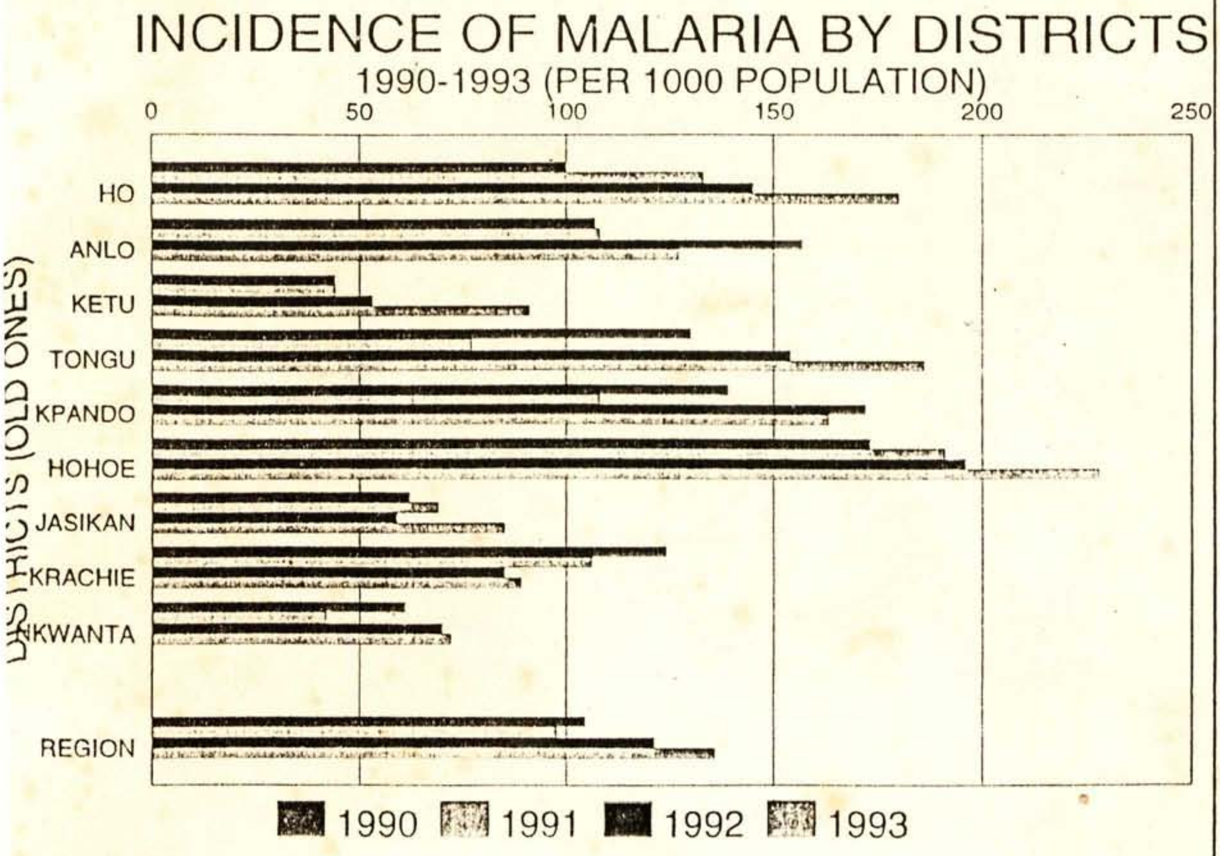
	Comboni Clinic, Sogakofe.
	New Hope Clinic, Viepe-Aflao.
Mineral oil	Fluka Chemie, Switzerland.
Mixed deoxyribonucleotide triphosphate (dNTPs).	Promega. Madison, W1 , USA.
Nusieve agarose	FMC Bio Products, Rockland USA.
Oligonucleotide primer (DIA 12,3,15 & 16,SP1,2 & 3)	Promega. Madison,W1, USA.
DNA from cultured *P.falciparum* clones (3D7, ItG2F6 & HB3).	Prof.F.N. Gyang, Department. of Biochem.Univ of Ghana,Legon.
Sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$; 156.01)	BDH chemical Ltd. Poole, England.
Sucrose	Hopkins & Williams, Essex, England.
Taq DNA polymerase.	Promega. Madison, W1 , USA. Gold Biochemical, Amsterdam, Netherlands.
Taq DNA polymerase buffer.	Promega. Madison, W1 , USA. Gold Biochemical, Amsterdam, Netherlands.
Tri-Sodium Citrate Dihydrate.	Fluka Chemie, Switzerland.
Tris-base.	Fluka Chemie, Switzerland.

2.1.1 **STUDY AREAS**

The study was carried out on samples obtained from two Southern Regions, Volta and Greater Accra, of Ghana (Fig. 10). Of 162 samples collected, 97 were from the Volta Region (Fig. 11) and the rest (65) from the Greater Accra Region. Seventy - six samples were from Tongu district (Adidome, Sogakofe and Bator) which in 1993 (Fig. 9) showed the highest incidence of malaria in Southern Volta Region . Ten samples (4 from Dzodze and 6 from Aflao) were from Ketu district in the Volta Region which experienced the lowest incidence of malaria in 1993 and 11 from Keta (Anlo district). Below are the sampling sites with their corresponding number of samples.

Table 1. Sampling sites and their corresponding number of samples.

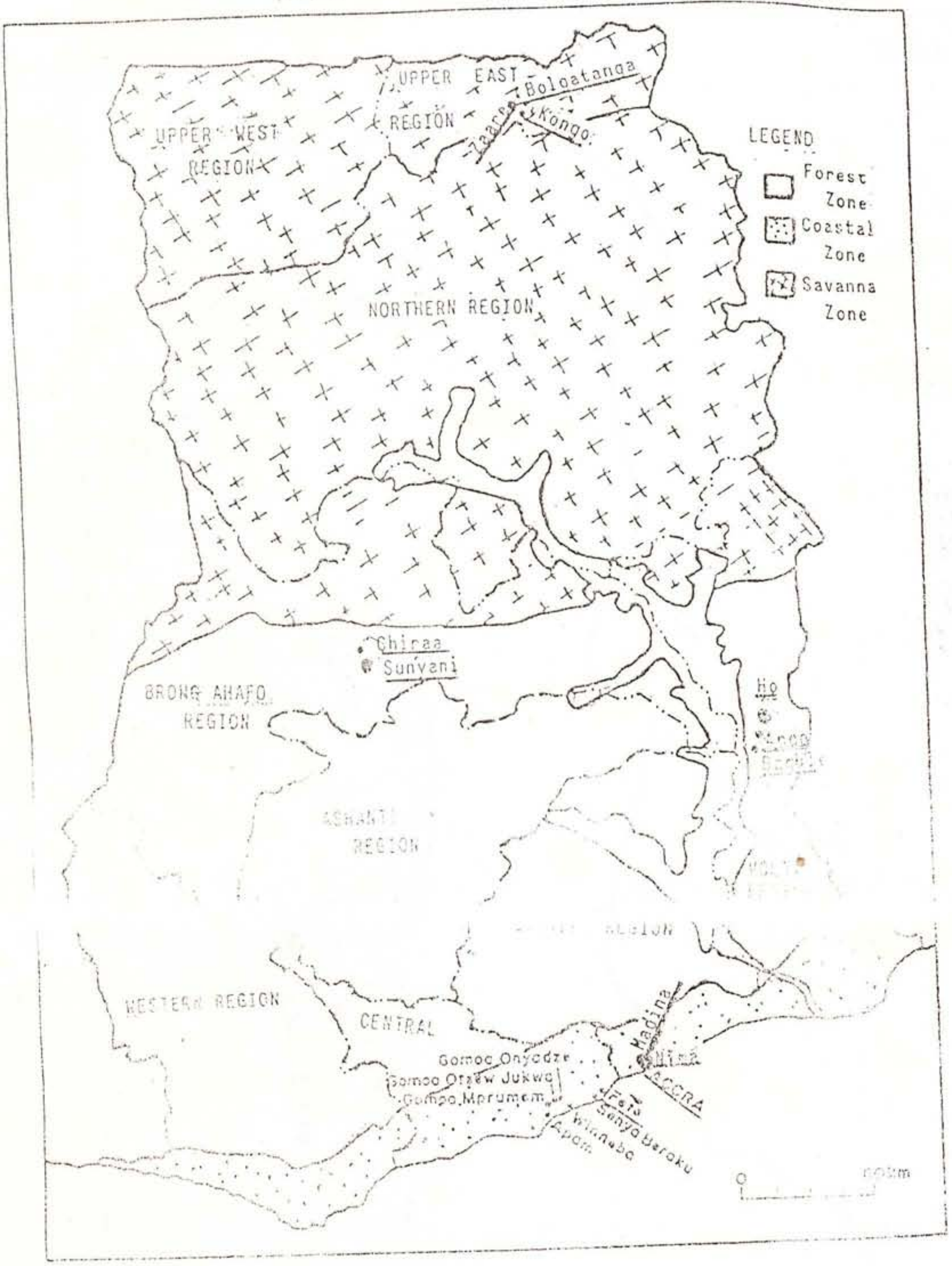
Region	District	Hospital	No.of samples	No.of samples by district	Total by Regions
Greater Accra	Accra West	Korle-Bu (KB)	59	59	65
	Accra East	Legon (L)	6	6	
Volta	Tongu	Adidome(A)	20	76	97
		Bator (B)	16		
		Sogakofe(S)	40		
	Anlo	Keta (K)	11	11	
	Ketu	Dzodze (D)	4	10	
		Aflao (AF)	6		
Total Samples					162



