"DETECTION OF DRUG RESISTANT *PLASMODIUM FALCIPARUM* BY POLYMERASE CHAIN REACTION USING MUTATION-SPECIFIC OLIGONUCLEOTIDE PRIMERS "

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ABSTRACT

Due to increased chloroquine resistance, the antifolate-sulfa combinations are becoming increasingly important in the chemotherapy of falciparum malaria. However, resistance to the antifolates exists and it is important to study it because they are still effective in the above combinations. Point mutations in the dihydrofolate reductase (DHFR) gene lead to resistance to the antifolate drugs. It was considered important to establish the prevalence of the six reported point mutations among Kenyan field isolates of *P. falciparum*, and look for a correlation between these mutations and resistance of these isolates to antimalarials.

Mutation specific polymerase chain reaction, (MSPCR), was carried out on 21 Kenyan *P. falciparum* isolates to detect point mutations. Optimal concentrations of each PCR component were established for each MSPCR reaction. Direct sequencing of the DHFR gene was also carried out to firstly confirm the above point mutations and secondly to look for any other base pair changes. ID_{50} values were calculated from drug sensitivity tests carried out by measuring ^{3}H Hypoxanthine uptake in the presence of increasing concentrations of the appropriate drug.

Out of the 21 Kenyan isolates five were found to be pyrimethamine sensitive and 16 were pyrimethamine resistant. Drug sensitivity results have shown that compared to reference strains, the Kenyan isolates examined are not resistant to the biguanides with respect to their ID_{50} values. At the same time none of the base pair changes associated with biguanide resistance (at

positions 16 and 108 in the DHFR gene) were found and neither was the change at 164 which confers cross resistance to both pyrimethamine and biguanides. Of the reported six mutations, we have so far only found three in Kenyan isolates. These are Ser-108 to Asn-108, Asn51 to Ile-51 and Cys-59 to Arg-59; all associated with pyrimethamine resistance. The results obtained were confirmed by direct sequencing. No other differences in the DHFR sequence were detected.

Thus the point mutations correlated well with absence or presence of pyrimethamine resistance and absence of cycloguanil resistance. MSPCR has the advantage over drug sensitivity tests of being specific, quicker and more reproducible. It should therefore be feasible to screen large numbers of patients to monitor the appearance, persistence and spread of anti-folate (e.g., pyrimethamine or biguanides) resistance of *P.falciparum* in a population.

1. INTRODUCTION

1.1.1 Literature review

Malaria, inspite of intensive efforts to reduce its transmission, is still the most serious and widespread protozoal infection in man. It is recognized as a serious health-care problem in tropical and subtropical regions of the world and one that has far-reaching medical, social and economic consequences for the countries in which it is found. Mortality due to malaria is high. Each year, approximately 2.5 million people die of malaria, most of whom are children (Sturchler, 1989). The population exposed to malaria infection is also growing. Malaria is now spreading to temperate regions where infected mosquitoes are carried in aeroplanes *en route* from tropical regions.

But the greatest problem remains in the developing world, where any strategy for the management of malaria is hampered by the generally poor standard of health care available. The worst affected region at present is Sub-Saharan Africa, which has to contend with rudimentary health-care services and a high transmission rate for *Plasmodium falciparum* - the most severe type of malaria.

According to the latest WHO estimates, 40% of the population of the world lives in areas where malaria is endemic, (WHO Reports, 1988). The size of the death toll from malaria is attributable partly to the size of the population at risk, and partly to the emerging problem of resistance to standard antimalarial treatments. Through continuous generation of new genetic variants, the malaria

parasites maintain barriers against control by chemotherapy or immunization.

1.1.2 Chemotherapy and drug resistance

Drug resistance is defined as the ability of a parasite strain to multiply or to survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication (WHO., 1965). Such resistance may be relative, yielding to increased doses of the drug that can be tolerated by the host or complete, when the parasite withstands maximum doses tolerated by the host.

Since the first report of chloroquine-resistant *Plasmodium* strains in Thailand 20 years ago, (Rieckman *et al.*, 1978) the problem has spread throughout Asia to South America and, more recently, to parts of Africa, (Peterson *et al.*, 1991). Furthermore, widespread resistance of *Plasmodium falciparum* to drugs, especially 4-aminoquinolines (chloroquine and amodiaquine), has been progressing with such a speed in many endemic malarious areas that therapy and prophylaxis procedures have been changing and new drugs or associations of them have been introduced.

At present, in Kenya about 50% of falciparum malaria is resistant to chloroquine treatment, (Watkins *et al.*, 1988). Although chloroquine remains the drug of choice, its use has been restricted to semi-immune people with uncomplicated malaria. However in combination with sulfa drugs, the dihydrofolate reductase (DHFR) inhibitors, such as pyrimethamine, (with either sulfadoxine (Fansidar; Watkins, 1988) or sulfalene (Metakelfin;

Oloo, personal communication), proguanil, chlorproguanil and trimethoprim, have been shown to be effective against chloroquine-resistant strains of *P. falciparum* and may succeed chloroquine and amodiaquine as the first line of treatment for falciparum malaria in Africa. Furthermore since these drugs are sporontocidal as well as schizontocidal they further reduce the transmission of the parasite and thus are of direct importance in the control of malaria.

Preliminary studies indicate that chlorproguanil in combination with dapsone is as effective as Fansidar in the treatment of non-severe falciparum malaria in Kenya and may exert less pressure for the selection of Dihydrofolate reductase resistance, an important consideration in areas of high transmission. A comparatively high *in vitro* activity of cycloguanil and chlorocycloguanil against pyrimethamine resistant Kenyan isolates has been noted (Watkins *et al.*, 1987). This may account for the greater effectiveness of chlorproguanil as a prophylactic in areas where pyrimethamine is quite ineffective (Bjorkman *et al.*, 1980).

1.1.3 Drug sensitivity studies

Longitudinal drug sensitivity studies were carried out in Kenya from 1980 through 1984 at a time when chloroquine-resistant falciparum malaria was emerging (Spencer *et al.*,1982; Spencer,1985). The results of these investigations illustrated the progression of chloroquine resistance, the response of *P. falciparum* to other antimalarial drugs and the potential

usefulness of the *in vivo* and *in vitro* tests. Pyrimethamine resistance was found to be widespread in Kenya (Spencer, 1985), and there was a good correlation between the *in vivo* response to Pyrimethamine and *in vitro* results using a modified 48 hr test (Nguyen-Dinh *et al.*, 1982).

Furthermore, *in vitro* drug susceptibility tests have been found useful for epidemiological purposes and for evaluating new drug candidates, (Desjardins *et al.*, 1979; Spencer *et al.*, 1984). *In vitro* and *in vivo* systems for evaluation of drug sensitivity against the same *P.falciparum* isolates have been found to produce comparable results. Spencer *et al.* (1984), found that the *in vitro* response to pyrimethamine /sulphadoxine could predict the *in vivo* response of the parasite to the drug. The predictive value of the *in vitro* response to the *in vivo* response was also reported by Nguyen- Dinh *et al.* (1982), Spencer *et al.* (1983) and Smrkovski *et al.* (1985).

1.1.4 Molecular mechanisms of drug resistance

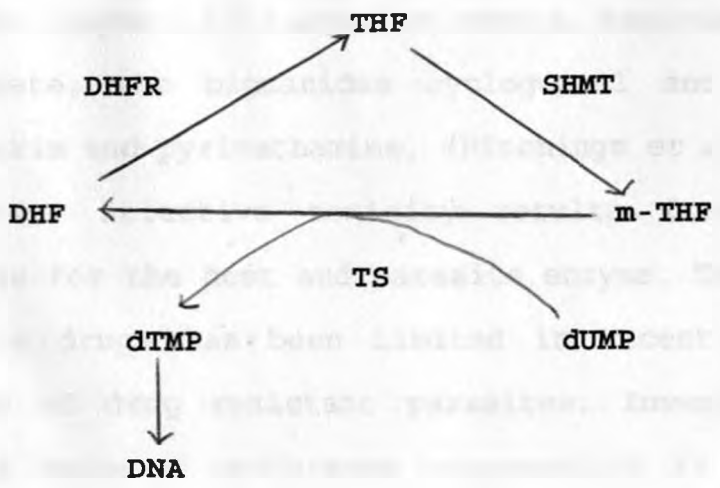


Diagram 1. Simplified representation of folate metabolism.

DHF: Designates dihydrofolate

THF: Designates tetrahydrofolate

m-THF: Designates N^5 N^{10}- methylene tetrahydrofolate

dTMP: Designates 2'deoxyribosylthymine 5'monophosphate

dUMP: Designates 2'deoxyribosyluracil 5'monophosphate

DHFR: Designates dihydrofolate reductase

SHMT: Designates serine hydroxymethyl transferase

TS: Designates thymidylate synthetase.

Antifolate drugs bind to and selectively inhibit dihydrofolate reductase (DHFR; 5,6,7,8 - tetrahydrofolate $NADP^+$ oxidoreductase EC 1.5.1.3) which plays an essential role in parasite growth. Dihydrofolate reductase catalyzes the NADPH- dependent reduction of dihydrofolate. The product, tetrahydrofolate, and other reduced folates serve as coenzymes in a number of 1-carbon transfers in purine, pyrimidine and amino acid biosynthesis (Blakely, 1984). Inhibition of dihydrofolate reductase results in depletion of cellular tetrahydrofolates, inhibition of DNA synthesis and cell death. The enzyme is a target of several important drugs including methotrexate, the biguanides cycloguanil and chlorcycloguanil, trimethoprim and pyrimethamine, (Hitchings *et al.*, 1965). With the latter two, selective toxicity results from their different affinities for the host and parasite enzyme. The effectiveness of antifolate drugs has been limited in recent years due to the emergence of drug resistant parasites. Investigations into the molecular basis of resistance is essential in the development of new strategies to counteract the resistance.

Several different molecular mechanisms have been implicated in the development of antifolate drug resistance in *P. falciparum*. A reduction in cell permeability to the drug is a possible mechanism although studies on *P. falciparum* isolates, FCR3 (pyrimethamine sensitive) and W2 (pyrimethamine resistant), showed identical drug uptake levels (Dieckman & Jung, 1986). A number of reports have suggested overproduction of the DHFR enzyme in cultured parasites (Kan & Siddiqui 1979; Inselberg *et al.*, 1987). Amplification of the DHFR gene leading to drug resistant mutants which overproduce the DHFR enzyme have been demonstrated in both mammalian lines (Schimke, 1984), *P. falciparum* cultured cell lines (Inselberg *et al.*, 1987; Tanaka *et al.*, 1990) and *P. chabaudi* (Sirawaraporn and Yuthavong, 1984). Other investigators have shown that the DHFR-TS gene is present as a single copy on chromosome 4 in both antifolate sensitive and resistant isolates (Cowman *et al.*, 1988; Snewin *et al.*, 1989). Furthermore, in pyrimethamine resistant field isolates, no evidence for overproduction of the enzyme (Chen *et al.*, 1987) or of DHFR gene amplification has been shown, (Cowman *et al.*,1988; Peterson *et al.*,1988; Khan et al, personal communication). Recent reports on the molecular basis of antifolate drug resistance in *P. falciparum* have implicated structural changes in the DHFR enzyme which affect drug binding (Cowman *et al.*, 1988; Peterson *et al.*, 1988; Peterson *et al.*, 1990; Foote *et al.*, 1990; Snewin, 1988). Evidence favoring such a mechanism has come from studies on a number of parasite isolates which display pyrimethamine and cycloguanil resistance associated with particular point mutations.

The amino acid residue at position 108 appears very important in this respect (Cowman *et al.*, 1988; Peterson *et al.*, 1988; Zolg *et al.*, 1989). All pyrimethamine resistant strains have been found to have a Ser 108 to Asn 108 mutation that produces only a small decrease in susceptibility of the parasite to cycloguanil (Peterson *et al.*, 1988). Furthermore parasites with a Ser 108 to Thr 108 mutation together with a Ala 16 to Val 16 mutation exhibit cycloguanil resistance but not pyrimethamine resistance. Ancillary point mutations at AA (amino acid) 51 and AA 59 have also been identified which result in a further increase in pyrimethamine resistance. Significant cross resistance to both pyrimethamine and cycloguanil has been reported to occur in parasites having mutations that include Ser 108 to Asn 108 and Ile 164 to Leu 164. Thus only 6 point mutations have been associated with antifolate drug resistance, with the rest of the enzyme sequence remaining invariant (Peterson *et al.*, 1988; Bzik *et al.*, 1987; Snewin, 1989).

The various mutations on the DHFR enzyme appear to have arisen in response to the different ways Cyc and Pyr bind to the DHFR. Alignment of the nucleotide sequence of DHFR from *P.falciparum* and other organisms shows that all of these mutations occur in conserved regions that border the active site cavity of the enzyme (Peterson *et al.*, 1990). Residue 108 occur in the C-alpha helix of the enzyme; residues 51 and 59 align near the beta-helix at the back of the cavity; and residues 16 and 164 in the A-beta strand and E-beta strand, respectively. The amino acid residues at or near

these positions have been shown to be involved in the binding of various inhibitors to avian, bacterial and mammalian DHFRs (Volz, *et al.*, 1982).

Since little is known about qualitative or quantitative aspects of the DHFR mutations in *P. falciparum* in Kenya, it is important to study the relationship between the DHFR gene sequence and their resistance to antifolate drugs.

1.1.5 Detection of single base changes in nucleic acids

Ever since the detection of the first mutations in *Escherichia coli*, and subsequently in human DNA (Flavel *et al.*,1978), efforts have been expended in defining changes in nucleic acid sequences of many organisms. This activity has been pursued in order to: (a) understand mechanisms of disease, (b) understand the structure and function of enzymes and nucleic acids (e.g. tRNA), (c) track micro-organisms in the environment, (d) track disease genes in people for diagnostic purposes, (e) define mutagenic substances, and (f) discover evolutionary relationships between species, (Flavel *et al.*,1978). The end point of a diagnostic study should be a method which will guarantee detection of all point mutations with a minimum of manipulation of the genetic material. However, there is a difference in the methodology depending on whether the position of the mutation is known. When the position of the mutation is unknown the method must screen kilobases of nucleic acid. Whereas, if the mutation position and type are known the operation is relatively simple.

The techniques for isolation of nucleic acids have been standardized over many years (Maniatis *et al.*,1982). This applies to microbial DNA, mRNA, viral RNA and DNA, and eukaryotic DNA. If larger quantities of these molecules or their copies (in the case of RNA) are needed for mutation characterization, amplification has usually been by cloning into plasmids and other vectors (Maniatis *et al.*,1982). However, this amplification has been achieved one or two orders of magnitude more quickly by a different approach which

relies on enzymatic rather than plasmid amplification of nucleic acids and is called the polymerase chain reaction (PCR), (Mullis *et al.*, 1987; Saiki *et al.*, 1986).

1.1.6 Mutation detection methods where the mutation position is unknown (Screening Methods)

Sequencing

Almost without exception the ultimate complete characterization of a base pair change will rely on sequencing of the target DNA or molecule which is based on strategies developed over 10 years ago (Maxam & Gilbert,1977;Sanger *et al.*,1977). This will define the new base and its exact position in the gene. However direct sequencing of PCR products can now be achieved without the need for M13 subcloning (Wong *et al.*,1987). The recent automation of sequencing promises to improve the rate and quality of sequencing, and thus the speed with which mutations can be detected.

1.1.6.1 Direct sequencing of enzymatically amplified DNA fragments

Application of PCR to amplify selected regions of genomic DNA flanked by a pair of oligonucleotide primers bypass DNA purification. Direct sequencing of the PCR product involves assymmetric PCR to produce single stranded DNA for sequencing reactions. We describe here PCR amplification of the DHFR gene of Kenyan field isolates of *P. falciparum*, rapid purification of the products, and direct sequencing using assymmetric PCR-mediated sequencing reactions.

The main problem associated with direct sequencing of PCR products derives from the ability of the two strands of the amplified fragment to rapidly reassociate, preventing the sequencing primer from annealing to its complementary sequence of blocking the primer template complex from extending. To reduce this problem either a variant of standard method for sequencing double-stranded DNA may be employed or single-stranded templates may be produced in the PCR (as is the case in this study).

1.1.6.2 Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes

Single base substitutions can be detected and localized by a simple and rapid method that involves ribonuclease cleavage of single base mismatches in RNA:DNA heteroduplexes. The resulting single base mismatch is cleaved by ribonuclease A, and the location of mismatch is then determined by analyzing the sizes of the cleavage products by gel electrophoresis. However, for technical reasons, only 20% of all possible substitutions can be detected directly in total genomic DNA, (Myers et al., 1985).