gure 9 Incidence of Malaria by districts in Volta Region 1990-93.

FIG 10

MAP OF GHANA



MAP OF VOLTA REGION SHOWING SAMPLE COLLECTION SITE

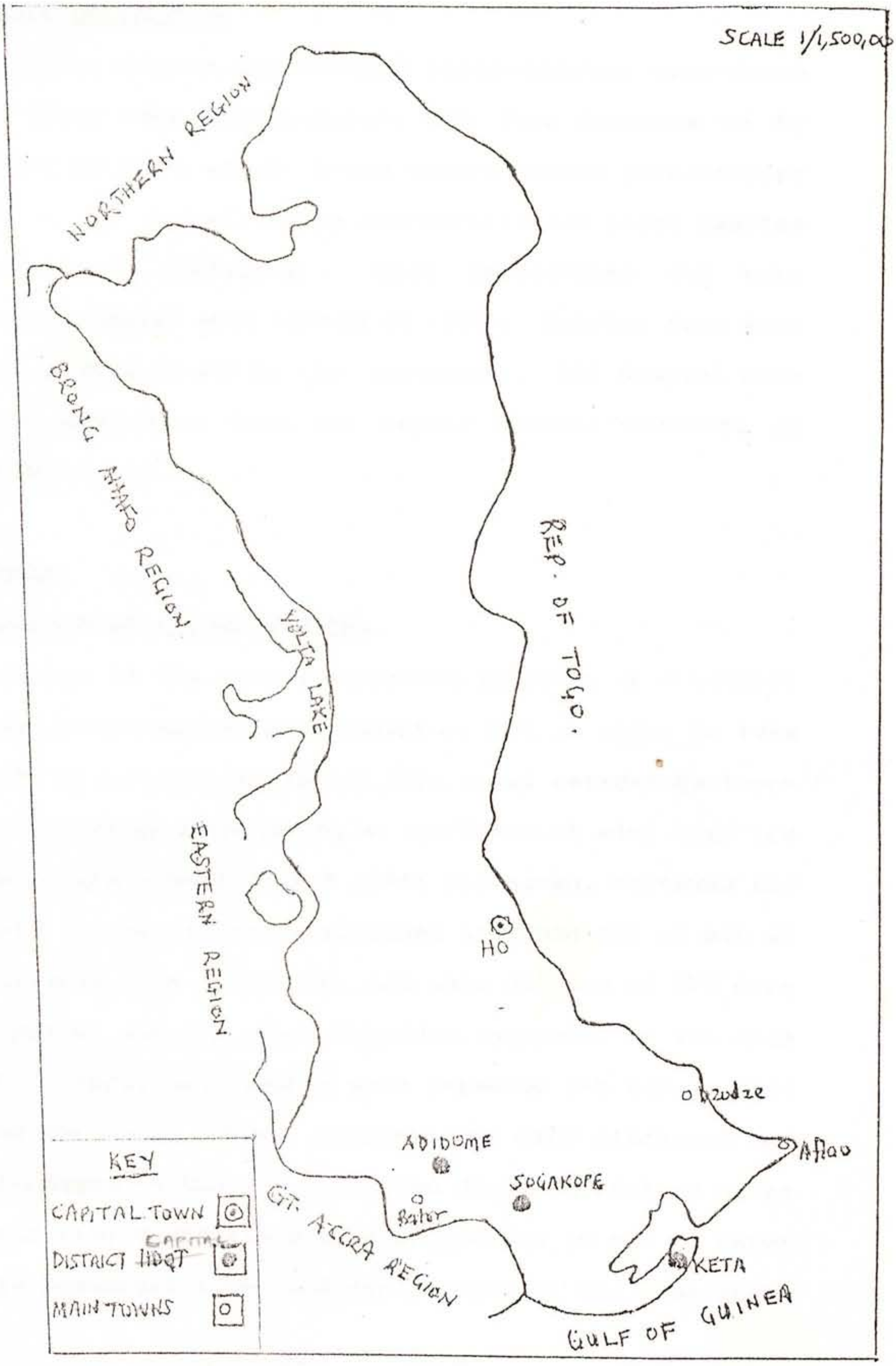


Fig. 11

2.1.2 SAMPLE COLLECTION

One mililitre malaria parasitized blood samples were drawn into sterile tubes with anticoagulant ACD (see Appendix A) by venipuncture from patients whose blood smears showed parasitemias ranging from +1 to +4. One mililitre non-parasitized blood samples were also collected similarly. Both parasitized and non-parasitized blood samples were stored at -20°C. Samples from Keta were mistakenly stored at 4°C by lab. personnel. All samples were collected upon permission from the Senior Medical Officers in charge of the Hospitals.

2.2 METHODS

2.2.1 ISOLATION OF *P.FALCIPARUM* DNA

A modification of the method described by Foley *et al.*,(1992) was used. Frozen blood samples were thawed at 37°C in order to lyse the cells and 200 ul portions dispensed into 12 ml centrifuge tubes with caps, sterilized by autoclaving at 121°C for 15 min. 10ml ice cold 5mM sodium phosphate buffer pH 8 (SP8) was added, vortexed for 30 s to resuspend the cells and centrifuged at 6000g for 10 min at 4°C. The supernatants were discarded, and same volumes of SP8 were added to the pellet and the centrifugation repeated at the same speed for 10 min. These cell washes were repeated 3-5 times until the pellets became clear. Final supernatants were discarded and the pellets resuspended in 200 ul sterilized double distilled water (SDDW). The resulting suspensions were boiled for 10 min on water path in sterile Eppendorf tubes and centrifuged at the same speed for 10 min. The supernatants containing the DNA were removed into 1.5 ml sterile Eppendorf tubes and stored at -20°C.