1.1.7 Mutation detection methods Where the mutation position is known

(Diagnostic Methods)

Mutation-specific or allele-specific amplification

(Polymerase Chain Reaction)

The PCR technique can accomplish efficient amplification of

any DNA sequences of interest. Two synthetic oligonucleotides are prepared using target DNA sequences. The oligonucleotides align on the target DNA one complimentary to each of the opposite strands. The DNA is denatured at high temperature (94°C) and then reannealled in the presence of a large molar excess of the oligonucleotide primers, oriented with their 3'ends pointing towards each other, hybridize to opposite strands of the target sequences and prime enzymatic extension along the nucleic acid template in the presence of the four deoxyribonucleotide triphosphates. The end product is then denatured again for another cycle. This leads to the enrichment of specific DNA sequences. This three step cycle is repeated 20 to 60 times, and amplification of upto a factor of 10^{12} can be achieved, (Saiki *et al.*,1988). The PCR provides alternative and improved methods of analysis. Mutation specific PCR determines whether previously characterized point mutations are present in a target sequence. Since efficient amplification occurs only when there is a perfect match between the target DNA and the 3' terminus of a diagnostic primer, a single nucleotide change can be detected by a PCR primer having a 3'terminal nucleotide complimentary to the mutation, (Gyang *et al.*, 1992). Two diagnostic primers and one common primer are required for each point mutation to be detected; one diagnostic primer complimentary to the 3'terminal nucleotide to the wild type sequence, the other complimentary to the mutated sequence, (Zolg *et al.*, 1989; Gyang *et al.*, 1992).

Sensitivity, speed and specificity make this assay an

attractive alternative to the *in vitro* drug testing as a means of monitoring the resistance of *P.falciparum* to inhibitors of the DHFR.

1.2 Overall Aim

The overall aim of this project is to correlate the susceptibility of *P.falciparum* isolates to cycloguanil and pyrimethamine with specific nucleotide changes in a DHFR gene.

1.2.1 Specific Objectives

1. To investigate the base pair change (from Ser-108 to Asn-108) in the DHFR gene implicated in pyrimethamine drug resistance.
2. To investigate the mutations (Cys-59 to Arg-59, Ser-108 to Asn-108 and Ile-164 to Leu-164) in the DHFR gene implicated in cross-resistance between pyrimethamine and cycloguanil.
3. To investigate the paired mutations (Ser-108 to Thr-108 and Ala-16 to Val-16) in the DHFR gene implicated in cycloguanil resistance in the absence of pyrimethamine resistance.

2. MATERIALS AND METHODS

2.1.1 Source of infected blood samples

Venous blood samples, seven from each region of Kenya, previously collected from malaria patients as part of field studies and cryopreserved in liquid nitrogen, were used. The infected blood samples were collected during the 1982 to 1990 period from malarious regions of Kenya namely: Nyanza Province (Kisumu and Saradidi), Rift Valley Province (Entaasopia) and Coast Province (Malindi, Jilore Primary School and Dindiri Primary School). Nyanza and Coast Provinces are areas of hyper- to holo-edemic malaria. The Rift Valley Province is an area of seasonal malaria following the rainy seasons. For cryo-preservation in liquid nitrogen, Rowe's cryo solution (2.5% glycerol, 3% sorbitol, 0.65 NaCl) (Rowe *et al.*, 1968) or dimethyl sulfoxide (DMSO) were used (Diggs *et al.*, 1977; Strome *et al.*, 1977).

2.1.2 *In Vitro* Cultivation of *Plasmodium falciparum* Isolates

Aseptic technique was used during the continuous cultivation of the malaria parasites. Glassware and plasticware used were sterile and all the preparations and filter sterilization of solutions done inside a laminar flow hood.

Among the human malaria parasites, only *Plasmodium falciparum* can be maintained in continuous *in vitro* culture

The parasite isolates were thawed out from liquid nitrogen (WHO, 1981; Trager and Jensen, 1986) and then cultivated in group O+ human erythrocytes by a modification of the methods of Trager

and Jensen (1976) and Haynes *et al.*, (1976) as described by Chulay *et al.* (1984) as follows :-

A 50% suspension of human type O+ erythrocytes was prepared by centrifugation in wash medium which consisted of powdered RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) diluted in sterile water with 25mM Hepes buffer (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; Calbiochem, La Jolla, California) and 25mM $NaHCO_3$ (GIBCO).

Stock cultures were maintained in 5ml tissue culture flasks as 6% erythrocyte suspension of RPMI 1640 medium diluted in sterile water with 25mM Hepes buffer, 25mM $NaHCO_3$ and 10% heat inactivated (40 minutes at 56°C) human serum. The flasks were flushed with a gas mixture consisting of 3% CO_2, 5%O_2 and 92% N_2 (East African Oxygen, Nairobi, Kenya) at 37°C.

During the continuous *in vitro* cultivation of the isolates, Giemsa-stained thin blood films of the parasite cultures were made to assess the parasite developmental stages, parasitaemias, growth rates and also to check if the cultures were free from bacterial, fungal or other contaminants.

Daily changes of the culture medium were carried out and fresh erythrocytes were added every three or four days. The dilutions were particularly necessary during the initial stages of culture adaptation of individual *P. falciparum* isolates and also for the preparation of the parasite isolates for *in vitro* drug sensitivity studies which required fast growing stock cultures. To obtain large numbers of the parasites for mutation-specific PCR and DNA

sequencing, 20 mls of fast growing parasites were used and double medium changes (one in the early morning, the other in the evening) carried out once the parasitemia was more than 3%.

Faster growth also resulted from putting cultures in the shaking incubator when the parasites were predominantly in the schizont stages.

2.1.3 *In vitro* test for *Plasmodium falciparum* sensitivity to antimalarial drugs

Microculture techniques were used to measure the activity of the antimalarial drugs to the isolates examined. The antimalarial drugs pyrimethamine and cycloguanil, were dissolved at required stock concentrations in ethanol and water.

The concentration range of the drugs used were as follows: pyrimethamine; 1.8×10^{-5} M /L to 1.5×10^{-13} M /L; Cycloguanil; 6.56×10^{-11} M /L to 6.5×10^{-12} M /L., (chlorcycloguanil and sulphadoxine were also tested although the results were not used during this particular study).

The microtiter plates contained 96 flat-bottom wells (see Figure 1), arranged in a matrix of eight columns (A through H) and 12 rows (1 through 12). When prepared , all wells of row 1 were used as controls (no drugs added). The first three wells: A, B, and C were used as UPRBC (Unparasitised Red Blood Cells) controls, the remaining wells of row 1, D,E,F,G,H were used as PRBC controls. All other wells of the rows 2 through 12 were charged with antimalarial drugs concentrations. The drugs were serially diluted three-fold

for the subsequent wells upto row 12 whose wells had the lowest drugs concentrations.

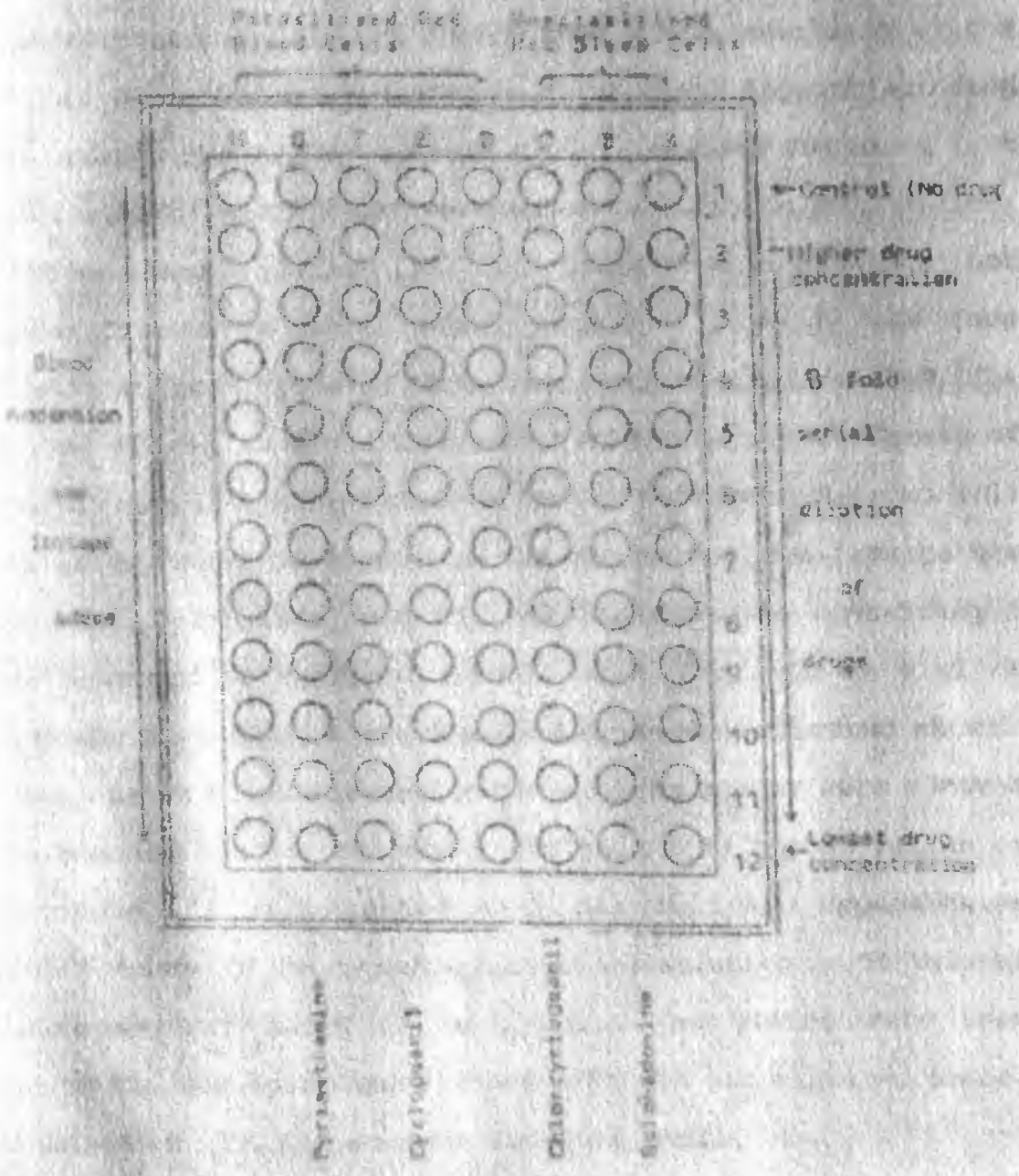


Figure 1. Drug sensitivity test plate.

In preparation for addition to the microtiter plates, fast growing stock cultures were further diluted in culture medium containing sufficient noninfected type O^+ human erythrocytes to yield a final hematocrit of 1.5% and parasitaemia of 0.4%. 200 ul of the parasitized erythrocytes were then added into each of the wells of the 96-well plate containing 25 ul of the appropriate drug solution, except the 3 UPRBC control wells (in which 200 ul of 1.5% hematocrit suspension of UPRBC were added). After the preparation, the plates were placed in a humidified airtight box (Instrumentation Department, Washington D.C.), which was then flushed with a gas mixture of 3%CO_2, 5%O_2 and 92%N_2 and sealed. The box was then placed in an incubator at 37°C for 48 hours. Uptake of [G^3H] hypoxanthine (Amersham/Searle Corp., Arlington Heights.III) was used as an index of growth of the parasites. The isotope was supplied as a lyophylate (6.2 ci/mMol) in ampoules containing 5 Mci. The contents of a single ampoule were dissolved in 5 ml of sterile water to provide a stock solution which was stored at 4°C.

After the 48 hours incubation period, the plates were removed from the box and 25ul of diluted radiolabel (the concentration of the radiolabel in each tested well was 1uci/ml) hypoxanthine solution (1 volume of G^3H-hypoxanthine stock solution in 50 volumes of culture medium) added to each well. The plates were then returned to the box again and flushed with the gas mixture, sealed and incubated at 37°C for an additional 18 hours.

At the end of the second incubation period, each plate was harvested on a MASH-II harvester (Microbiological Associates,

Bethesda, MD.). This instrument aspirated and deposited the particulate contents of each of the wells onto small disks of filter paper (No.934-AH, Whatman, Inc., Clifton, N. J.), which were then washed three times with deionized water. Each disk was dried and placed in a glass scintillation vial containing 1ml of scintillation fluid, and counted in a Beckman model LS 1801 liquid scintillation counter for two minutes.

This scintillation counter measures the incorporation of ^{3}H-hypoxanthine into parasite nucleic acids, with the results expressed as counts per minute (CPM).

The regression function, log (counts per minute) = a + b (log drug concentration), was calculated using one data point above and one below the value midway between the mean counts per minute in infected and uninfected control erythrocytes. The drug concentration causing 50% inhibition of radioisotope incorporation (ID_{50}) was estimated by interpolation. This method of estimating the ID_{50} gives a value equal to 100.5% $\pm$ 8.9% (mean $\pm$ SD) of the value estimated by nonlinear regression as described by Desjardins *et al.*, (1979).

2.1.4 Lysate preparation

Non-synchronous parasites were harvested at 5-10% parasitaemia, washed in 10mM Tris.Hcl, pH8, 0.95% (w/v) NaCl, 1mM EDTA, (TNE) and resuspended in the same buffer. Parasites were lysed from host erythrocytes by incubation with 0.1% saponin at 37°C for 5 minutes, pelleted and washed in TSE (1M Tris pH 8.0, 10%

SDS, 0.5M EDTA) at 4°C. The lysate was frozen and used as required.

2.1.5 DNA extraction

Lysates were incubated overnight at 37°C in TNE (50 Mm Tris-Cl pH 7.5, 50mM EDTA Ph 8.0, 100mM NaCl) buffer containing Proteinase K at 250ug/ml and 1% sodium dodecyl sulphate. The suspension was extracted twice with phenol:chloroform (1:1) and once with chloroform. The DNA was then precipitated by the addition of ethanol, vacuum-dried and finally resuspended in distilled water. One ul of 200ug/ml RNAse A was added to the final DNA volume, and incubated at 37°C for 30 minutes. The main purpose of the RNAse is to digest mRNA, tRNA and rRNA. The DNA samples, normally stored at 4°C, were used for both mutation-specific PCR and dideoxy DNA sequencing.

2.1.6 Ethidium bromide fluorescent quantification of the amount of double-stranded DNA

This is a rapid way of estimating the amount of DNA because the amount of fluorescence is proportional to the total mass of DNA.The quality and quantity of DNA was confirmed by agarose gel electrophoresis. Lambda DNA digested with Hind III was used as a standard. One hundred ng of the lambda Hind III marker was electrophoresed in 1% agarose gel (ultrapure,BRL) together with 1ul of the genomic DNA, 2 ul of 6x loading buffer (0.25% bromophenol blue .025% xylene cyanol and 40% (w/v) sucrose in water H_2O), and

made upto 12ul with ddH_2O. The gel was electrophoresed at 120V until bromophenol blue migrated to about 3 cm from the bottom (15x10 cm gel). The gel was stained with 0.5 ug/ml of ethidium bromide for five minutes and after the run the gel was destained with water for 15 mins. DNA was visualized under UV light (Transilluminator UVP) and photographed with Polaroid film Type 55.

2.1.7 Oligonucleotide primers and phosphoramidite method of oligonucleotide chemistry

Oligonucleotide primers for use in the mutation specific primer technique and for amplifying the DHFR gene were prepared on an Applied Biosystems 381A automatic synthesizer using phosphoramidate method of oligonucleotide synthesis chemistry. Synthesis of the oligonucleotides was done at International Laboratory for Research on Animal Diseases, ILRAD.

Beta-cyanoethyl was used as the phosphoramidite. The primary advantage of beta-cyanoethyl phosphoramidite was that thiophenol treatment was unnecessary making deprotection simple.

The synthesis was performed with the growing nucleotide chain attached to a solid support so that excess reagents which are in the liquid phase can be removed by filtration. The starting material was the solid support derivatized with the nucleoside which will be the 3'-hydroxyl end of the product. The nucleoside was bound to the silica solid support through a spacer arm attached at the 3'-hydroxyl. The 5'-hydroxyl was blocked with a

dimethoxytrityl group. The use of the solid support was essential for the automation of DNA synthesis because it eliminates the need for purification steps between base additions.

The first step of the synthesis cycle was treatment of the derivatized solid support with acid to remove the trityl group. This frees the 5'-hydroxyl for the addition reaction. The next step, activation , creates a highly reactive nucleoside derivative which reacts with the hydroxyl group. This activated intermediate was created by simultaneously adding the phosphoramidite derivative of the nucleoside to be coupled and a weak acid, tetrazole, to the reaction column. The tetrazole protonates the phosphoramidite, making it susceptible to nucleophilic attack. The phosphoramidite was blocked at the 5'-hydroxyl end with the dimethoxytrityl group. The next step, capping, terminates any chains which did not undergo addition. Since the unreacted chains have a free 5'-OH, they can be terminated or capped by acetylation. This reaction was accomplished with acetic anhydride and dimethylaminopyridine. Since the chains which reacted with the phosphoramidite in the previous step are still blocked with dimethoxytrityl group, they were not affected by this step. Although capping was not required for DNA synthesis, it minimized the length of the impurities and thus facilitated purification.

After oxidation, the dimethoxytrityl group was removed and the cycle was repeated until chain elongation was completed. At this point, the oligonucleotides were still bound to the support. They have beta-cyanoethyl protecting groups (adenosine and cytosine are

protected by a benzoyl group and guanosine by an isobutyryl group; thymidine is unreactive and does not need protecting), on the phosphates and protecting groups on the exocyclic amines of the bases Adenine, guanine and cytosine. Ammonium treatment alone removed the beta-cyanoethyl phosphate protecting groups and cleaved the DNA from the support. The crude DNA solution was then treated at 55°C for 8 to 15 hours to remove the protecting groups of the bases. The subsequent purification steps were done manually using Oligonucleotide Purification Cartridges (OPC).

The crude mixture was cooled to room temperature and applied directly to the OPC. No prior evaporation and re-dissolution was necessary, thereby preserving the fragile DMT (dimethoxytrityl) protecting group attachment. Prior to loading, the OPC cartridge was washed with 5ml each of acetonitrile and 2.0M TAE-Ac (pH 7.0).

The crude, 5'-trityl-on oligonucleotide in ammonium was applied to OPC using a syringe and male to male Luer. The eluant was then reapplied as above. The eluant from the loading contained substantial amounts of the crude oligonucleotide mixture and therefore was saved for future use.

The OPC was washed with dilute (1:10) ammonium hydroxide (3 X 5ml) to elute all the 5'-trityl-off contaminants under denaturing conditions, then washed with deionized water (2 X 5ml). The 5'-trityl-on oligonucleotide was then detritylated on the OPC with an aqueous solution of TFA (Triflouro acetic acid) (2 X 5ml). This 5'-trityl-off product was then eluted with the aqueous acetonitrile solution after an aqueous wash (2 X 5ml) to remove the TFA salts.

This solution eluted the oligonucleotide but not the trityl alcohol that was a by-product of the acidic cleavage.

One hundred ul to 500 ul of the deprotected oligonucleotide in eppendorf tube was vacuum-dried for 2-3 hours, making sure that the lid was perforated. The rest of the deprotected oligonucleotide was stored at -20°C. The pellet was resuspended in 200ul distilled deionized water and the $O.D._{260}$ UV was read using Spectrophotometer-20 with distilled water as the blank. The absorbance reading was converted to concentration using the formula: OD_{260} unit of single stranded DNA = 33 ug/ ml.

Fig. 2 a., shows a schematic presentation of phosphoramidite method of oligonucleotide chemistry.

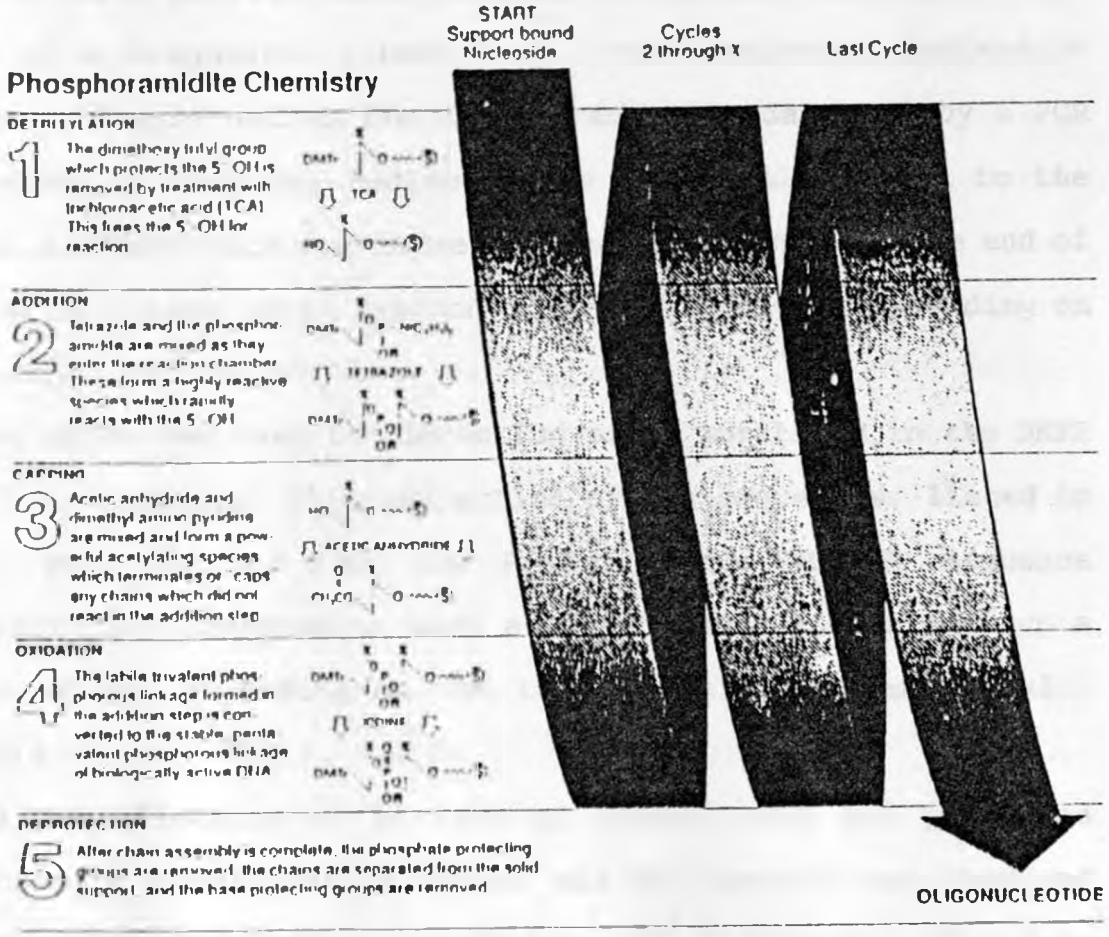


Figure 2 a: Schematic presentation of phosphoramidite method of oligonucleotide chemistry; (this article was obtained from Applied Biosystems Protocol).

2.1.8 Mutation specific PCR assay

Fig. 2 b., shows a diagrammatic presentation of mutation specific oligonucleotide PCR. Efficient amplifications occur only when there is a perfect match between the target DNA and the 3′ terminus of a diagnostic primer, e.g., with resistant isolate in Fig. 2 b; a single nucleotide change cannot be detected by a PCR primer whose 3′ terminal nucleotide is not complementary to the mutation, as shown with sensitive isolate. The result at the end of a reaction is either amplification or no amplification depending on the nature of the target DNA.

This assay was used for detecting point mutations in the DHFR gene of *P. falciparum*. Oligonucleotide primer sequences, listed in table 1, were derived from the *P. falciparum* DHFR/TS sequence (Bzik, 1987) and designed in such a way so as to either pick up a mutation or not depending on the target DNA, (Peterson *et al.*, 1990; Zolg *et al.*, 1991).

PCR amplification of 25-50ng of genomic DNA was performed under standard conditions. A master mix of reagents was prepared for all samples (PCR buffer [500 mM Kcl, 100 mM Tris-Hcl- pH 9.0 at 25°C, 1% Triton X-100], dNTPS [dATP, dGTP, dCTP, dTTP], primers and *Taq* polymerase) and then aliquoted into the individual tubes. The target DNA was added last. Such mixes minimized reagent losses on pipette tips and increased accuracy. To reduce evaporation or refluxing, the mix was overlayed with 25 to 50 ul of mineral oil (SIGMA, Part No. 8042-47-51). The oil did not interfere when withdrawing samples. The reaction was carried out using the Perkin

Elmer Cetus DNA Thermal Cycler. Since the method distinguishes a single base pair difference, annealing had to be done at the appropriate temperature to avoid false positives and false negatives, since at too low an annealing temperature, non-specific priming occurs leading to amplification of non-specific DNA fragments throughout and when the annealing temperature is too high no priming occurs, thus leading to no amplification. Amplification parameters for the different DHFR primers used are shown in table 2. Specificity of priming could also be increased by varying the magnesium chloride and enzyme concentration in the buffer. Different primer sets distinguish different point mutations (see table 3). The chosen primer pairs that distinguished resistant and sensitive isolates, besides giving a positive and negative answer, also gave products of different sizes, thus further helping to distinguish resistant and sensitive isolates. The products of the assay were visualized by gel electrophoresis using a 1% ethidium-stained agarose gel.

TABLE 1:

Sequences of the DHFR primers

OLIGONUCLEOTIDE	SEQUENCE
RP4	5'GTTATGGGAAGAACAAA 3'
P1	5'TTAAGCAGCCATATCCATTGA 3'
SP-1	5'ATGGGAAGAACAAC 3'
P2	5'TAATTTCTTCGTAGTTAATAATGG 3'
SP2	5'GGAATGCTTTCCCAGC 3'
P3	5'ATGATGGAACAAGTCTGCGAC 3'
DIA-16	5'TTATGCCATATGTGC 3'
SP-12	5'ACATTTTATTATTCGTTTTC 3'
DIA-15	5'TTTATGCCATATGTGT 3'
SP-13	5'TTTAATTTCCCAAGTAAAAC 3'
DIA-14	5'CAACGGAACCTCCTAA 3'
SP-11	5'ATGATGGAACAAGTCTGCGAC 3'
DIA-13	5'CAACGGAACCTCCTAT 3'
DIA-12	5'TATGATGGAACAAGTCTGCGACGTTTTCGAT 3'
SP-10	5'CTATACGGAAATTTCTTACTACTGTTTCTATG 3'
Asn-51	5'GTATTACCATGGAAATGTAAT 3'
Ile-51	5'GTATTACCATGGAAATGTAT3'
DIA-59	5'ATGTTGTAACTGCACG 3'
DIA-60	5'ATGTTGTAACTGCACA 3'

DEN: Designates the denaturation step, during which time the two strands of DNA are separated.

ANN: Designates the annealing step, during which time the base pairing of complementary sequences to the opposite strands of DNA takes place.

EXT: Designates the extension step, during which time the primers are extended in the 5′ to 3′ direction.

TABLE 2

Amplification parameters for the DHFR gene primers.

AMPLIFICATION PARAMETERS, FINAL CONC IN 25 ul VOLUME	16	51	59	108	164
$MgCl_2$ mM/L	1.5	2.5	1.5	1.5	1.5
dNTP uM	200	200	200	100	200
Primer uM	0.5	0.5	0.5	0.5	0.5
Taq U	1.25	1.25	1.25	1.25	1.25
DNA ng	50	25	50	50	50
CYCLES	35	30	35	35	35
DEN: ° C /1min	94	94*	94	94	94
ANN: ° C /1min	47	52*	54	54	56
EXT: ° C /1min	72	72*	72	72	72
Extension 72°C File: (min)	10	10	10	10	10

* The denaturing, annealing and extension times spent in this case is 30 secs each.

TABLE 3

Sizes of the PCR product generated by the primer pairs on the DHFR gene

Primer Set	Codon	PCR product size (bp)
1. DIA16/SP12	Ala-16 (GCA)	667
2. DIA15/SP13	Val-16 (GTA)	438
3. DIA14/SP11	Leu-164 (TTA)	505
4. DIA13/SP14	Ileu-164 (ATA)	505
5. RP4/P1	Asn-108 (AAC)	1521
6. RP4/DIA13	Asn-108 (AAC)	206
7. SP1/P2	Thr-108 (ACC)	776
8. SP2/P3	Ser-108 (AGC)	301
9. DIA59/P3	Arg-59 (CGT)	190
10.DIA60/P3	Cys-59 (TGT)	190
11.WILD TYPE 51/SP12	Asn-51 (AAT)	563
12.RESISTANT TYPE 51/SP12	Ile-51 (ATT)	563

2.1.9 Optimization of PCR

The various parameters and buffer components of the PCR listed below need to be adjusted for this. The effect of each component is described. Optimal concentrations of each component were established for each of the 11 reactions and are summarized in Table 2.

2.1.9.1 *Taq* concentration

A recommended concentration range for *Taq* DNA polymerase was between 1 and 2.5 units per 100 ul reaction when other parameters were optimum. However, enzyme requirements may vary with respect to individual target template or primers. When optimizing a PCR reaction, it is recommended that testing be performed of enzyme concentration ranging from 0.5U to 5U /100 ul and assaying the results by gel electrophoresis. If the enzyme concentration is too high, non-specific PCR products may be made, and if the amount of enzyme is too low, an insufficient amount of desired PCR product may be made.

2.1.9.2 Deoxyribonucleotide-triphosphates

Deoxyribonucleotide-triphosphates, (dNTP) concentrations between 20 and 200 uM each resulted in the optimal balance among yield, specificity and fidelity. The four dNTPS (dATP, dTTP, dCTP and dGTP), were used at the same concentrations to minimize misincorporation. Low DNTP concentrations minimize mispriming at non-target sites and reduce the likelihood of extending misincorporated nucleotides (Innis *et al.*, 1988).

2.1.9.3 Magnesium chloride

The magnesium concentration may affect all of the following- primer annealing, strand dissociation temperature of the template, product specificity, primer-dimer formation, enzyme activity and fidelity. *Taq* DNA polymerase requires free magnesium in addition to that bound by template DNA, primers and dNTPS. Accordingly polymerase chain reaction should contain 0.5 to 2.5 mM magnesium.

2.1.9.4 Primer Annealing

The temperature and length of time required for primer annealing depended upon the base composition, length and concentration of the amplification primers. Increasing the annealing temperature reduces non-specific annealing of primers to the target DNA.

2.1.9.5 Primers

Primer concentrations between 0.1 and 0.5 uM were generally optimal. Higher primer concentrations promoted mispriming and accumulation of non-specific product and increased the formation of template-independent primer-dimers.

Non-specific products and primer-dimer are themselves substrates for PCR and compete with the desired product for enzyme, dNTPS and primers, resulting in a lower yield of the desired template. Typical primers were 18 to 28 nucleotides in length having 50% GC composition. One should avoid complementarity at the 3′ ends of primer pairs as this promotes the formation of primer-dimer artifacts.

2.1.9.6 DNA concentration

High concentrations of non-target DNA, can result in non-specific amplification. Fifty ng of the template DNA was found to be optimal amount for a 25ul PCR reaction. For the 51 codon the amount of the template DNA was 25 ng.

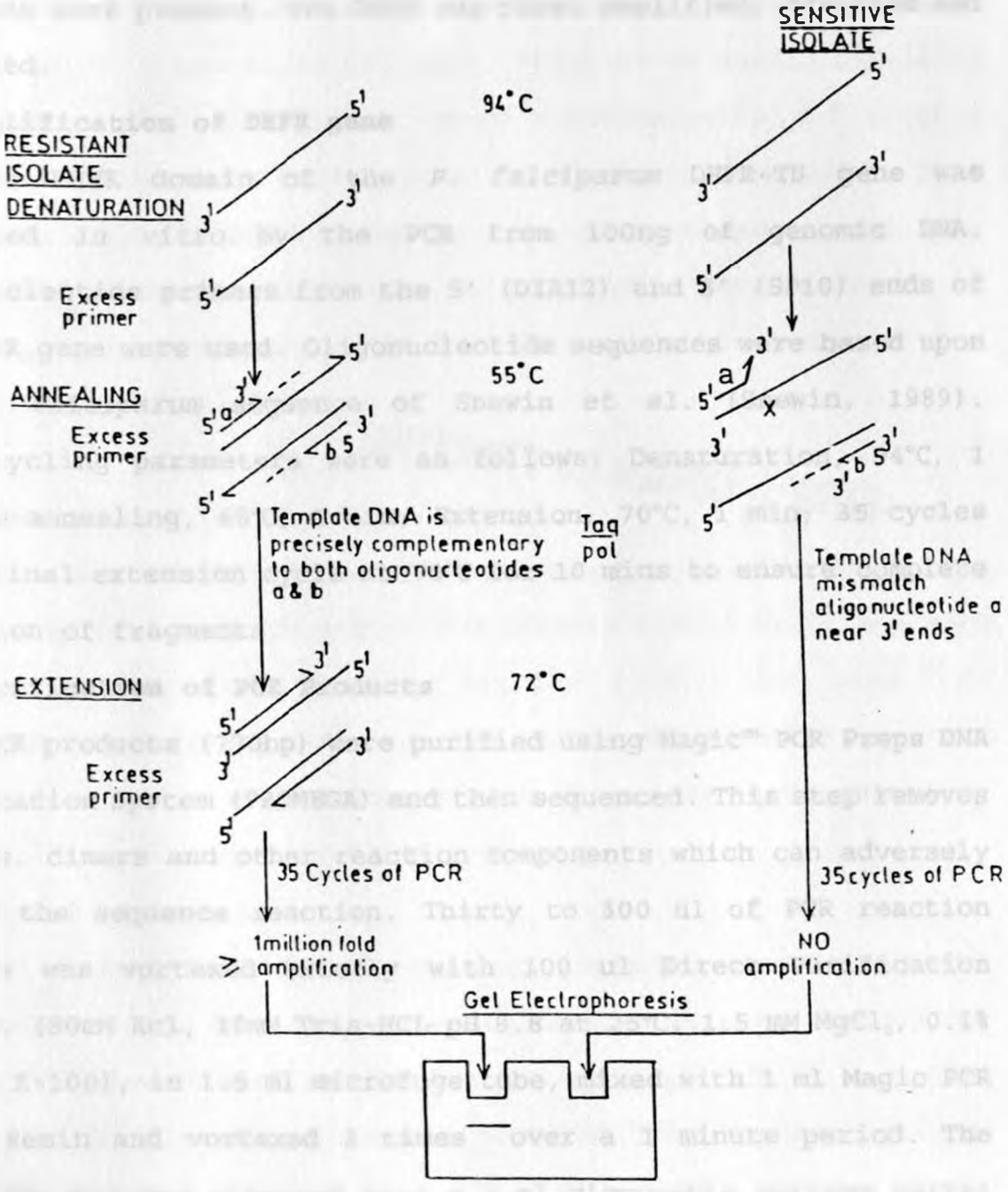


Figure 2 b: Diagramatic presentation of Mutation-specific polymerase chain reaction.