Five non-parasitized blood samples were also processed similarly as the parasitized samples and used as controls. Separate sets of pipettes and different laboratories were used for blood processing, PCR assays and electrophoresis.

2.2.2 SPECTROPHOTOMETRIC DETERMINATION OF DNA CONCENTRATIONS

Absorbance readings of the prepared DNAs were taken at wavelenths 260 and 280nm. Fifty microlitres of the DNA preparations were diluted with 550 ul SDDW and the absorbance read at 260nm and 280nm in a silica cuvette against a SDDW blank in a double-beam spectrophotometer (Shimadzu UV 190 Double-Beam). From the absorbance readings, the concentrations and the purities of the DNA preparations were calculated (Appendix B). The readings at 260nm allow for calculation of the concentrations of nucleic acids and the absorbance ratios A260/A280 provide estimates of the purity of the nucleic acids. For pure DNAs, the ratios should be between 1.8 to 2.0 and ratios less than 1.8 indicate significant contamination of the DNA with proteins and phenols (Maniatis *et al.*,1982). Absorbance at 260nm of 1 corresponds to approximately 50 ug/ml double stranded DNA (Maniatis *et al.*, 1989).

2.2.3 PCR ASSAY

Two - 10 ul of parasite DNA preparations of concentrations ranging from 360-400 ng/ul were amplified by PCR assay systems using mutation specific oligonucleotide (Diagnostic) primers:

DIA 12 5'-GGA-AAT-GCT-CTT-TCC-CAG-T-3' (specific for Asn-108),

DIA 3 5'-GAA-TGC-TTT-CCC-AGC-3' (specific for Ser-108),

DIA 15 5'-TTT-ATG-CCA-TAT-GTG-T -3' (specific for Val-16), and

DIA 16 5'-TTA-TGC-CAT-ATG-TGC-3' (specific for Ala-16)

Diagnostic primers DIA 12 and DIA 3 were used with common primers SPI 5'-ATG-ATG-GAA-CAA-GTC-TGC-GAT-3' while diagnostic primers DIA 15 and DIA 16 were combined with

SP3 5'-TTT-AAT-TTC-CCA-AGT-AAA-AC-3' and

SP2 5'-ACA-TTT-TAT-TAT-TCG-TTT-TC-3' respectively.

Amplification mixtures, 50 ul, consisted of 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 0.01% detergent mix, 0.2 mM mixed dNTPs (dATP,dGTP, dCTP, and dTTP), 5 ug primers, 8-10 ug DNA template and 1.5 units *Taq* DNA polymerase. 50 ul mineral oil was layered on the 50 ul PCR mix in 1.5 ml sterile Eppendorf tubes to prevent evaporation. The Eppendorf tubes 5'-TTT-ATG-CCA-TAT-GTG-T were inserted into the heat block holes of the thermo cycler (Techne PH2-Dri Block $^{(R)}$) containing drops (1 in each hole) of mineral oil to ensure thermal contact.

Forty cycles of amplification were employed each consisting of denaturation at 94°C for 30s, annealing at 50 C for 30s and primer extension at 74°C for 45s.

DNA templates from 3 *P.falciparum* clones whose responses to antifolates had been well established (Table 2 , Peterson *et al.*, 1990, Cowman *et al.*, 1988) were used as either positive or negative controls in each PCR assay (Fig. 12). 3D7 clone encodes Ser 108

(AGC), ItG2F6 clone encodes Val-16 (GTA) and HB3 clone encodes Asn-108 (AAC).

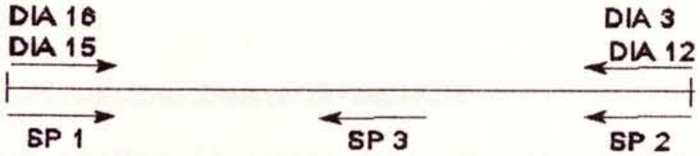
Table 2. Point mutations and antifolate susceptibility in *P.falciparum* (Peterson *et al.*, 1990).

Clone or isolate		Drug susceptibility, (ng/ml) F/P-free* RPMI		Amino acid residue				
Name	Origin	Pyr	Cyc	16	51	59	108	116
3D7	The Netherlands	0.02	0.02	Ala	Asn	Cys	Ser	Ile
ItG2F6	Brazil	2.10	55.00	Val	Asn	Cys	Thr	Ile
HB3	Honduras	22.00	0.45	Ala	Asn	Cys	Asn	Ile

Boxes identify mutations producing resistance to cycloguanil (Cyc) or pyrimethamine (Pyr).

* Folate - and PABA - free RPMI 1640 medium supplemented with 10% undialyzed human serum.

Fig. 12 Strategy for detecting *P.falciparum* DHFR mutations by mutation specific PCR amplification.



Primer pairs DIA15/SP3 and DIA16/SP2 produce amplified products when the *P.falciparum* DHFR gene has Val-16 and Ala-16 mutations respectively. Primers DIA3 and DIA12 with SP1 amplify in the presence of wild-type Ser-108 and Asn-108 codons respectively.

DIA12/SP1 was used with those test templates which did not give amplified products with DIA13/SP1, for Asn-108 detection. This was repeated for Val-16 detection by using DIA15/SP3 following DIA16/SP2 use.

Second PCR assays were conducted with samples which did not produce amplified product after the first PCR reaction to check if low template concentrations were responsible for PCR failures. Second PCR reactions were carried out by adding 2 ul of the 50 ul PCR product (as templates) to 48 ul PCR reaction mixture containing PCR buffer, primer, dNTPs, *Taq* DNA polymerase and SDDW and amplified for 32 cycles at the same temperatures.

2.2.4 OPTIMIZATION OF PCR ASSAY

Positive controls were used in the optimization reactions to establish that no non-specific amplification occurred and also to obtain only one PCR product. The amplification components examined were (1) units of *Taq* DNA polymerase and (2) various cycling parameters. Optimization reactions were based on a reaction volume of 50 ul.

2.2.4.1 UNITS OF *TAQ* DNA POLYMERASE

The stock enzyme (5 units/ul) was diluted 5 times with SDDW and various volumes, 1.0, 1.5, 2.0, 2.5 and 3.0 ul were used with a positive control (3D7 DNA as template and DIA3/SP1 primers).

2.2.4.2 CYCLING PARAMETERS

For denaturation, temperatures 97°C, 96°C and 94°C for 30s and 1min were tried and primer extension temperatures of 76°C and 74°C for 45s and 2 min were also tried using 3D7 DNA as template in each case. Number of cycles of extension were also varied as 32, 40 and 45.

The final modification which was used to process the blood samples is denaturation (94°C for 30s), annealing (50°C for 30s) and primer extension (74°C for 45s) for 40 cycles.

2.2.5 GEL ELECTROPHORESIS OF PCR PRODUCTS

The electrophoresis was carried out in a minicell EC 370M electrophoretic gel system. The electrophoresis chamber was placed near the power supply (Biorad Model 200/2.0) and levelled using the built-in bulls eye level and its adjustable feet. Both ends of the gel tray (10 x 6.5 x 0.5 cm) were sealed with the outer and inner molding rubber pieces and the tray placed across the chamber. The depth gauge surface to be used was selected and the slot former carefully positioned to ensure that it was level and also in the correct position for gel casting.