2.2 DNA Sequencing of DHFR

DNA sequencing of the DHFR gene was carried out in order to confirm point mutations detected by MSPCR and to see if novel point mutations were present. The DHFR was first amplified, purified and sequenced.

2.3 Amplification of DHFR gene

The DHFR domain of the *P. falciparum* DHFR-TS gene was amplified *in vitro* by the PCR from 100ng of genomic DNA. Oligonucleotide primers from the 5' (DIA12) and 3' (SP10) ends of the DHFR gene were used. Oligonucleotide sequences were based upon the *P. falciparum* sequence of Snewin *et al.* (Snewin, 1989). Thermocycling parameters were as follows: Denaturation, 94°C, 1 min; Re-annealing, 60°C, 1 min; Extension, 70°C, 1 min; 35 cycles and a final extension cycle at 70°C for 10 mins to ensure complete extension of fragments.

2.4 Purification of PCR Products

PCR products (770bp) were purified using Magic™ PCR Preps DNA Purification System (PROMEGA) and then sequenced. This step removes primers, dimers and other reaction components which can adversely affect the sequence reaction. Thirty to 300 ul of PCR reaction mixture was vortexed briefly with 100 ul Direct Purification Buffer, (50mM Kcl, 10mM Tris-HCL pH 8.8 at 25°C, 1.5 mM $MgCl_2$, 0.1% Triton X-100), in 1.5 ml microfuge tube, mixed with 1 ml Magic PCR Preps Resin and vortexed 3 times over a 1 minute period. The resin/DNA mix was pipetted into a 3 ml disposable syringe barrel attached to the Luer-lock extension of a Magic Minicolumn and

slowly pushed in the Minicolumn with a syringe plunger. The column was washed by carefully pushing through 80% isopropanol.

The Minicolumn was transferred to a 1.5 ml Microfuge tube and centrifuged at 12000 g to dry the resin. The column was eventually transferred to a new microfuge tube. Fifty ul of double distilled water was added to the column using a micropipette and after 1 minute the column was centrifuged for 20 seconds at 12000 g to elute the bound DNA fragment. An aliquot was used for quantification of the PCR product by ethidium bromide-stained 1.5% agarose gel electrophoresis. The rest of the sample was stored at -20°C until needed.

2.5 Sequencing of DHFR gene

This was performed using the Promega fmol DNA Sequencing System. Ten pmol of each sequencing primers DIA12, SP12, and SP10 were end-labelled in a labelling reaction with 10 pmol gama ^{33} P-Adenosine Triphosphate using 5 units T4 polynucleotide kinase and incubated at 37°C for 30 minutes. The kinase was inactivated by heating the reaction at 90°C for 2 minutes.

2.6 Sequencing reactions

The Promega fmol™ DNA sequencing system was used to sequence PCR products. The fmol DNA sequencing system is a new method for enzymatic sequence analysis which takes advantage of the intrinsic properties of the DNA polymerase isolated from *Thermus aquatics* (*Taq* DNA polymerase).

For a set of four 0.5 ml microfuge tubes labelled A,T,C,G, 2

ul of appropriate d/ddNTP mix was added and kept on ice. For a sequencing reaction, 20 ng DNA, 1.5 pmol end-labeled primer, fmol sequencing 5X buffer (Promega, Cat No. Q407B)- { 250mM Tris-Hcl, pH 9.0 at 25°C; 10mM $MgCl_2$ } were added together, volume made up to 16 ul with distilled water, and 5 units (1 ul) of Sequencing Grade *Taq* DNA Polymerase added. Four ul of this was transferred to each of the four tubes containing d/ddNTP mix, a drop of mineral oil layered on top of each reaction in a tube which were then placed in a Perkin Elmer DNA thermal cycler preheated to 95°C.

The sequencing reaction cycle profile consisted of 95°C for five seconds, 55°C for 30 seconds, 72°C for 1 minute, in a total of 30 cycles performed. Samples were cooled to 4°C and 3 ul of fmol™ sequencing stop solution (Promega, Cat No. Q408B) added. These samples were heated at 70°C for 2 minutes and 4 ul loaded on a 6% Sequencing gel.

The principle behind the dideoxy chain termination DNA sequencing method is illustrated by the diagram shown in Fig. 2 c. Regions covered in bases by DIA12, SP12 and SP10 sequencing primers showing overlapping of sequences is shown in Fig. 2 d.

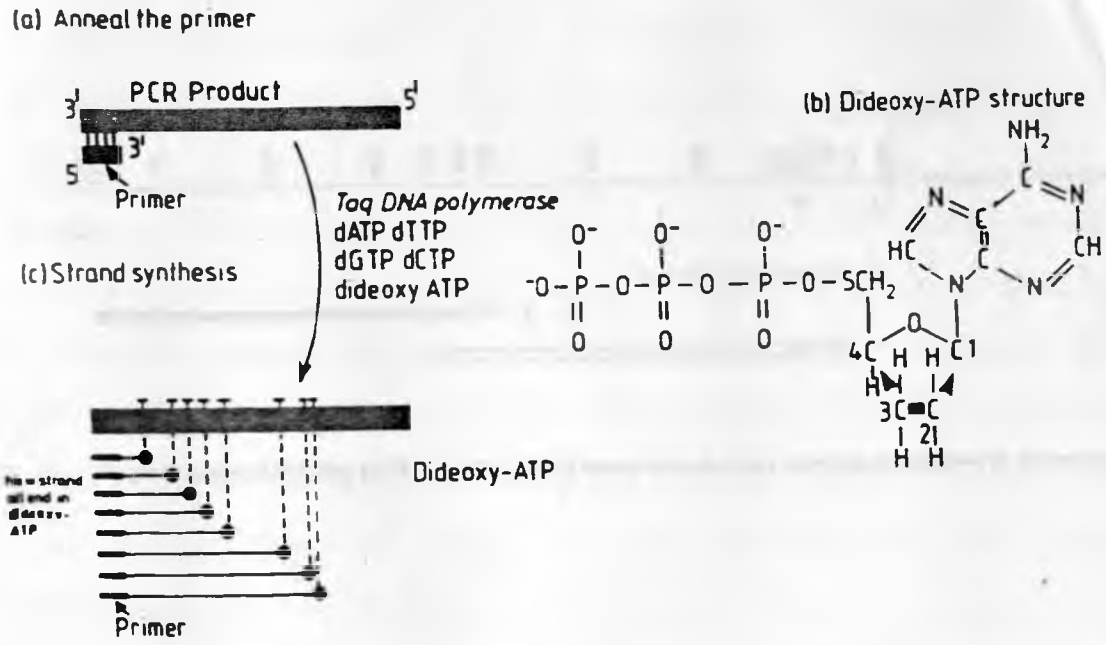


Figure 2 c: Dideoxy chain termination method of sequencing of DNA.

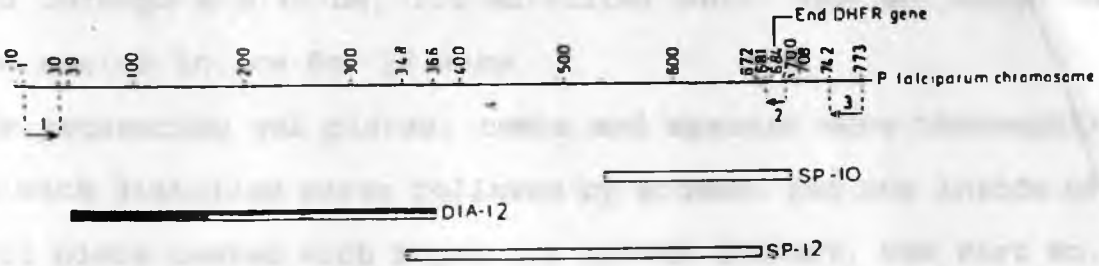


Figure 2 d: Regions covered in bases by DIA12. SP12. SP10 sequencing primers showing overlapping of sequences

2.7 Gel-preparation

The sequencing gel was a 6% polyacrylamide gel (42 gm ultrapure urea, 15 ml of 40% acrylamide, 10 mls of 10X TBE (Tris base 1M, Boric acid anhydrous 1M, $Na_2EDTA.2H_2O$ 20mM, pH 8.3) and autoclaved water). One hundred mls of the above gel solution was prepared. The sequencing gel plates can take 50 mls of gel solution but 100 mls was prepared to allow for spillage.

The gel solution was warmed in a microwave to dissolve, at medium setting for 5 minutes. After dissolving the solution was made upto 100 mls with autoclaved distilled water. The solution was filtered through a 0.45 uM, 115 ml-filter unit. The gel solution was then cooled in ice for 15 mins.

The sequencing gel plates, combs and spacers were thoroughly cleaned with distilled water followed by ethanol and the inside of the small plate coated with Sigmacote (SIGMA COMPANY, USA Part No. 31H-6176), which allows the gel to stick only to the big plate so as to facilitate the subsequent steps of gel drying and fixing. The plates with spacers were taped at the bottom and the combs assembled on top of the plates

Six hundred ul of 10% ammonium persulfate (APS) followed by 100 ul of TEMED was added to the cooled acrylamide gel solution and mixed well. The APS facilitates the polymerization reaction. The contents of the gel were immediately but quickly poured in between the plates using a 60 ml syringe. Constant tapping of the gel-plates was done when the gel contents were poured to ensure no air bubbles were trapped.

2.7.1 Polyacrylamide gel electrophoresis

The combs were kept in position and the tape removed from the bottom of the gel plates. The whole set up was then clamped in place in the electrophoresis tank (Sequencing gel electrophoresis tank, Model S2- Life Technologies Inc., BRL) containing 1X TBE electrophoresis buffer in the top and bottom compartments.

The gel was pre-heated for 30 minutes at a constant wattage of 65 W and 2100 Volts (Pharmacia power pack, LKB. ECPS 3000 /150). This is done to test for possible leakage that might occur and also to allow for a uniform run of the samples.

The PCR samples were denatured at 94°C for 2 mins before loading into the polyacrylamide gel. After denaturing the PCR products were kept on ice to prevent reannealing of the DNA strands. The plates were marked according to the loading of the samples, i.e., the template and the primers used.

Prior to loading of the samples, the wells were thoroughly washed with 1X TBE to remove urea that might interfere with the loading of the samples.

Four ul of the PCR samples were loaded into each well. The samples were allowed to run for three and a half hours, for the long run, followed by a short run of one and a half hours.

2.7.2 Gel fixing

After electrophoresis the running buffer from the top compartment was allowed to run into the bottom compartment of the electrophoresis tank. The small plate was removed and the longer plate having the gel, was put in a tray having an acetic acid,

methanol water mixture: (1:1:8) for fixing the gel. It was fixed for 30 minutes and then rinsed with double distilled water for 20 minutes. 3 MM Whatman paper, 46 X 57 cm (Whatman, Cat No. 3030917) was gently applied onto the gel and the gel lifted off the plate and onto the Whatman paper. The gel was covered with a cling film, and placed on a drier (Electrophoresis ATTD) and dried for 40 to 60 minutes. After drying, the gel was put in a cassette containing an X-ray film 34 X 43 cm (X- OGRAPHIC LTD), and allowed to develop for 72 hours at room temperature.

2.7.3 Radiographic film processing

The X-ray film was developed for five to seven minutes in a mixture of acetic acid, diethylene glycol, ethylene glycol, glutaraldehyde, hydroquinone, phenidone and potassium hydroxide in appropriate concentrations.

The autoradiograph (autorad) was then rinsed in water to remove all traces of the above chemicals. The next step involved fixing of the autorad for four to five minutes in a mixture of acetic acid, aluminium chloride and ammonium thiosulfate. The autorad was again rinsed in a tank of water so as to remove all traces of the fixative. The autorad was then allowed to dry and the sequences of the bases read manually starting from the bottom of the gel which represents the 5′ end of the sequenced molecule.

3. RESULTS

3.1.1 *In vitro* response of *P.falciparum* isolates to antimalarial drugs

The activities of the two antimalarial drugs studied against 21 *P.falciparum* isolates from Kenya are shown in Table 4. For the antimalarial drug pyrimethamine 100 nanoMoles was chosen (McCutchan, *et al.*, 1988; Chen, *et al.*, 1987) to be the cut-off point between pyrimethamine sensitive (<100 nM) and resistant (>100 nM) isolates.

TABLE 4

In Vitro Sensitivities of Kenyan isolates of *Plasmodium falciparum* to Pyrimethamine and Cycloguanil antimalarial drugs. ID_{50} values for 21 isolates

Pyr^R : Designates that the parasite isolate is resistant to pyrimethamine.

Pyr^S : Designates that the parasite isolate is sensitive to pyrimethamine.

All the Kenyan *P. falciparum* isolates so far studied in this project have shown to be sensitive to cycloguanil in comparison to reference strains.

ISOLATES	ID_{50} nM /L		$Pyr^{R/S}$
Nyanza Province	**Pyr**	**Cyc**	
1.K39	581± 210	13.5± 2.5	R
2.K67	0.3± 0.2	0.9± 0.1	S
3.S104	347± 68	12.2± 2.2	R
4.S136	5.1± 0.4	1.4± 0.1	S
5.S158	431± 163	17± 8.4	R
6.KIL9	352± 123	12.6± 3.1	R
Rift Valley Province			
7.ENT7	532± 176	9.9± 1.9	R
8.ENT11	349± 150	11.0± 5.5	R
9.ENT22	3.6	1.7	S
10.ENT24	583± 112	17.2± 4	R
11.ENT30	300 /86	NOT DONE	R/S
12.ENT36	231± 128	3.6± 2.0	R
13.ENT37	785± 113	22.2± 0.6	R
14.ENT41	290 /0.3	0.4/17	R/S
Coast Province			
15.M24	2.3±1.1	.9± 0.5	S
16.JP1	183± 80	7.9±1.6	R
7.JP17/A	184± 34	18.5± 3.0	R
18.JP78	175± 45	9.9± 1.9	R
19.JP119	246± 50	11.7± 4.2	R
20.JP62	1.7± 0.6	2.5± 0.5	S
21.D7/22	312± 100	8.5± 0.6	R

3.1.2 Results of DNA concentration in ng/ ul by ethidium bromide flouresence quantification tests

From the first four lanes of Fig. 3 it was possible to determine the concentration of DNA, in ng/ ul, present in each of the DNA samples prepared from the four isolates shown i.e., JP62, K39, JP119 and S104. One hundred ng of lambda DNA digested with Hind III was loaded into the first lane as the standard marker. To estimate how much DNA was present from the fifth to the eighth lane proved to be difficult since as is evident from the photograph, alot of the DNA had not dissolved or it could be because alot of DNA was loaded into the lane. The idea of using the ethidium-bromide flouresence quantification tests was to standardize the amount of DNA used in the PCR reactions and also to check for the state of the DNA sample; for example a smear on a gel is indicative of DNA degradation. Ethidium-bromide stained 1% agarose gels were used. Each fragment size of the marker used, in terms of ng/ ul, was obtained from the available data. In the first lane JP62 ethidium bromide stained-DNA flouresence was three times as intense as the first fragment size of the standard marker. And the amount of DNA present in this fragment was 47 ng. Therefore 3 times 47 is equivalent to 141 ng. Thence concentration of the DNA from the first four lanes from Fig. 3, i.e., JP62, K39, JP119 and S104 were :-

Isolate:	DNA conc. ng/ ul:
1. JP62	141
2. K39	141

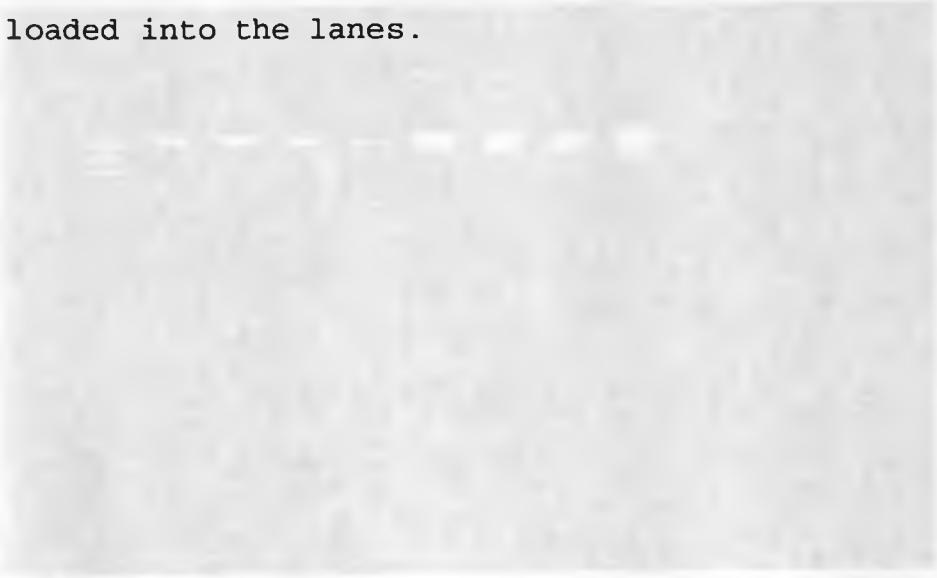
3. JP119 94

4. S104 47

The concentrations of DNA prepared from each of the isolates studied was obtained using the above method after redissolving and re-run.

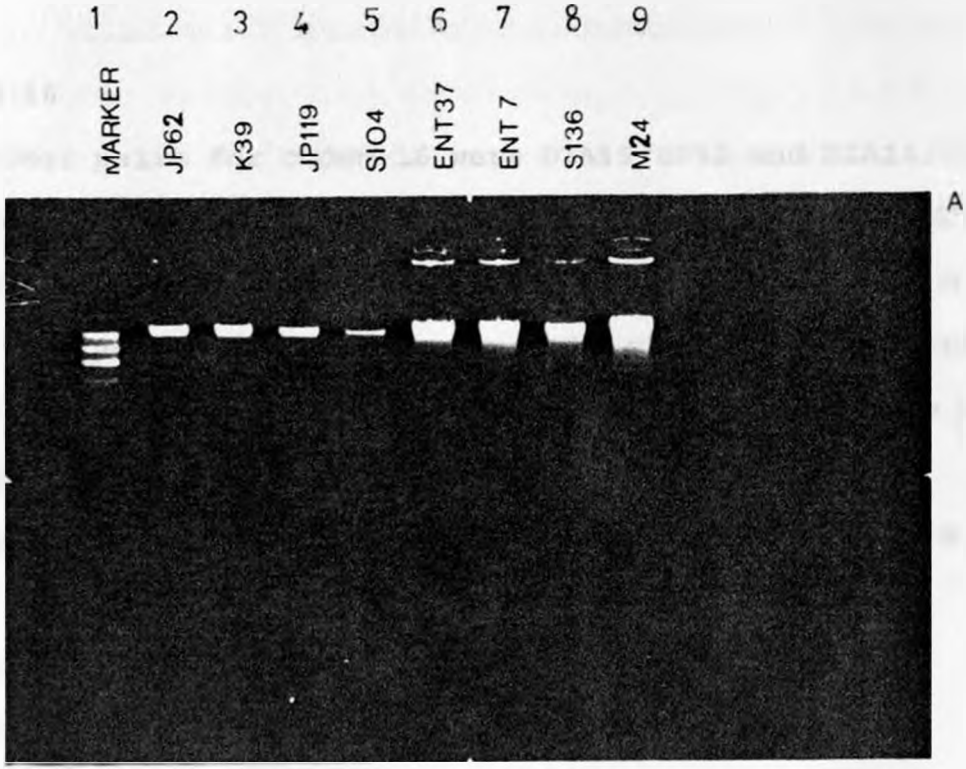
FIGURE 3

The figure shows results of ethidium-bromide fluorescent quantification tests for dsDNA. One hundred ng of lambda DNA digested with Hind III was loaded into the first lane and used as the standard marker. Lanes 2, 3, 4 and 5 shows the DNA of 4 *P. falciparum* isolates which was quantified by the ethidium-bromide fluorescence test. Lanes 6, 7, 8 and 9 shows the DNA of the other *P. falciparum* isolates which had not completely dissolved or had excess DNA loaded into the lanes.



QUANTIFICATION

Figure 3 Ethidium-bromide flourescent quantification tests for dsDNA.



DNA CONC. BY ET-BR FLOURESCENCE

3.1.3 Optimizing conditions for PCR

A total of 11 different mutation-specific primer pairs were used to distinguish the 6 different point mutation in the DHFR gene. The amplification conditions for each primer pair used were unique.

3.1.4 CODON 16

The primer pairs for codon-16 were DIA15/SP13 and DIA16/SP12. The expected PCR product size when using the primer pair (DIA16 /SP12) that would amplify the target DNA sequence with no nucleotide change is 667 bp and conversely when using the primer pair (DIA15 /SP13) that would amplify the target DNA sequence with a nucleotide change is 438 bp.

Initially, see Fig. 4, the amplification parameters were:

STEP 1

PCR-Cycles:

94°C for 1 min- Denaturation temperature

45°C for 1 min- Annealing "

72°C for 1 min- Extension "

In reaction Buffer:

Magnesium chloride- 1.5 mM

dNTP mix - 200 uM

DNA - 50 ng

The results showed that the annealing temperature of 45°C was too low to allow for efficient amplification to occur, see Fig. 4; amplification was observed in some of the lanes and not in others. It was decided to alter the conditions.

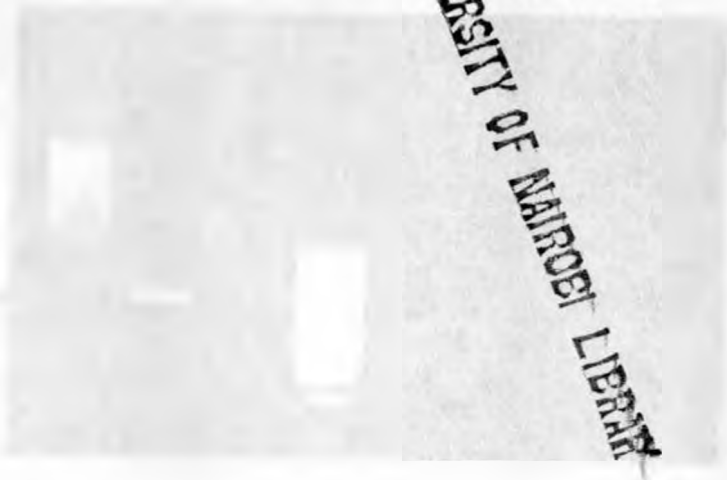
STEP 2

In the next step the annealing temperature was raised to 47°C but using the same amplification parameters, i.e., Mg++, dNTP, *Taq*. This resulted in specific reaction for both primer pairs.

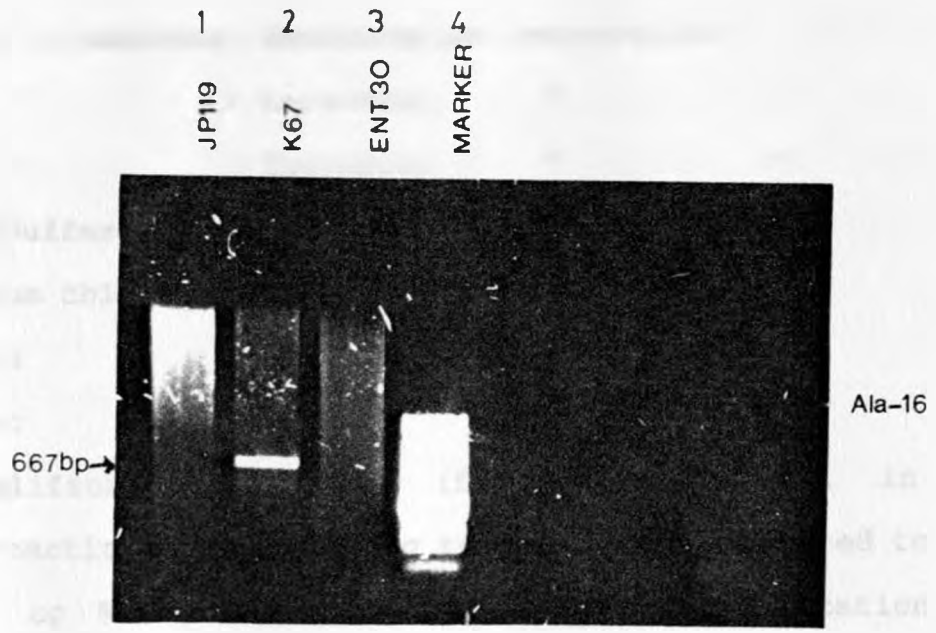
Therefore, the final optimal amplification parameters for the codon 16 primers was established to be : 1.5 mM $MgCl_2$, 200uM dNTP, 50 ng DNA and annealing temperature of 47°C, for 1 minute. The results indicate the correct PCR product sizes expected, i.e., 667 bp for DIA16/ SP12 and 438 for DIA15/ SP13 primer pairs as shown in the Figs. 9 and 10.

FIGURE 4

The figure shows part of the results obtained when optimizing conditions for codon 16. The standard marker used in lane 4 was Phi X DNA digested with Hinc II. Lane 2 shows smears and the expected PCR product size of 667 bp Lanes 1 and 3 show only smears which could be due to non-specific reactions as a result of the sub-optimal MSPCR conditions used. The conditions were finally made optimal with more subsequent reactions, (see text and Fig. 9 and 10).



CODON-16



PRIMERS: DIA16/SP 12

ANNEALING TEMP: 45 deg C, 1min

Figure 4 Optimization conditions for codon 16.

3.1.5 CODON 51

The primer pairs for the 51-codon were Asn-51/ SP12 and Ile-51/SP12. The expected PCR product size when using the primer pair (Asn 51/ SP12) that would amplify the target DNA sequence with no nucleotide change is 563 bp and conversely when using the primer pair (Ile 51/ SP12) that would amplify the target DNA sequence with a nucleotide change is also 563 bp.

The initial amplification parameters used were:

STEP 1:

94°C for 30 seconds- Denaturation temperature.

56°C " - Annealing "

72°C " - Extension "

In reaction buffer:

Magnesium chloride - 2.5 mM

dNTP mix - 200 uM

DNA conc - 50 ng

No amplification occurred (figure not shown). In two subsequent reactions the annealing temperature was reduced to 54°C and finally to 52°C. In the former case no amplification was observed, (figure not shown), in the latter case at an annealing temperature of 52°C non-specific bands was observed.

STEP 2:

The next step of the reaction was to titrate the Mg++ concentration at 1.5, 2.0 and 2.5 mM and annealing was performed at 52°C.

It was still possible to observe non-specific amplification,

(figure not shown).

The PCR products obtained when the reaction was performed with magnesium chloride at 2.5 mM were more bright.

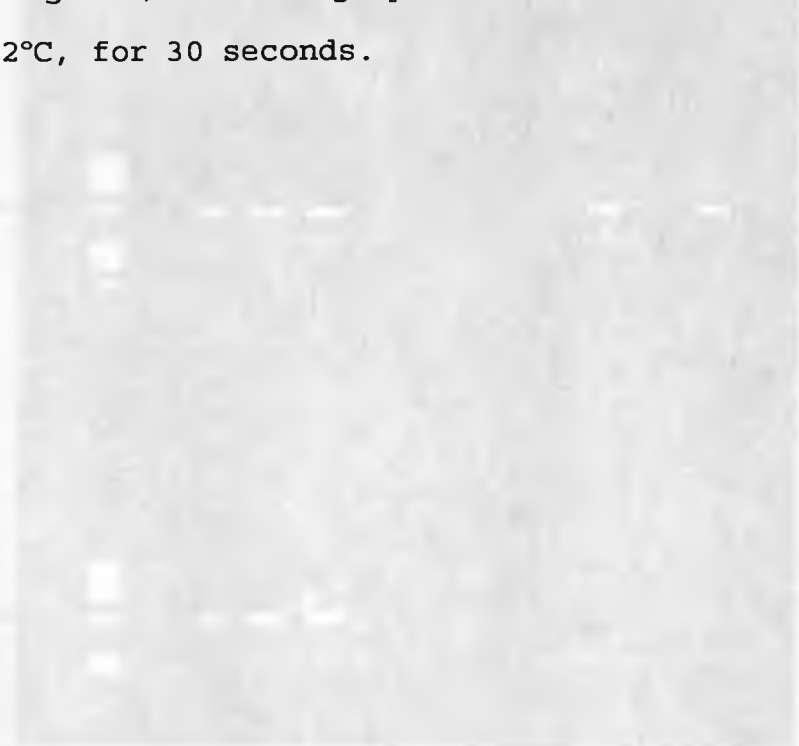
STEP 3:

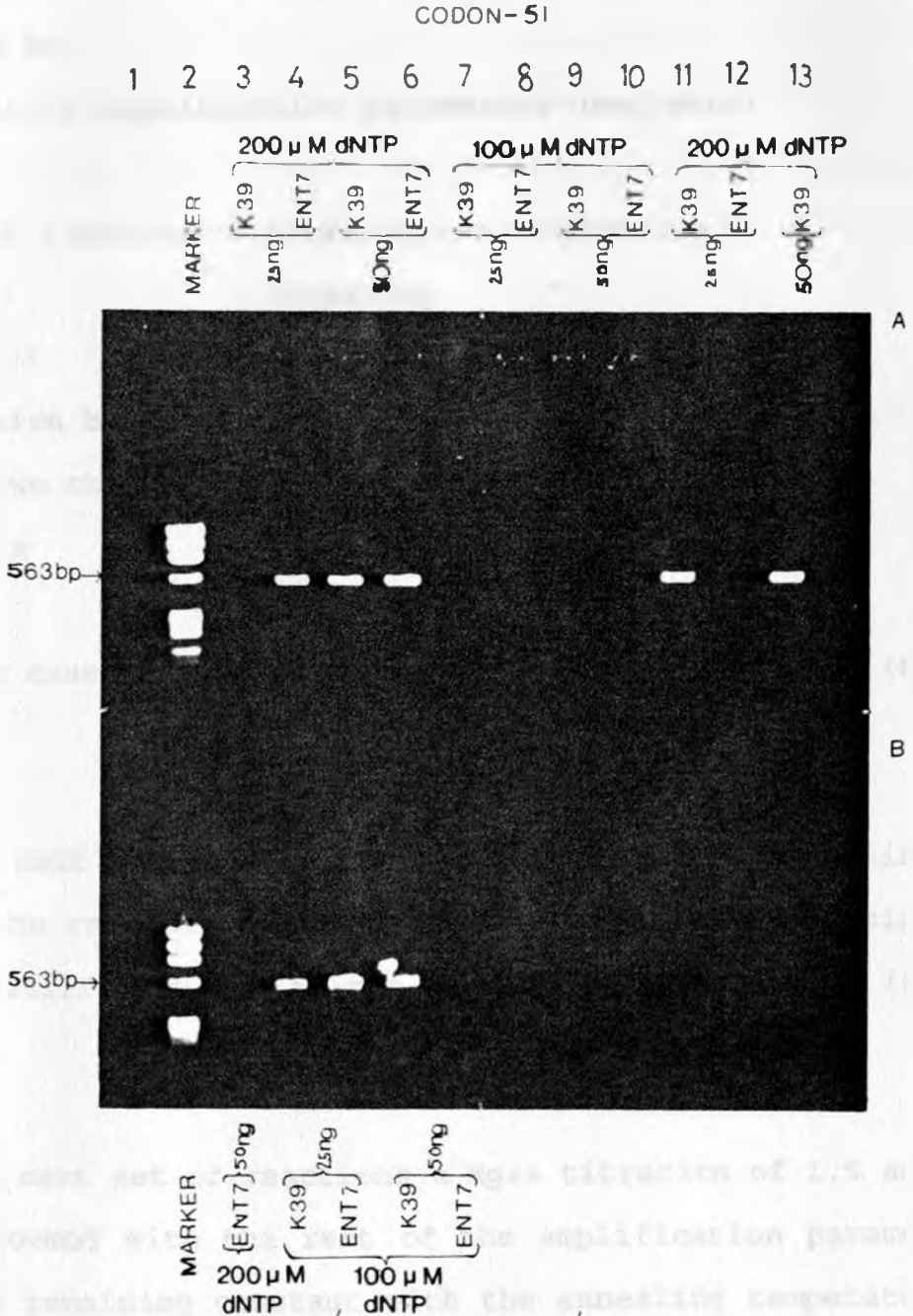
In the third step a DNTP titration versus DNA titration was done. Deoxyribonucleotide triphosphates, (dNTP) concentrations at 100 uM and 200 uM and DNA at 25 and 50 ng were used. The rest of the amplification parameters included: 2.5 mM Mg++ concentration and annealing temperature of 52°C for 30 seconds.