Enough electrophoresis buffer (0.5xTBE see appendix A) to fill the electrophoresis chamber and to prepare the gel was prepared. 100 ml of 0.5 x TBE was added to 1 g agarose and 2 g nusieve in a conical flask to prepare 1%/2% agarose nusieve composite gel. The slurry was heated in a boiling water bath with swirling from time to time, until the gel was completely dissolved. The solution was cooled to a temperature a few degrees above the setting point of the gel. To 30 ml portion of the gel solution was added 1.5 ul ethidium bromide (Appendix A) to give a final concentration of 0.5 ug/ml and the mixture thoroughly mixed. The molten gel solution was poured into the gel tray and any air bubble removed with a plastic probe. When it had set, the slot former and the outer and inner molding rubber pieces were removed. The gel was positioned in the chamber with the sample slots oriented closest to the cathode. The chamber was slowly filled with electrophoresis buffer to submerge the gel to a depth of 1 mm to ensure that the slot did not dry out during the run.

The PCR products (40 ul) mixed with 5 ul gel-loading buffer (Appendix A) were carefully loaded into the slots of the submerged gel using a micropipetter. The first slot was loaded with DNA molecular weight marker and the rest with PCR products. The cover was placed onto the chamber with both plugs and the leads connected to the electrophoresis power supply so that the DNA migrated towards the anode. A voltage of 100V was applied and the gel run until the bromophenol blue tracking dye had migrated two-thirds the length of the gel. The current was turned off and the safety cover removed from the chamber. The gel was placed on the UV-transilluminator (Ultra-lum UVA 40 Dual intensity) with the blocking shield in place and examined.

2.2.6 PHOTOGRAPHING THE GEL

Photographs of the gels were taken using the transmitted light from the UV transilluminator. The camera with hand grip and orange filter attached was loaded with the film (polaroid type 667) according to the manufacturer's instructions. The hood (model QSP Hood No. 14) was now attached and the whole set up placed completely over the gel (with UV luminescence ruler by the side), allowing it to rest on the UV transilluminator. The appropriate shutter speed and lens aperture were set, the light source was turned on and the trigger squeezed. The film was pulled from the camera slowly and developed for 30s (Fig. 14&15).

2.2.7 MEASUREMENT OF DNA SIZE

The mobilities of the standard DNA fragments (Øx174 RF1 DNA digested with Hinf1, Fig. 16) as well as those of the PCR products were measured from the original gel photographs with the gel slots as the origin. Three fragment sizes of the standard (L_1 L_2 and L_3) with their mobilities (M_1,M_2 and M_3) were used to calculate mo, a correction factor to fit a straight line: $L=K_1/(m-mo)+K_2$.

The sizes of the PCR products were determined from a plot of L against 1/ (m-mo), Fig. 16, by interpolation (Appendix B).

CHAPTER 3

3.0 RESULTS

3.1 ISOLATION OF *P.FALCIPARUM* DNA

P. falciparum DNAs were isolated from all 162 smear positive blood samples collected. Only 3 samples from each Hospital except 4 from Korle-Bu Teaching Hospital, with their corresponding concentrations and purities are given in Table 3.

The DNA concentrations varied from 63 to 274 ug/ml with almost 100-250 ug/ml from all communities except Keta where *P.falciparum* DNA concentrations ranged from 63-81 ug/ml.

The lowest ratios of 0.9 were obtained from Keta Government Hospital samples which had been stored at 4°C instead of -20°C.

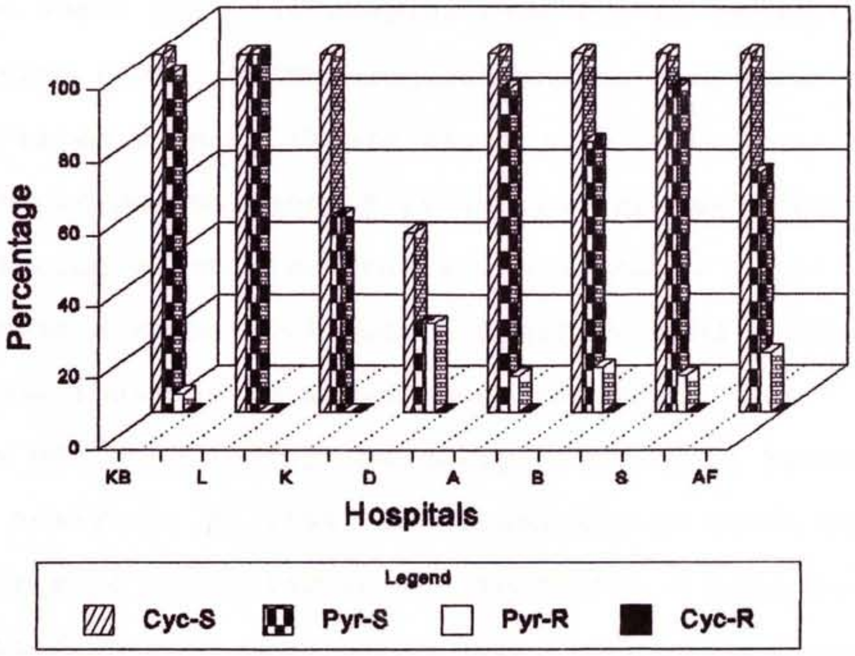
3.2 PCR ASSAY

PCR reactions were used to analyze the smear positive samples collected after determining optimum conditions under which the PCR assay could work. Table 4 shows the proportions of pyrimethamine-sensitive and - resistant *P.falciparum* isolates from the communities.

At 97°C denaturation temperature, no amplification was observed. Amplification for 45 cycles at 94°C denaturation temperature gave rise to distribution of broad amplification bands (non-specific amplification).

Pyrimethamine-resistant *P.falciparum* strains were present in 13 (8.6%) of the 151 samples analyzed, 10 were from Volta Region

Fig. 13. SENSITIVITY PATTERN OF *P. FALCIPARUM* FROM THE COMMUNITIES



Cyc-S - Cycloguanil-sensitive *P. falciparum* isolates.

Cyc-R - Cycloguanil-resistant *P.falciparum* isolates.

Pyr-S - Pyrimethamine-sensitive *P.falciparum* isolates.

Pyr-R - Pyrimethamine-resistant *P.falciparum* isolates.

KB - AF represent Hospitals where parasitized blood samples were obtained (see Table 3).

with the rest (3) from Greater Accra Region (Table 4). Of 151 samples, 133 (88.0%) contained pyrimethamine-sensitive *P.falciparum* isolates, 71 (47.0%) from Volta Region and 62 (41.0%) from Greater Accra Region.

Ten smear positive samples gave no PCR products. Second PCR assay using these 10 DNA preparations as templates showed only one amplified product (Table 6).

Various percentages of pyrimethamine-resistant parasites were detected in samples from all Hospitals. (Fig. 13).

Tables 5 shows cycloguanil-sensitive and -resistant *P.falciparum* isolates obtained from the communities. No resistant parasite was seen in any community. Out of 50 parasitized samples analyzed, 42 (95%) contained Ala-16 codon GCA characteristic of cycloguanil - sensitive *P.falciparum*. Six samples out of 11 from Keta Government Hospital contained parasites susceptible to cycloguanil, (Table 5). Smear positive samples from all communities except 2 from Dzodze showed 100% sensitive *P.falciparum* isolates.