Specific reaction was observed at 200 uM DNTP mix, 25 ng or 50 ng DNA, 2.5 mM $MgCl_2$ concentration and annealing temperature of 52°C, for 30 seconds. These were the final optimal amplification conditions. The data obtained under these conditions is shown in Fig. 5 lanes 11, 12, 13 and 14.

FIGURE 5

Phi X DNA digested with Hae III was used as the standard marker in lane 2. The expected PCR product size was 563 bp for both primer pairs. Mg++ concentrations being tested were 1.5 mM in lanes 3 to 10 and 2.5 mM in lanes 11 to 18. The dNTP and DNA concentration were being tested at 200/ 100 uM and 25/ 50 ng titrations respectively. Expected results were only observed in lanes 11, 12, 13 and 14 where the final optimal MSPCR conditions were 200 uM dNTP mix, 25 ng or 50 ng DNA, 2.5 mM $MgCl_2$ concentration and annealing temperature of 52°C, for 30 seconds.





PRIMERS Asn 51/ sp12 AND Ile 51/SP12

ANNEALING TEMP 52 deg C. 30 Secs

Figure 5 Optimization conditions for codon 51

3.1.6 CODON 59

The primer pairs for the codon 59 were DIA 60/ P3 and DIA 59/ P3. The expected PCR product size when using either primer pair was 190 bp.

The initial amplification parameters used were:

STEP 1:

94°C for 1 minute - Denaturation temperature.

52°C " - Annealing "

72°C " - Extension "

In the reaction buffer:

Magnesium chloride - 2.5 mM

dNTP mix - 200 uM

DNA - 50 ng

In this case non-specific amplification was observed, (figure not shown).

STEP 2:

In the next reaction the annealing temperature was raised to 54°C, with the rest of the amplification parameters remaining the same. This still resulted in non-specific amplification, (figure not shown).

STEP 3:

In the next set of reactions a Mg++ titration of 1.5 and 2.5 mM was performed with the rest of the amplification parameters, named above remaining constant with the annealing temperature of 52°C. The result was non-specific amplification, (figure not shown).

STEP 4:

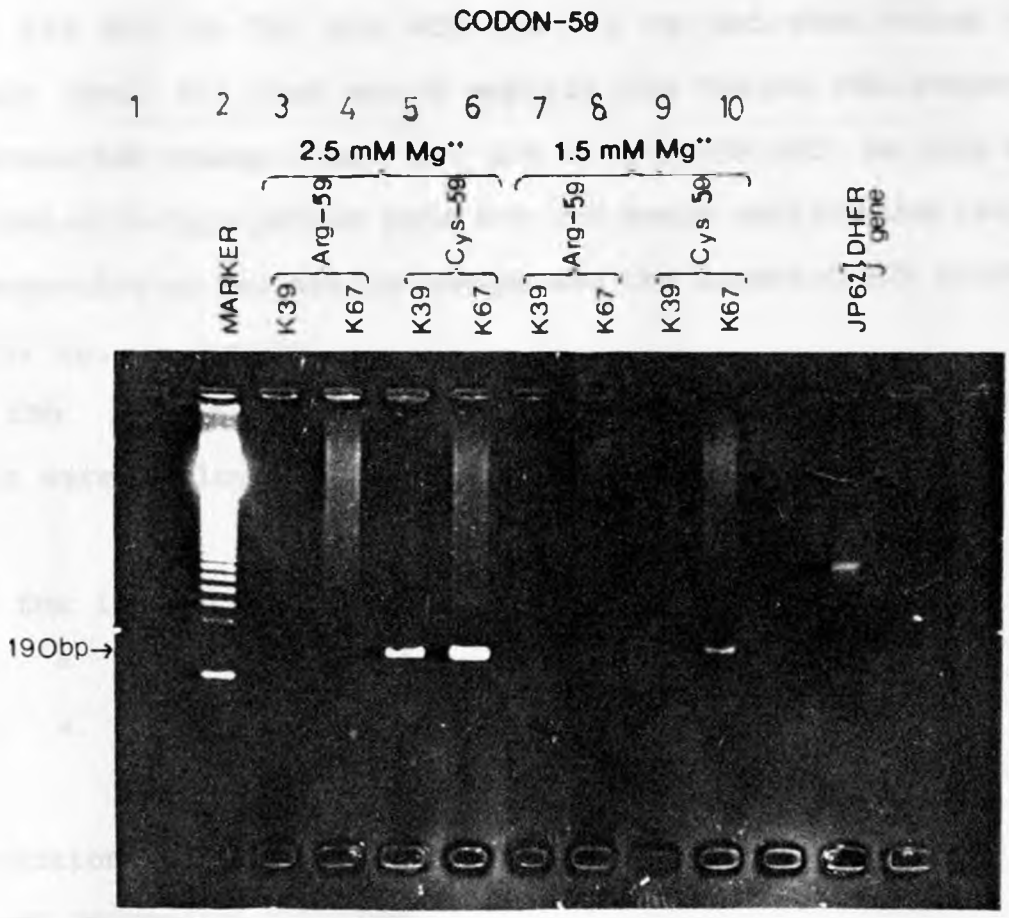
In the subsequent reaction a Mg++ titration at 2.5 and 1.5 mM was done but with the annealing temperature of 54°C for 1 minute.

The end result of this was that more specific reaction was observed for the 59 primer pairs at 1.5 mM Mg++.

Therefore the final optimal amplification parameters for the codon 59 was established to be : 1.5 mM $MgCl_2$, 200uM dNTP mix, 50 ng DNA and annealing temperature of 54°C for 1min. The data obtained under these conditions are shown in figure 6. It is evident that these primer pairs when used under these conditions will reveal the mutation affecting codon 59 (figure 6 lanes 3, 4, 5 and 6).

FIGURE 6

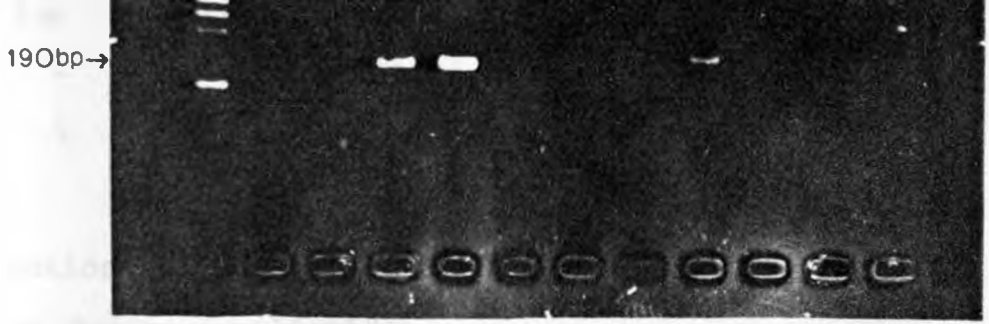
The figure indicates part of the optimization procedures for codon 59. A 123 bp ladder was used as the standard marker in lane 2. Optimal MSPCR reaction were observed at 1.5 mM Mg++ concentration. The expected PCR product of 190 bp was observed in lanes 5 and 6. No amplification occured in lanes 3 and 4 as was expected. The rest of the lanes gave unexpected results at 2.5mM Mg++ concentration.



PRIMERS: DIA59/P3 AND DIA60/P3

ANNEALING TEMP: 54 degC, 1min

Figure 6 Optimization conditions for codon 59.

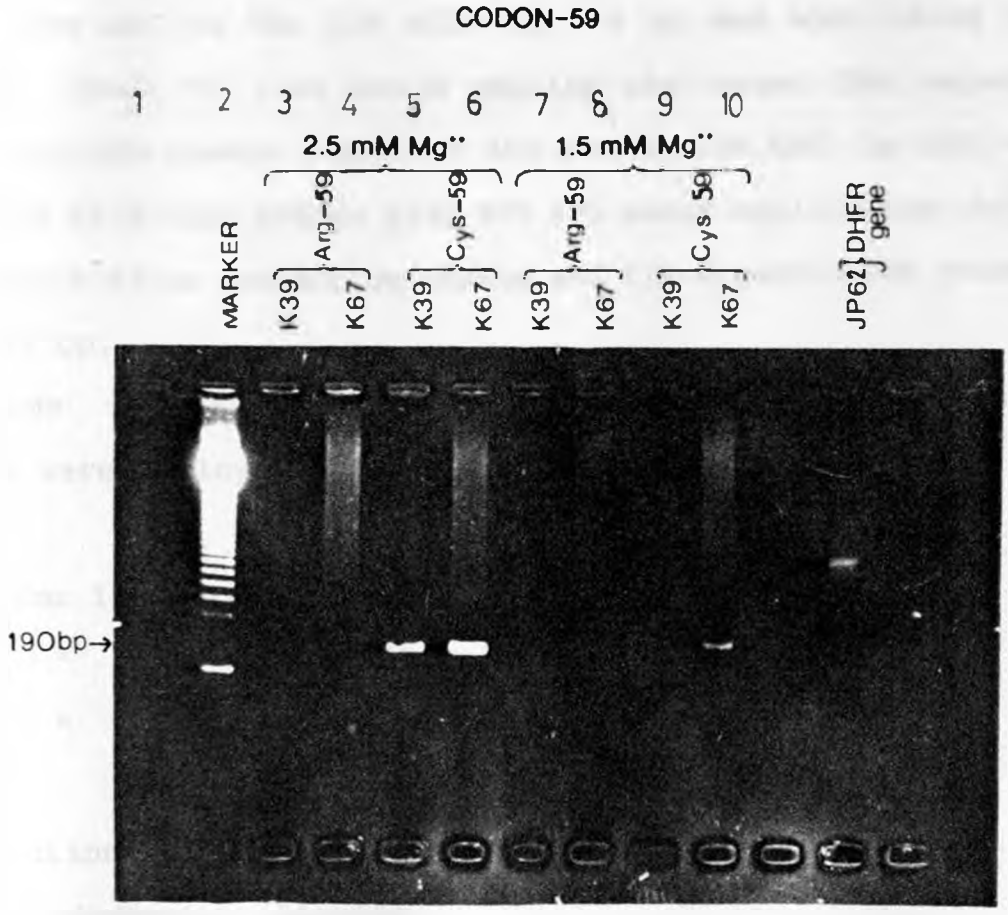


PRIMERS: DIA59/P3 AND DIA60/P3

ANNEALING TEMP: 54 degC, 1min

Figure 6 Optimization conditions for codon 59.


CODON-59
1
2
3
4
5
6
7
8
9
10
2.5 mM Mg++
1.5 mM Mg++
MARKER
K39
Arg-59
K67
K39
Cys-59
K67
K39
Arg-59
K67
K39
Cys 59
K67
JP62
DHFR gene



PRIMERS: DIA59/P3 AND DIA60/P3

ANNEALING TEMP: 54 degC, 1min

Figure 6 Optimization conditions for codon 59.

3.1.7 CODON 108

The primer pairs included: SP1/P2, RP4/P1 and SP2/ P3. The expected PCR product size when using the primer pair (SP1/ P2) that would amplify the target DNA sequence with a nucleotide change (from Ser 108 AGC to Thr 108 ACC) is 776 bp and when using the primer pair (RP4/ P1) that would amplify the target DNA sequence with a nucleotide change (from Ser 108 to Asn 108 AAC) is 1521 bp. However, the wild-type primer pair SP2 /P3 would amplify the target DNA sequence with no nucleotide change and the expected PCR product size is 301 bp.

In the initial amplification reaction, the following parameters were employed:

STEP 1:

94°C for 1 minute - Denaturation temperature

50°C " - Annealing "

72°C " - Extension "

In the reaction buffer:

1.5 mM Magnesium chloride

200 uM dNTP mix

50 ng DNA

This resulted in non-specific amplification, (figure not shown).

STEP 2:

This involved increasing the annealing temperature to 54°C for 1 minute but maintaining the rest of the amplification parameters constant. The outcome was still non-specific amplification for all

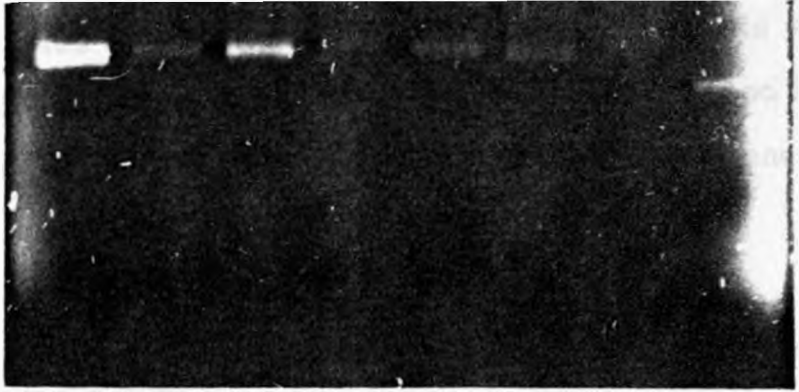
the primer pairs used, (figure 7 lanes 1, 2, 3, 4, 5 and 6).

STEP 3:

In the subsequent reaction the dNTP concentration was reduced from 200 uM to 100 uM amounts. This resulted in specific reactions for all the primer pairs used. The final optimal PCR conditions used were 1.5 mM $MgCl_2$, 100 uM dNTP mix, 50 ng DNA and an annealing temperature of 54°C, for 1 minute. It is evident that these primer pair when used under these conditions will reveal the mutation affecting codon 108 (figure 15, panel A, lanes 2, 4, 6, 8 and 9).

FIGURE 7

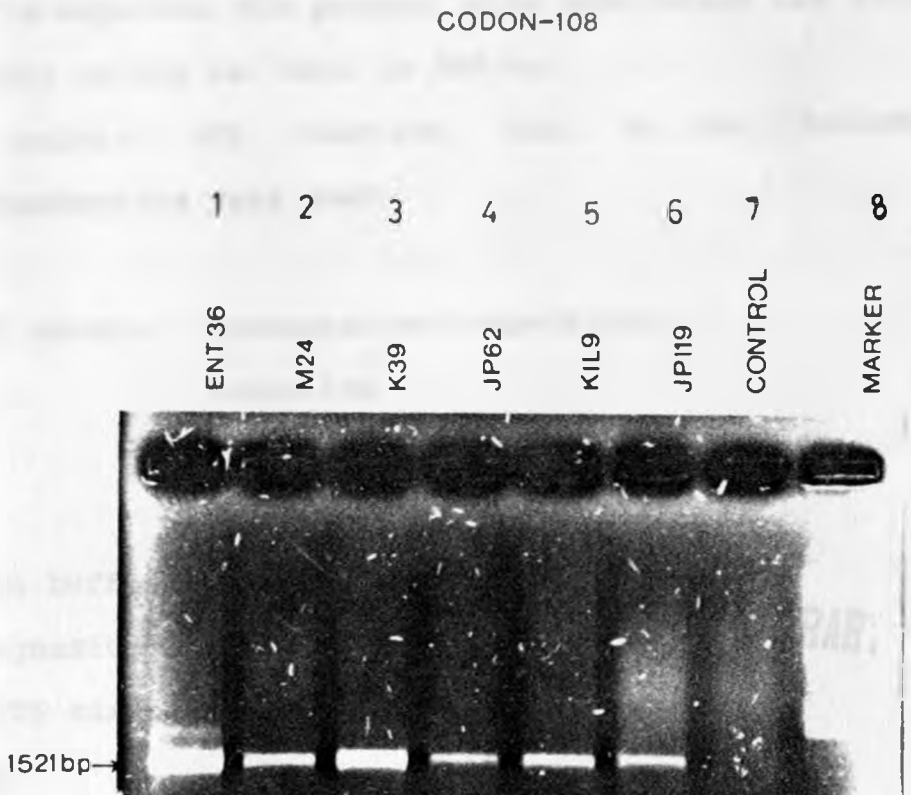
Lambda DNA digested with Hind III was used as the standard marker in lane 8. A master mix with no DNA was used as the negative control whereas K39 was used as the positive control. 1521 bp was the expected PCR product size for the RP4 /P1 primer pair. A non-specific PCR product of approximately 800 bp was also resolved. However, the conditions were finally made stringent in subsequent reactions and the 800 bp PCR product eliminated, (see text and Fig. 15).



PRIMERS: RP4/P1

ANNEALING TEMP: 54 deg C, 1min (at 200μM dNTP)

Figure 7 Optimization conditions for codon 108


CODON-108
1
2
3
4
5
6
7
8
ENT 36
M24
K39
JP62
KIL9
JP119
CONTROL
MARKER
1521bp

3.1.8 CODON 164

The primer pairs used for codon 164 were DIA14/ SP11 and DIA13/ SP14. The expected PCR product size when using the primer pair DIA 13/ SP14 or DIA 14/ SP11 is 505 bp.

In the initial PCR reaction, Fig. 8 the following amplification parameters were used:

STEP 1:

94°C for 1 minute - Denaturation temperature

58°C " - Annealing "

72°C " - Extension "

In the reaction buffer:

1.5 mM Magnesium Chloride

200 uM dNTP mix

50 ng DNA

The result of this PCR reaction was that faint bands were observed. The annealing temperature of 58°C, was probably too high for efficient amplification to occur, (figure 8, panel A, lanes 1-10) .