Table 3. Purity and Quantity of *P.falciparum* DNA from the communities (Appendix B).

Samples	A260nm	A280nm	Conc.,ug/ml	A260/A280
KB1	0.221	0.203	132.6	1.1
KB2	0.200	0.198	120.0	1.0
KB3	0.241	0.230	144.6	1.0
KB4	0.166	0.168	99.6	1.0
K1	0.115	0.122	69.0	0.9
K2	0.135	0.149	81.0	0.9
K3	0.105	0.111	63.0	0.9
S1	0.176	0.177	105.6	1.0
S2	0.286	0.290	171.6	1.0
S3	0.296	0.294	177.6	1.0
B1	0.224	0.213	135.0	1.1
B2	0.177	0.177	106.2	1.0
B3	0.457	0.436	274.2	1.0
A1	0.173	0.171	103.8	1.0
A2	0.168	0.174	100.8	1.0
A3	0.328	0.305	204.0	1.1
D1	0.157	0.145	94.2	1.1
D2	0.275	0.273	165.0	1.0
D3	0.334	0.354	200.4	0.9
AF1	0.200	0.193	120.0	1.0
AF2	0.298	0.281	179.0	1.1
AF3	0.226	0.224	135.6	1.0
L1	0.176	0.175	105.6	1.0
L2	0.200	0.189	120.0	1.1
L3	0.176	0.168	103.8	1.0

DNA concentrations and purities (A260/A280) of 3 samples from each Hospital except 4 from Korle-Bu Teaching Hospital are given. Calculation of DNA concentrations of parasites is shown in appendix B. KB and K stand for Korle-Bu Teaching Hospital and Keta Government Hospital while S, B and A for Sogakofe Comboni Clinic and Health Centre, Bator, Catholic Hospital and Adidome, E.P. Church Hospital respectively. D stands for Dzodze St.

Anthony's Hospital, AF for Aflao New Hope Clinic and L for Legon, University Hospital.

Table 4. ASSESSMENT OF SENSITIVITY OF *P.FALCIPARUM* TO PYRIMETHAMINE.

Communities	No. of samples	Pyr-S	%Sensitive	Pyr-R	%Resistance	PNS
KB	59	56	94.9	3	5.1	0
L	6	6	100.0	0	0.0	0
K	11	6	54.5	0	0.0	5
D	4	1	25.0	1	25.0	2
A	20	18	90.0	2	10.0	0
B	16	12	75.	2	12.5	2
S	40	36	90.0	4	10.0	0
AF	6	4	66.6	1	16.6	1
Total Samples	162	133		13		5

Data on the samples from Keta Government Hospital are omitted because they have an additional variable (different storage temperature). Determination of Pyr-R is shown in appendix B.

Pyr-S —Pyrimethamine-Sensitive *P.falciparum* isolates.

Pyr-R —Pyrimethamine-resistant *P.falciparum* isolates.

PNS —PCR negative samples, samples which did not produce amplified product.KB —AF represent Hospitals where parasitized blood samples were obtained (see Table 3).

Table 5. ASSESSMENT OF SENSITIVITY OF *P.FALCIPARUM* TO CYCLOGUANIL

Communities	No. of Samples	Cyc-S	%Sensitive	Cyc-R	%Resistance	PNS
KB	8	8	100	0	0	0
L	6	6	100	0	0	0
K	6	6	100	0	0	0
D	4	2	50	0	0	2
A	6	6	100	0	0	0
B	6	6	100	0	0	0
S	8	8	100	0	0	0
AF	6	6	100	0	0	0
Total Samples	50	42		0	0	2

Data concerning the Keta samples are omitted because they have an additional variable (different storage temperature).

Determination of Cyc-R is similar to that of Pyr-R (appendix B). In this case DIA 15/SP3 primer pair specific for resistance of parasites to cycloguanil was used (Gyang *et al.*,1992).

Cyc-S ––Cycloguanil-Sensitive *P.falciparum* isolates.

Cyc-R ––Cycloguanil-resistant *P.falciparum* isolates.

PNS ––PCR Negative samples.

KB ––AF represent Hospitals where parasitized blood samples were obtained (see Table 3).

Table 6. Nested PCR

Communities	PNS	PPS
K	5	1
D	2	0
B	2	0
AF	1	0

Nested PCR reactions were performed with DNA templates which did not produce amplified products in the first PCR reactions. Only one DNA template (sample) produced PCR product after the second amplification. PNS ––PCR Negative samples,PPS ––PCR positive samples.

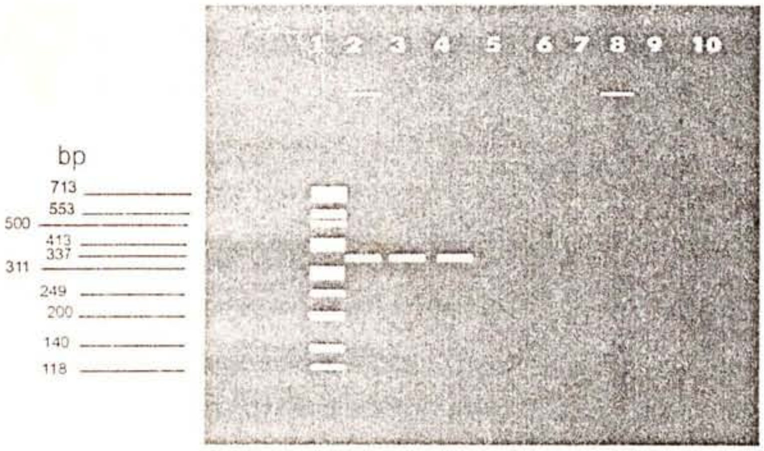
3.3 GEL ELECTROPHORESIS OF PCR PRODUCTS

Figure 14 shows separation and characterization of mutation specific PCR amplified products using purified genomic DNA from 3D7 or DNA prepared from blood obtained from malaria patients with primer pair DIA3/SP1. All DNA templates used gave 337 bp PCR amplified products on ethidium bromide-stained 1% agarose /2% nusieve gel.

Three lanes 2,3,4 after lane 1 (marker) showed PCR products when 3D7 (positive control), KB1 and KB2 templates were used. No amplified product was seen in lanes 5,6,9 and 10. High molecular weight fragments were seen in lane 8 (template control; no primers were used in the PCR assay) and lane 2.

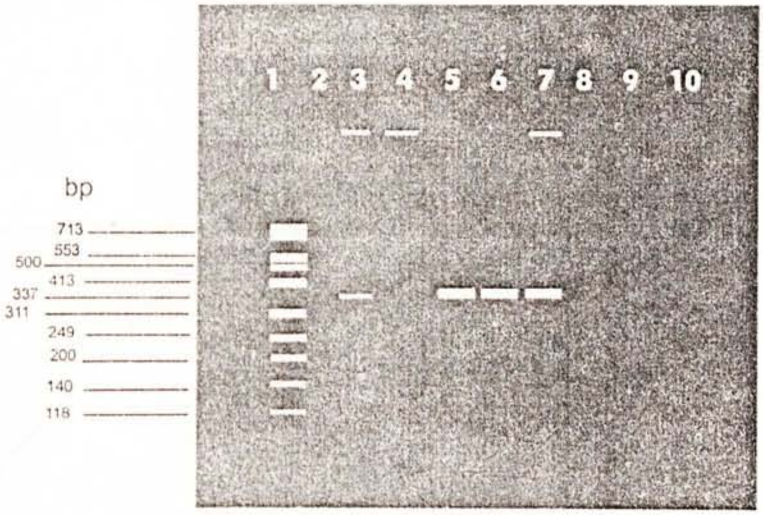
Separation and characterization of mutation specific PCR amplified products using purified genomic DNA from HB3 clone or

Fig. 14 Diagram of PCR products of *P. falciparum* isolates and DIA3 \ SP1 primer pair specific for Ser-108 on gel



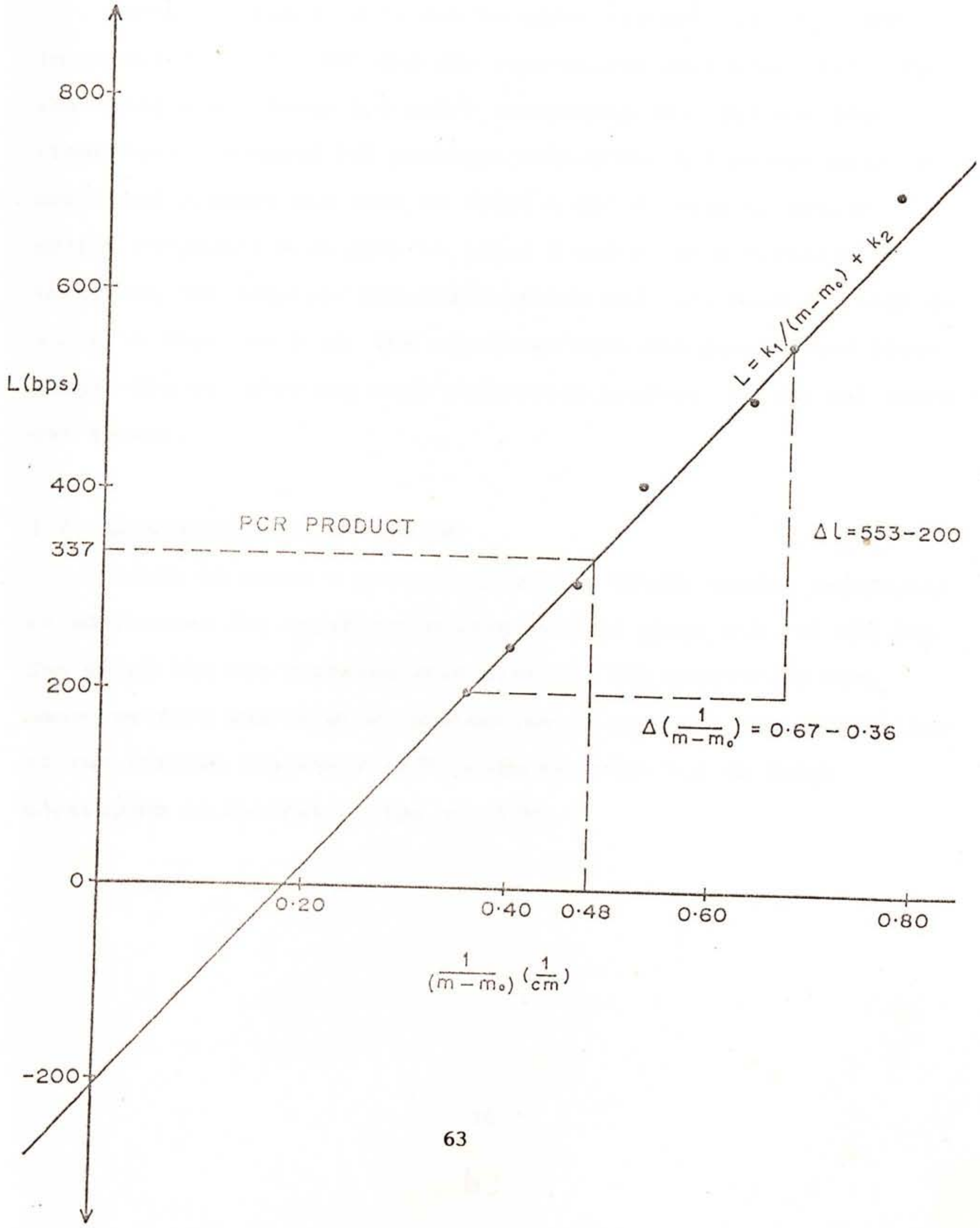
Legend: Lane 1 contained marker (ϕ X 174 RFI DNA digested with Hinf 1). Lanes 2-7 contained DNA from 3D7 (positive control), KB1, KB2, HB3(negative control), KB3 and KB4 respectively, lane 8, HB3 (template control - no primer) and lane 9 contained water control (no template), lane 10 had no PCR product.

Fig. 15 Diagram of PCR products of *P. falciparum* isolates and DIA 12 \ SPI primer pair specific for Asn-108 on gel



Legend: Lane1 contained marker (ϕ X 174 RFI DNA digested with Hinf I).
Lanes 2-7 contained DNA from 3D7 (negative control), KB1, KB2, HB3 (positive control), KB3 and KB4respectively. Lane 8 contained water control (no template). Lanes 9 and 10 contained no PCR product.

Fig. 16 A PLOT OF MOLECULAR WEIGHT VERSUS RECIPROCAL OF MOBILITY FOR RESTRICTION FRAGMENTS OF PHAGE Ø x 174 RFI DNA



test DNA prepared from malaria patients as templates with primer pair DIA 12/SP1 is shown in Fig. 15. Also 337 bp amplified products were observed.

Lanes 2,3 and 4 contained no amplified products when 3D7 (negative control), KB1 and KB2 were primed with DIA12/SP1. On the other hand, lanes 5,6 and 7 containing HB3, KB3 and KB4 (templates) produced PCR products with DIA12/SP1 primer pair. No amplified product was seen in lanes 8 and 9. High molecular weight fragments were seen in lanes 3 and 4. No mispriming of incorrect DNA template occurred because only one band was seen as shown in Figs. 14 & 15. PCR reactions with non-parasitized blood sample did not give any band (amplified product) on the gel (data not shown).

3.4 MEASUREMENT OF DNA LENGTH

Figure 16 shows a plot of molecular weight versus reciprocal of mobilities for restriction fragments of phage ϕ x 174 RF1 DNA. The phage DNA was digested with Hinf I. The mobilities were measured from the original photograph of the gel. The mobilities of the unknown fragments (PCR products) were 2.5 cm which correspond to molecular size of 337bp.

CHAPTER 4

4.0 DISCUSSION

P.falciparum DNA has been isolated from whole parasitized blood samples from patients (Table 3). The DNA was released by boiling which also inactivated parasites' proteases and nucleases (Foley *et al.*, 1992). Several washes in sodium phosphate buffer pH 8 (SP8; Appendix A) removed haemoglobin which inhibits *Taq* DNA polymerase activity (Barker, 1992).

Isolation of parasites' DNA from 200 ul infected blood samples gave enough DNA used as templates for PCR assays while those from 20 ul blood samples did not, no band was seen on ethidium bromide-stained gels (data not shown). Also resuspension of pellets when using 1ml blood needs a longer time of vortexing and breaking of DNA may be probable.

DNA concentrations (63-81 ug/ml) obtained from infected blood samples stored at 4^0C by laboratory personnel at Keta Government Hospital were lower than that obtained from other Hospitals where samples were stored at -20^0C (Table 3). *P.falciparum* DNA may have been degraded in the Keta samples.