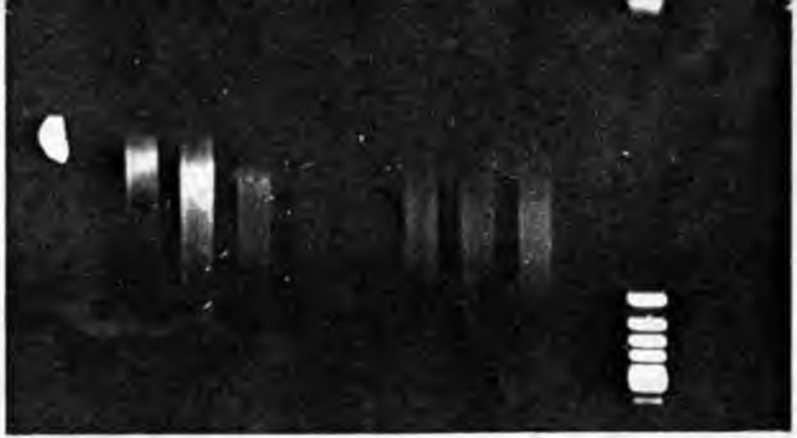
STEP 2:

In the next step the annealing temperature was reduced to 56°C with the rest of the amplification parameters remaining constant. This resulted in specific amplification.

Therefore the optimal PCR conditions for the 164 primer pairs was 1.5 mM $MgCl_2$, 200 uM dNTP mix, 50 ng DNA and an annealing temperature of 56°C, for 1 minute, (figure 19 and 20).

FIGURE 8

Phi X DNA digested with Hae III was used as the standard marker in lane 12, panel A and B. VI/S was used as the positive control in lane 11. VI/S is a reference strain that had a base pair change at codon 164 coding for AA Leu in the DHFR gene. As is evident from the figure, lanes 1-10 panel A, shows that non of the Kenyan *P. falciparum* isolates had the AA residue Leu 164. The faint bands present in some of the isolates explains the fact that the reaction was not stringent. However, with more subsequent reactions the conditions were finally made optimal, (see text and Fig. 19 and 20). Optimal conditions for the primer pair, DIA 15/ SP13, that primed the amplification of DNA adjacent to the AA Leu 164 in the DHFR gene were also established, (see Fig. 20).



PRIMERS: DIA13/SPI4 AND DIA14/SPI1

ANNEALING TEMP: 58 deg C, 1 min

Figure 8 Optimization conditions for codon 164

505bp
ENT37 1
JP1 2
JP78 3
S136 4
JP17/A 5
S104 6
ENT11 7
D7/22 8
ENT24 9
ENT7 10
CONTROL 11
MARKER 12
CODON-164
A

3.1.9 MS-PCR on 21 Kenyan *P.falciparum* isolates and reference strains

Electrophoresis in 1% agarose gels stained with ethidium bromide was used to resolve, according to size, the PCR products for all the codons except for codon 59. The PCR products for the latter codon were difficult to resolve with a 1% agarose gel. Therefore a low molecular weight 6% Nusieve: Agarose gel was used. The gels were run in 1 X TAE buffer with ethidium bromide. The markers used included Phi X 174 DNA digested with Hinc II, 123 base pair ladder and Phi X 174 DNA digested with Hae III. Two reference strains of *P. falciparum*, FCB and VI/S, were used. The figures below show the different sizes of PCR products obtained depending on the primer sets. As is evident from the figures, the outcome of the MSPCR reaction under stringent PCR parameters was the presence or absence of a PCR product. For example a diagnostic primer is able to pick out a mutant sequence compared to a wild type primer set thereby producing a PCR product in the former case and not in the latter case, (see figure 11). However, two isolates namely ENT41 and ENT 30, showed exceptions to the above case,(figure 11, 12, 13, 14). The results obtained from *in vitro* drug tests, MSPCR and sequencing indicated the presence, in ENT 30 and ENT 41, of mixed isolates whereby there was presence of both pyrimethamine sensitive and resistant isolates in the culture.

Where smears were observed it was thought to be due to non-specific amplification that occured during the initial amplification cycles. Standard reference strains were used as the

positive controls and mastermixes with no DNA was used as the negative control.

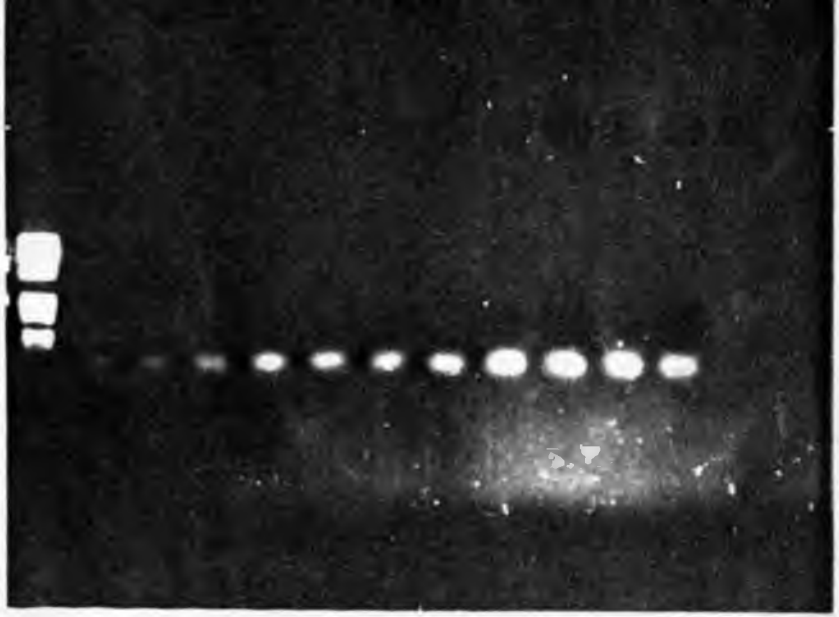
3.2 CODON 16

Alanine is the amino acid residue at position 16. A base pair change from GCA to GTA results in a change from amino acid Ala to Val.

Figures 9 and 10 show the results of the 21 isolates studied obtained under optimal MSPCR conditions.

FIGURE 9

Phi X DNA digested with Hae III was used as the standard marker in lane 1, in both panel A and B. A mastermix with no DNA was used as the negative control. Six hundred and sixty seven was the expected PCR product; see lanes 2-11. Therefore none of the Kenyan *P. falciparum* isolates shown in this figure had a nucleotide change at codon 16 in the DHFR gene. The latter statement is confirmed further by the results shown in panel B since no PCR product was resolved in this section. The bands observed at the bottom of the gel are the free primer region. Four hundred and thirty eight was the expected PCR product for AA Val at codon 16 in the DHFR gene.



PRIMERS: DIA16/SP12 AND DIA15/SP13

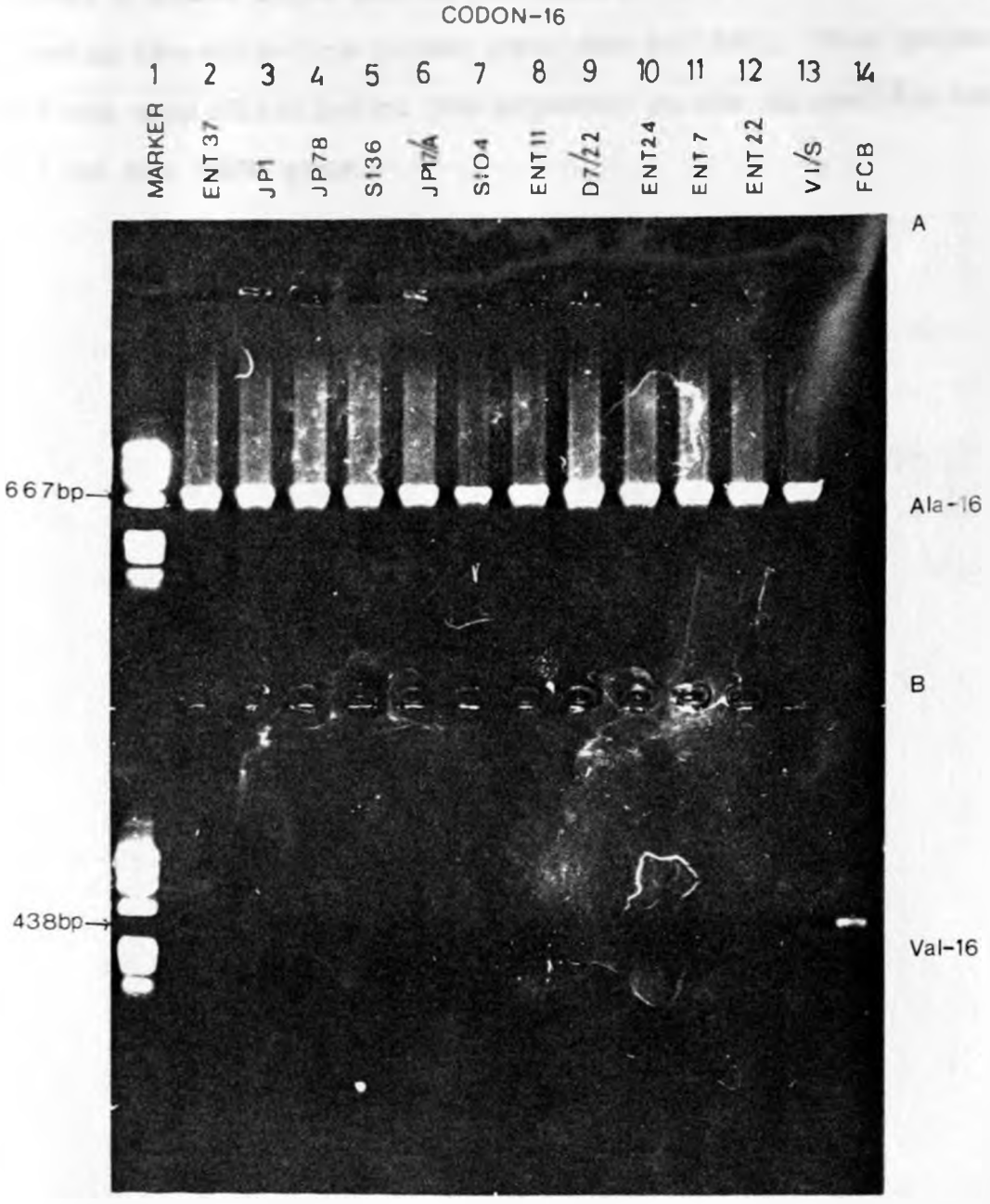
ANNEALING TEMP: 47 deg C. 1min

Figure 9 Results of MS-PCR for codon 16

667 bp
CODON-16
1 MARKER
2 JP62
3 JP119
4 M24
5 KIL9
6 K67
7 K39
8 S158
9 ENT30
10 ENT36
11 ENT41
12 CONTROL
A
Ala-16

FIGURE 10

Phi X DNA digested with Hae III was used as the standard marker in lanes 1, in both panel A and B. Panel A resolved PCR products when using the wild-type primer pair, DIA16/ SP12; panel B resolved PCR products when using the mutant primer pair, DIA15/ SP13. Two reference strains, VI/S and FCB were used as positive controls. The expected PCR product size, in panel A, was 667 bp which was resolved in lanes 2-12. The results indicated that none of the Kenyan *P. falciparum* isolates shown in this figure had a nucleotide change at codon 16 in the DHFR gene. From lanes 2-12 in panel B, no PCR products were resolved, stating further that none of the Kenyan *P. falciparum* isolates had the AA Val in the DHFR gene. Lane 13 shows a PCR product of 438 bp when amplifying the FCB DNA with the mutant primer pair, DIA15/ SP13. This confirmed that FCB has an AA Val at codon 16 in the DHFR gene.



PRIMERS: DIA16/sp12 AND DIA15/sp13

ANNEALING TEMP: 47 deg C 1min

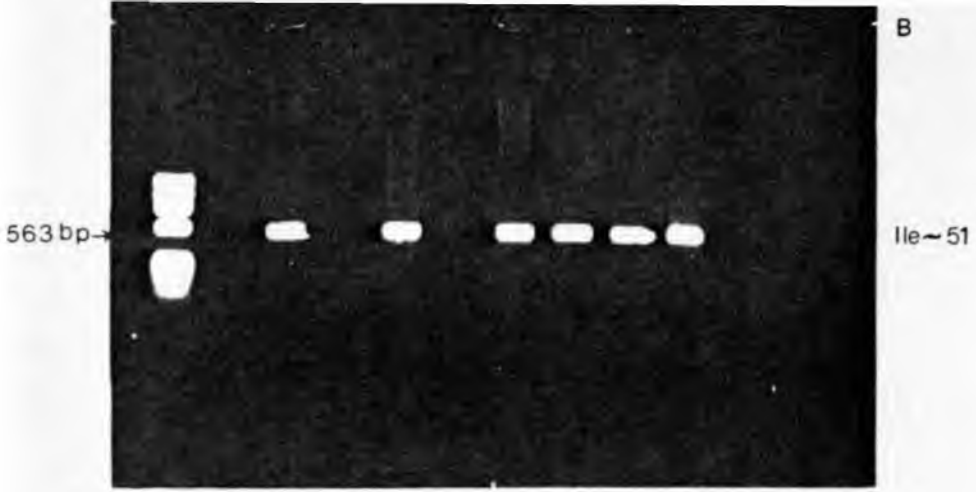
Figure 10 Results of MS-PCR for codon 16

3.2.1 CODON 51

Figure 11 shows the MS-PCR results of 9 out of the 21 isolates studied. Panel A shows three out of the nine isolates whose DNA was amplified using the wild-type primer pair Asn 51/ SP12. This primer pair primed the amplification of DNA adjacent to the AA residue Asn at codon 51 in the DHFR gene.

FIGURE 11

Phi X DNA digested with Hae III was used as the standard marker in lane 1. Reference strain, FCB, was used as the positive control in lane 11 and a mastermix with no DNA was used as the negative control in lane 12. Panel A shows three out of the nine isolates whose DNA was amplified using the wild-type primer pair Asn 51/ SP12. Amplification was observed in panel A, lanes 2, 4, 6, 9 and 11, where the DNA of these isolates amplified with the sensitive primer pair Asn 51/ SP12; conversely panel B shows the PCR products as a result of amplification when using the mutant primer pair Ile 51/ SP12. In the latter case specific PCR products were resolved only in lanes 3, 5, 7, 8, 9 and 10. ENT 30, is a mixed isolate hence PCR products were resolved with both sensitive and mutant primer pairs, (see lane 9).



PRIMERS: Asn 51/sp11 AND Ile-51/sp12

ANNEALING TEMP: 52 deg C, 30 Secs

Figure 11 Results of MS-PCR for codon 51.

CODON-51

1 2 3 4 5 6 7 8 9 10 11 12

MARKER
JP62
JP119
M24
KIL9
K67
K39
S158
ENT30
ENT36
FCB
CONTROL

563 bp→

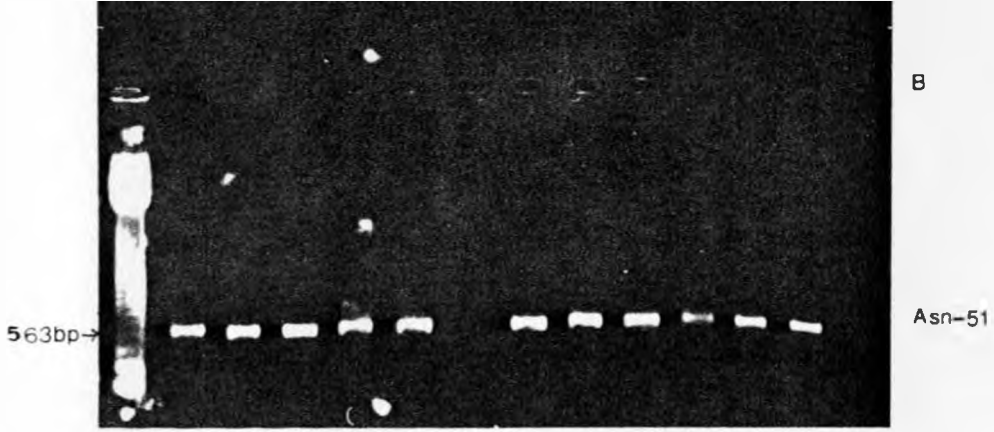
A

Asn-51

PCR products obtained from the DNA of 12 out of the 21 remaining isolates of *P. falciparum* studied are shown in Fig. 12. Only the DNA of one isolate, S104, in this category amplified with the mutant Ile51 /SP12 primer pair which picked out the mutant Ile 51 AA. ENT41 showed the characteristics of a mixed culture i.e., where PCR products were observed with both wild and mutant-type primer pairs.