Low DNA absorbance ratios (A260/A280) of 0.9-1.1 were obtained (Table 3) because SP8 could not remove proteins from the DNAs as absorbance readings at 280nm were as high as those at 260nm. Proteins absorb maximally at 280nm (Maniatis *et al.*,1989). Absorbance ratios less than 1.8 indicate that the DNAs are significantly impure (Maniatis, *et al.*, 1989).

The lowest absorbance ratio of 0.9 in Table 3, Keta samples and D3 from St. Anthony Hospital, Dzodze may be due to contamination of samples with microorganisms since these samples were not stored at -20^{0}C

The DNAs prepared from all samples did not show a high degree of purity. This notwithstanding, PCR reactions with them were successful. For instance, all *P.falciparum* DNAs prepared from Korle-Bu Teaching Hospital infected blood samples; KB1 KB2, KB3 and KB4 (Table 3) gave PCR products. This observation is in line with the findings that PCR DNA templates need not be pure for amplification to take place (Sjobring *et al.*, 1990; Saiki *et al.*, 1986).

No PCR amplification was observed at 97^{0}C because *Taq* polymerase has a lower half-life at this temperature than at 94^{0}c. It may be probable that after few cycles of amplification the enzyme activity left would not be enough to catalyse the synthesis of the product and that the PCR amplification which had taken place resulted in too small PCR product to be detected on ethidium bromide-stained gels.

The observation of non-specific amplification at or above 45 cycles of amplification was due to the formation of primer dimers (double stranded DNA fragments of two primers) which became predominant and overwhelmed the PCR amplification (data not shown).

In this study, the reduction of denaturation, annealing and primer extension times from 1 min, 1 min and 2 min to 30s, 30s and 45s respectively did not make any difference in amplification efficiency. An explanation may be that once primer template duplexes had been formed the *Taq* polymerase would catalyze the polymerization step spontaneously.

For the data to be statistically significant, standard error of 4% and confident interval of 95% were considered in estimating the sample size of 162 (Appendix B).

PCR analyses detected 96.7% (146/151) of samples collected when compared to the thick blood films (100%). This difference in detection (3.3%) is due to 5 smear positive samples (Table 4) which were consistently negative with the PCR assays. This observation is consistent with the reports that PCR specimens are occasionally negative (5-7%) in samples in which organisms were found microscopically (Sethabutr *et al.*, 1992).

PCR failures may be due to low concentration of DNA templates (63-81 ug/ml) as nested PCR (Table 6) showed amplification of one DNA template. This means that the product after the first PCR reaction was not enough to be detected on ethidium bromide-stained gels. Also some samples may have threonine rather than serine or asparagine at position 108 in the DHFR-TS protein (Peterson *et al.*, 1990) to which the primers used in the study could not anneal perfectly and could avoid detection. Alternatively, slides may have been stained in bulk and parasites washed off one onto another (Frimpong and Kankam, 1989). In addition, stained slides may have been misinterpreted by Laboratory personnel to have contained malaria parasites. Contamination of DNA templates may also be possible since 5 of the 10 PCR negative samples came from Keta Government Hospital where samples were stored at 4^{0}C and breakdown of the target sequence by microorganisms might have taken place. The target sequence of the rest(5) PCR negative DNA templates may have been fragmented during DNA isolation. For example, 2 out of 4 samples collected from St. Anthony's Hospital, Dzodze lacked the target sequence as nested PCR also failed to produce products (Table 6) giving the lowest pyrimethamine-sensitive and cycloguanil-sensitive percentages of 25 and 50 respectively (Table 4 & 5).

Thirteen (8.6%) pyrimethamine-resistant *P.falciparum* isolates detected (table 4) contained Asn-108 codon AAC of HB3 clone type since DIA12/SP1 primer pair was used (Fig. 15). DIA12/SP1 is diagnostic for Asn-108 while DIA3 is for Ser-108 (Gyang *et al.*, 1992).

The higher percentage of pyrimethamine-resistance 6.2% (10/162) in the Volta Region (table 4) may be due to self-medication and inaccurate dosage by the patients as drugs are bought without prescription in the rural areas of Volta Region. Prescription habits of Doctors and high use of drugs with pyrimethamine combination or alone as prophylactics may be responsible for the higher level of resistance observed in the Volta Region. These explanations may also account for the different percentages of pyrimethamine-resistance observed in all communities; Sogakofe 10% Dzodze 25% and Aflao 16.6%.

Of 151 samples, 88.0% (133/151) contained pyrimethamine-sensitive *P.falciparum* isolates. Of this number 47.0% (71/151) isolates were from Volta Region and the rest 41.0% (62/151) from Greater Accra Region (Table 4). This may be because more samples 60.3% (91/151) were collected from Volta Region than the 39.7% (60/151) from the Greater Accra Region.

The 337bp PCR products obtained (Fig. 14 & 15) is consistent with the results of Peterson *et al.*(1991) who used *P.falciparum* DNA with the same primers used in this study. Primer pair DIA3/SP1 produced amplified products with KB1 and KB2 (Fig. 14, lanes 3 and 4) while no amplified product was seen in Fig 14, lanes 6 and 7 with KB3 and KB4 as templates respectively. KB1 and KB2 hence contained Ser-108 codons AGC which were complementary at their 3′ ends to the diagnostic primer DIA 3 used. KB3 and KB4 may not contain Ser-108 to perfectly match with the DIA3 for PCR amplification to take place.

No PCR product was seen in Fig. 14, lanes 8 and 9 because lane 8 was loaded with a PCR reaction product of 3D7 as template with no primers (template control) while lane 9 (water control) lacked template. These reactions were used to eliminate mispriming and contamination of PCR reactions with DNA from sources other than from *P.falciparum* isolates. Instead of PCR products, a high molecular weight fragment was seen in Fig.14, lane 8. This is the 3D7 genomic DNA since Fig.14 lane 2 containing 3D7 DNA as template also gave a similar fragment size.

Fig.15 lane 5 contained PCR product of pyrimethamine-resistant HB3 with DIA12/SP1 primer pair specific for Asn-108 codon AAC (positive control). Since KB3 and KB4 also gave amplified products with DIA12/SP1 it indicates that their template DNAs contained Asn-108 (Fig.15, lanes 6 and 7). No band was seen in Fig.15 lane 4 loaded with KB2 - DIA12/SP1 PCR reaction product. It may be definite that KB2 lacked Asn-108 codon.

A faint band was seen in Fig.15 lane 3 loaded with PCR product of KB1 and DIA12/SP1. The same KB1 gave amplified product with DIA3/SP1 (Fig. 14, lane 3). It may be probable that double infection of pyrimethamine-sensitive and -resistant *P.falciparum* isolates were present in the KB1 sample. This supports the reports of Webster *et al.*(1985), that mixtures of parasites with different drug responses occur commonly in natural infections.

High molecular weight fragments seen in Fig. 14 lanes 3,4 and 7 may probably be parasite genomic DNAs since their mobilities were the same as that of 3D7 in Fig. 14 lane 2.

PCR assays for the detection of cycloguanil-sensitivity were performed with DNA templates from 50 out of the 162 smear positive samples. This is because the DIA16/SP2 and DIA15/SP3 primer pairs that detect cycloguanil-sensitive and-resistant malaria parasites respectively were not enough to assay for all the samples.

Similar gel pictures were seen when DIA15/SP3 specific for Val-16 codon GTA and DIA16/SP2 specific for Ala-16 codon GCA

(Gyang *et al.*, 1992) were combined with the isolated *P.falciparum* DNAs (data not shown). Here DNA purified from ItG2F6 clone harbouring Thr-108 and Val-16 mutations (Peterson *et al.*, 1990) was used as template (positive control) with DIA 15/SP3 as primer pair.

PCR products were seen when the test DNAs had been combined with DIA16/SP2 primer pair but no product was seen with DIA15/SP3 primer pair. This shows that all *P.falciparum* isolates examined contained Ala-16 codon GCA instead of Val-16 codon GTA. Unlike pyrimethamine, proguanil may have been used on a limited basis as a prophylactic antimalarial by patients. This explains why cycloguanil-resistant strains of the parasites were not detected. However, due to shortage of the appropriate primers for the cycloguanil sensitivity assessment no attempt is being made to put much emphasis on the result. It is recommended that further study involving larger sample size be conducted to make the results significant.

Results of this work provide strong evidence that Asn-108 mutation in the DHFR-TS gene which confers pyrimethamine-resistance of *P.falciparum* exists and is wide-spread in Southern Ghana.The occurrence of this mutation implies that the potential augmentative effects of sulfadoxine in fansidar combination will be ineffective against the pyrimethamine-resistant *P.falciparum* strains. This problem might be counteracted in some cases by using proguanil since the data, though limited, shows no cycloguanil- resistant *P.falciparum* strains.

For management of severe infections proguanil and pyrimethamine could be combined especially in areas where resistance is low to either drug so as to decrease the frequency of resistance and hence prolong the effective life-time of both drugs.

CHAPTER 5

5.0 CONCLUSION

Results of this study indicate that *P.falciparum* mutation which is responsible for parasites' resistance to pyrimethamine is wide spread in Southern Ghana.

Of 151 smear positive samples analyzed, 13 (8.6%) contained Asn-108 codon AAC that confers pyrimethamine resistance, 133 (88.0%) samples contained only the wildtype Ser-108 codon AGC. Out of the 13 pyrimethamine resistant cases, 10 were found in samples obtained from the Volta Region while the rest (3) were from Korle-Bu Teaching Hospital in the Greater Accra Region. Of 50 smear positive samples used for cycloguanil sensitivity testing because of inadequacy of diagnostic primers, none contained the Val-16 codon GTA responsible for cycloguanil resistance.

These data serve as baseline for continual monitoring of changes in parasites' sensitivity to antifolate antimalarial drugs in the country. The use of proguanil (paludrine) on a limited basis may be encouraged. However the total dependence on paludrine will lead to the spread of additional DHFR mutations. There is therefore the need to educate and continue to educate both patients and health professionals on the appropriate use of antimalarial drugs.

PCR amplification of the DHFR-TS gene presents a rapid alternative to *in vitro* drug susceptibility testing for the assessment of *P.falciparum* resistance to antifolate antimalarials.

CHAPTER 6

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CHAPTER 7.

7.0 APPENDIX A

7.0.1 SOLUTIONS AND BUFFERS

5x Tris-borate, EDTA (TBE):

54 g Tris base and 27.5 g boric acid: Dissolve in 900 ml of sterilised double distilled water (SDDW) and add 20 ml of 0.5M EDTA (pH 8.0). Make up to 1 litre with SSDW.

Ethidium bromide (10 mg/ml):

Add 10 ml SDDW to 0.1 g ethidium bromide powder, stir for several hours to ensure that the dye completely dissolves. Wrap the container in aluminum foil and store at room temperature. Wear gloves to avoid contamination.

6x Gel-loading buffer:

0.25 g bromophenol blue and 4 g sucrose. Dissolve in 10 ml SDDW.

Sodium phosphate buffer pH 8 (SP8):

Add 93.2 ml IM Na_2HPO_4(a) to 6.8 ml IM NaH_2Po_4(b) and dilute to 1 litre with SDDW. to produce stock 0.1M SP8.

Acid citrate dextrose (ACD):

8 g citric acid, 22 g sodium citrate and 24.5 g glucose. Dissolve in 1 litre of SDDW

7.1 APPENDIX B

7.1.1 CALCULATIONS

SAMPLING

Sample size (n) should satisfy the following conditions: (1) standard error (SE) of 4% or 0.04 and (2) confident interval (C.I) of 95% to be statistically significant.

Using $\{SE\ (\hat{}p)\}^2 = 1/4n \leq d^2$ (Prof. Odoom, Dept. of Statistics Univ. of Ghana, Legon) where p is proportion of resistance to antifolate and d is deviation.

$$\longrightarrow \sqrt{1/4n} \leq 0.04$$

$$\longrightarrow n \geq 162.$$

DNA CONCENTRATION

Dilute 50 ul DNA preparation to 600 ul with SDDW and read the absorbance at 260nm. A260 ≈1 is equivalent to 50ug/ml for double stranded DNA (Maniatis *et al.*, 1982). eg A260 of 0.262 equals:

[600/50 (0.262) 50] ug/ml which is 157.2 ug/ml.

DETERMINATION OF DNA LENGTH

Using $L=K_1/(m-mo)+K_2$ (1) (Southern^b, 1979) where m values are mobilities of DNA fragments while mo is a factor calculated to give the best fit straight line of the data. K_1 and K_2 are constants whereas L is molecular weight in terms of base pairs (bp). A plot of L vrs 1/(m-mo) gives slope as K_1 and intercept on the L-axis as K_2(ref. to graph, Fig. 16).

DETERMINATION OF Pyr-R

Blood samples whose *P.falciparum* DNA gave PCR products with DIA 12/SPI primer pair specific Asn-108 were taken as Pyr-R. Asn-108 confers on parasites resistance to pyrimethamine (Gyang *et al.*,1992).

STANDARD FRAGMENTS

Fragments	L(bps)	mobilities,m.(cm)
1	713	1.7
2	533	1.9
3	500	2.0
4	413	2.3
5	311	2.6
6	249	2.9
7	200	3.2

The unknown which is the PCR product has m of 2.5cm.

Consider fragments 2,5, and 7 as L_1, L_2, and L_3, respectively and their corresponding mobilities as m_1, m_2, and m_3.

Hence,

L(bps)	m(cm)
L_1(533)	m_1 (1.9)
L_2(311)	m_2 (2.6)
L_3(200)	m_3 (3.2)

From (1) $Mo = m_3 - m_1A/(1-A)$.

$$A = \frac{L_1 - L_2}{L_2 - L_3} \times \frac{(m_3 - m_2)}{(m_2 - m_1)}$$ (Southern[b], 1979).

$$\longrightarrow A = \frac{553-311}{311 - 200} \times \frac{3.2 - 2.6}{2.6 - 1.9}$$

$$A = \approx 1.869$$

$$Mo = \frac{3.2 - 1.9\ (1.869)}{1 - 1.869}$$

$$\approx 0.404$$

Table 7. DNA fragment lengths with their corrected reciprocal mobilities.

L (bps)	m (cm)	m-mo (cm)	1/(m-mo) (1/cm)
713	1.7	1.296	0.77
553	1.9	1.496	0.67
500	2.0	1.596	0.63
413	2.3	1.896	0.53
311	2.6	2.196	0.46
249	2.9	2.496	0.40
200	3.2	2.796	0.36

Mobility of the unknown fragment = 2.5 cm =m.

$$1/(m-mo) = 1/2.5-0.404 = 0.48\ cm^{-1}$$

$0.48\ cm^{-1}$ from the graph (Fig. 16) gives L value as 337bps which is consistent with the results of Peterson *et al.*(1991), who used *P.falciparum* DNA with the same primers.