FIGURE 12

A 123 base pair ladder marker was used as the standard marker in lane 1. For controls see figure 11. Panel A shows the PCR products as a result of amplification when using the wild-type primer pair Asn 51/ SP12 and conversely panel B shows the PCR products as a result of amplification when using the mutant primer pair Ile 51/ SP12. PCR products were resolved in lanes 6 and 12. In the latter case specific PCR products were resolved in all the lanes except in lane 2. ENT 41 is a mixed isolate i.e., PCR products were observed with both wild and mutant-type primer pairs.



PRIMERS: Ile51/sp12 AND Asn-51/sp12

ANNEALING TEMP: 52 deg C. 30 Secs

Figure 12 Results of MS-PCR for codon 51

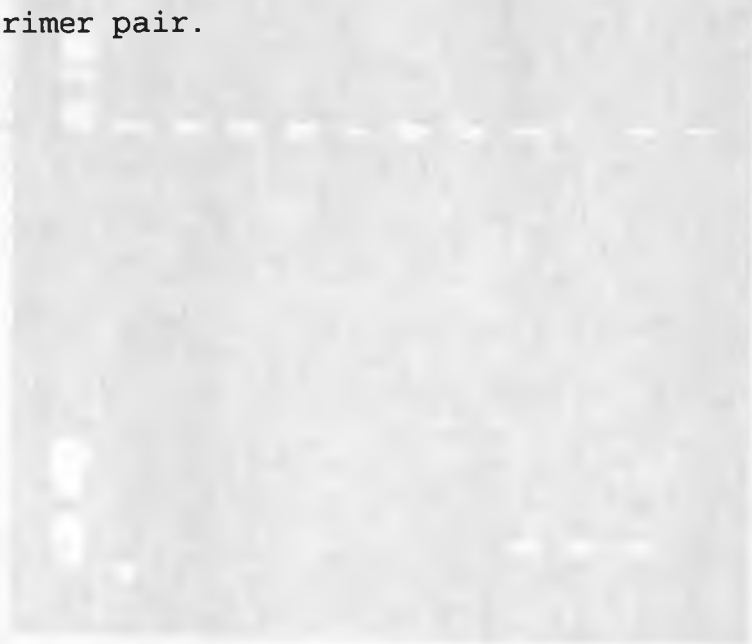
563bp
MARKER
ENT 37
JP1
JP78
S136
JP17/A
S104
ENT11
D7/22
ENT24
ENT 7
ENT22
ENT41
1 2 3 4 5 6 7 8 9 10 11 12 13 14
CODON-51
A
Ile-51

3.2.2 CODON 59

PCR products obtained from the DNA of 10 out of the 21 isolates studied are shown in this Fig. 13. Panel A shows PCR products when using the wild-type primer pair DIA60 /P3 that primed the amplification of DNA adjacent to the AA residue Cys at codon 59 in the DHFR gene in all except one isolate, ENT36. However, two cases of mixed culture were observed i.e., ENT30 and ENT41. In both of these cases amplification occurred when using the wild and mutant-type primer pairs as seen in Panels A and B.

FIGURE 13

Phi X DNA digested with Hae III was used as the standard marker in lane 1, a reference strain, FCB, was used as the positive control in lane 12, a mastermix with no DNA was used as the negative control in lane 13. One hundred and ninety bp was the expected PCR product when using both the wild- and mutant primer pairs. The DNA of two mixed isolates, ENT30 and ENT41 amplified with both primer pairs. FCB in lane 12 indicated that it has the AA residue Cys at codon 59 in the DHFR gene. This was so with all the DNA of isolates shown in the figure except ENT36 in lane 10 whose DNA amplified with the mutant primer pair.



PRIMERS:DIA 60/P3 AND DIA 59/P3

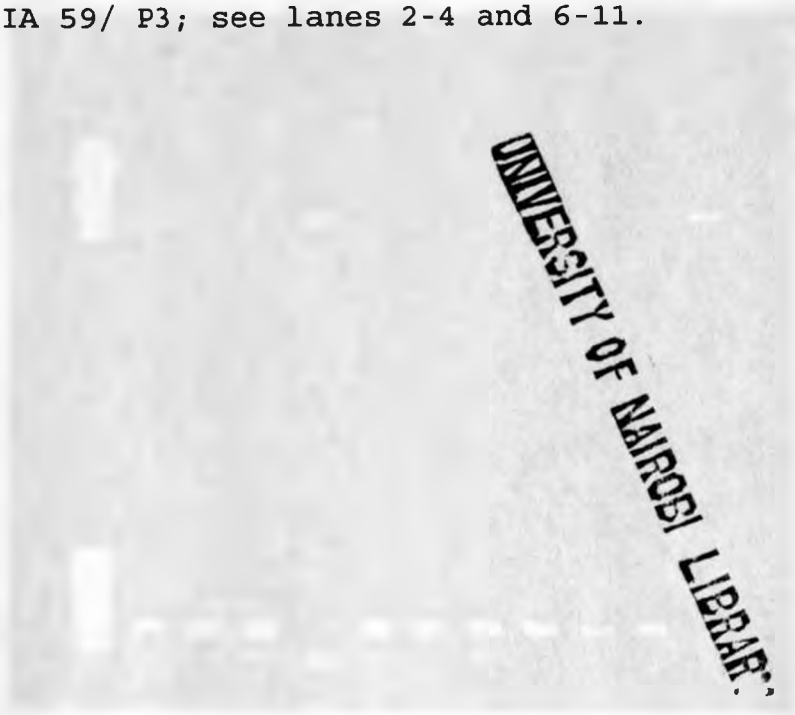
ANNEALING TEMP: 54 deg C 1min

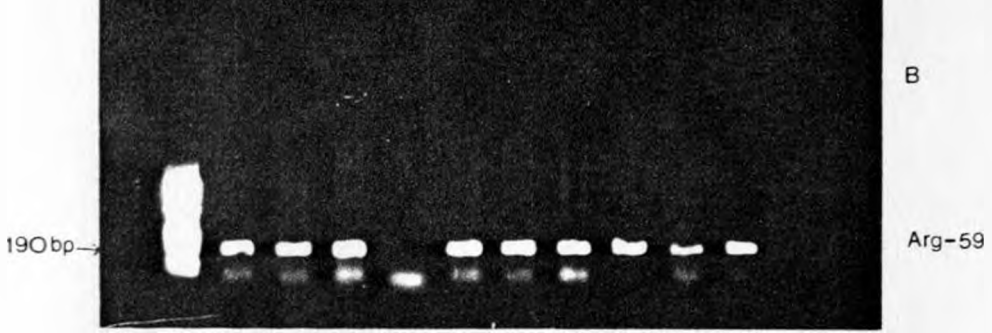
Figure 13 Results of MS-PCR for codon 59.

CODON-59
1 2 3 4 5 6 7 8 9 10 11 12 13
MARKER
JP62
JP119
M24
KIL9
K67
K39
S158
ENT30
ENT36
ENT41
FCB
CONTROL
190 bp
A
Cys-59

FIGURE 14

Lane 1 is Phi X DNA digested with Hae III used as the standard marker; lane 13 is a mastermix with no DNA used as the negative control. One hundred and ninety bp was the expected PCR product size when using both the wild- and mutant primer pairs. SI36, in lane 5 and ENT22, in lane 12 indicated that they both had the AA residue Cys at codon 59 in the DHFR gene. Conversely the DNA of the remaining isolates, except these two, amplified with the mutant primer pair, DIA 59/ P3; see lanes 2-4 and 6-11.





PRIMERS: D1A 60/P3 AND DIA 59/P3

ANNEALING TEMP. 54 deg C. 1min

Figure 14 Results of MS-PCR for codon 59.

190 bp

MARKER	1
ENT 37	2
JP1	3
JP78	4
S136	5
JP17/A	6
S104	7
ENT11	8
D7/22	9
ENT24	10
ENT 7	11
ENT22	12
CONTROL	13

CODON-59

A

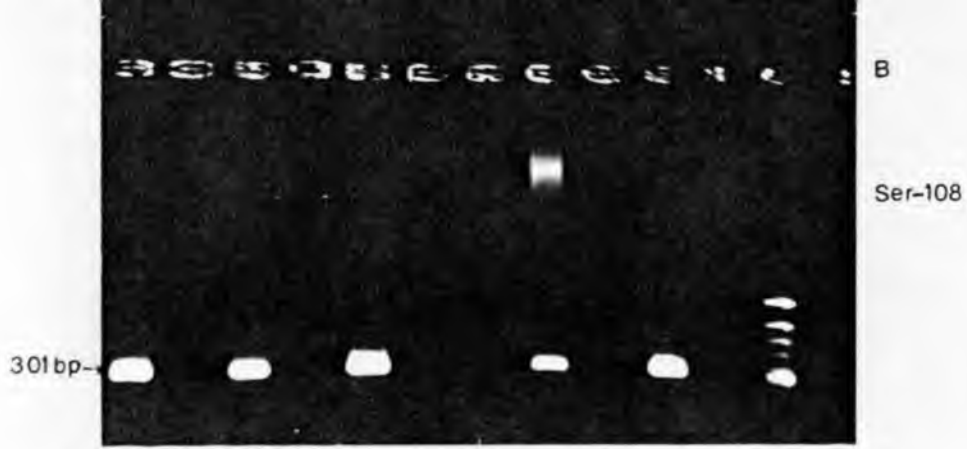
Cys-59

3.2.3 CODON 108

Fig. 15 shows the PCR products obtained from 10 out of the 21 isolates studied. One isolate S158, not shown in the Fig 16 was repeated under the same amplification parameters for the mutant RP4/ DIA13 and wild-type SP2 /P3 primer pair and the results are shown in Fig 18.

FIGURE 15

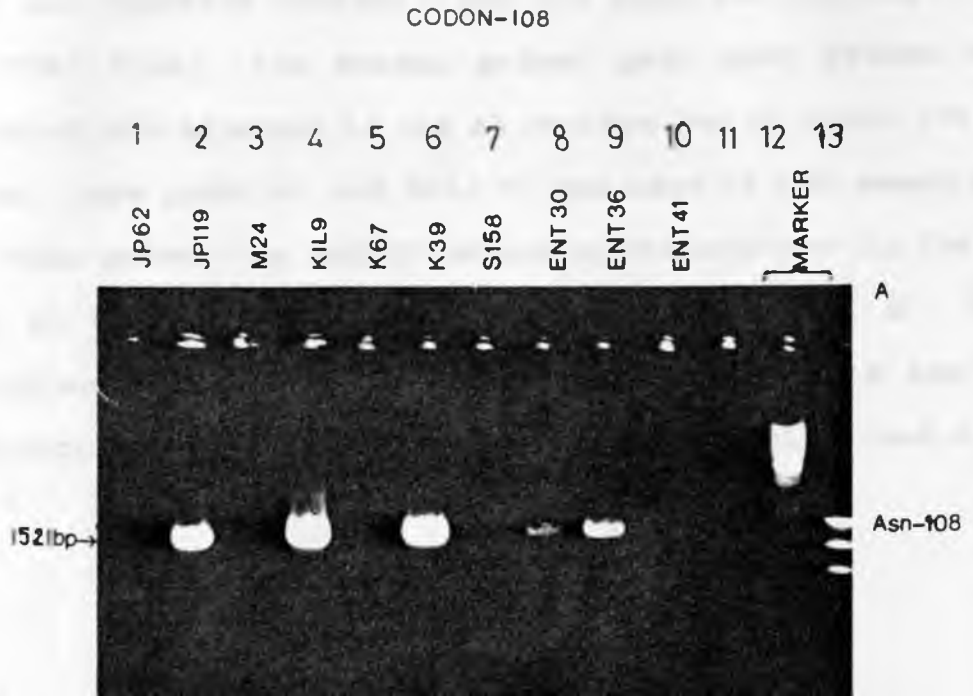
The figure shows results of MSPCR for codon 108. Lambda DNA digested with Hind III was used as the standard marker in both panels A and B. Panel A shows the expected PCR product size, (1521 bp), when the mutant primer pair RP4 /P1, was used and panel B shows the expected PCR product size, (301 bp), when the sensitive primer pair was used. The isolates are arranged from lane 1-10 in the order of pyrimethamine sensitivty followed by pyrimethamine resistance; e.g., lane 2 is a pyrimethamine sensitive isolate, JP62 and in lane 3 is a pyrimethamine resistant isolate, JP119. Panel A shows the results of amplification with mutant primers which was resolved in only pyrimethamine resistant isolates and conversely for the pyrimethamine sensitive isolates in panel B.



PRIMERS: RP4/ P1 AND SP2/P3

ANNEALING TEMP: 54 deg C. 1min

Figure 15 Results of MS-PCR for codon 108.


CODON-108
1
2
3
4
5
6
7
8
9
10
11
12
13
JP62
JP119
M24
KIL9
K67
K39
S158
ENT 30
ENT 36
ENT 41
MARKER
A
152 bp→
Asn-108

ENT37 JP1 JP78 S136 JP17/A S104 ENT11 D7/22 ENT24 ENT7 CONTROL MARKER

PRIMERS: SP2/P3 AND RP4/D1A13

ANNEALING TEMP: 54 deg C, 1min.

Figure 12 Results of MS-PCR for codon 108

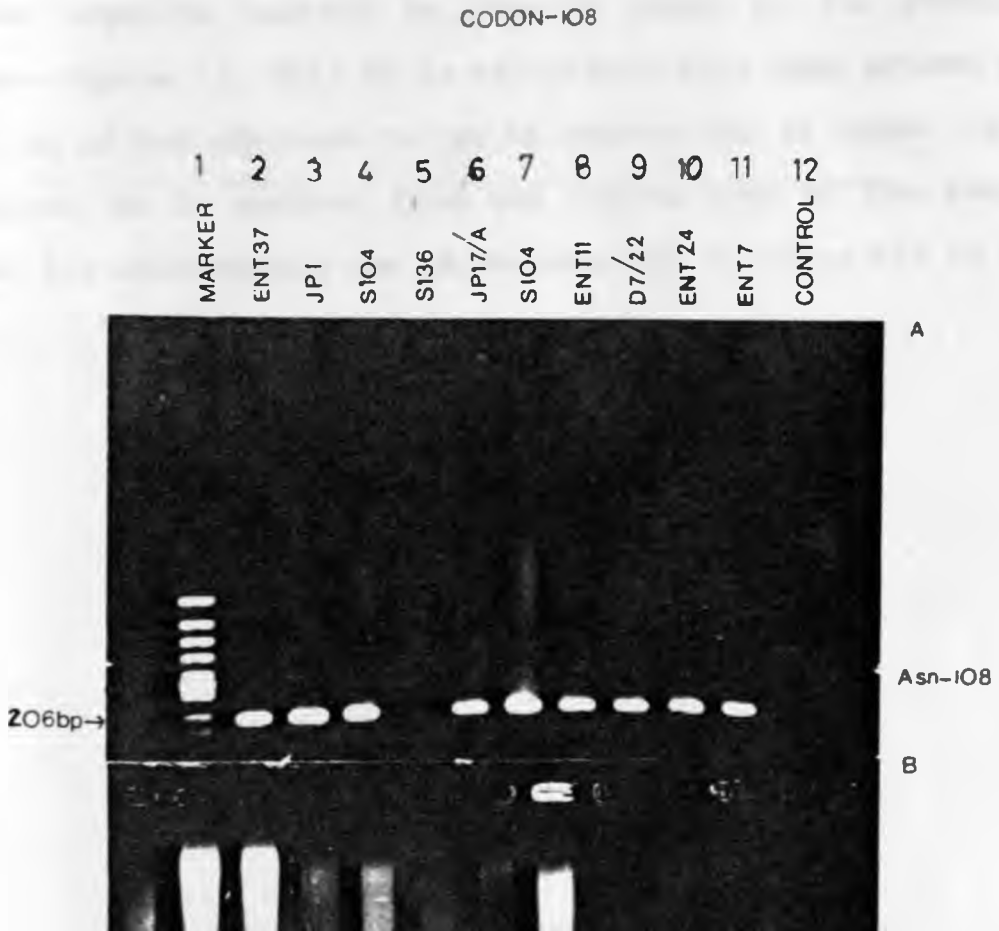

CODON-108
1 MARKER
2 ENT37
3 JP1
4 S104
5 S136
6 JP17/A
7 S104
8 ENT11
9 D7/22
10 ENT24
11 ENT7
12 CONTROL
A
Asn-108
206bp→
B

FIGURE 17

Phi X DNA digested with Hae III was used as the standard marker in lane 12 panel A and in lane 11 panel B. A mastermix with no DNA was used as the negative control in lane 11 panel A. For positive controls see figure 18. SP1/ P2 is the primer pair that primed the amplification of DNA adjacent to the AA residue Thr at codon 108 in the DHFR gene. As is evident from the figure none of the Kenyan isolates so far studied had the AA residue Thr at codon 108 in the DHFR gene.

ENT37 JP1 JP78 S136 JP17/A S104

PRIMERS: SP1/P2

ANNEALING TEMP: 54 deg C.

Figure 17 Results of MS-PCR for

Thr-108

ENT11

D7/22

ENT24

1min

codon 108

CODON-108

1	JP62
2	JP119
3	M24
4	KIL9
5	K67
6	K39
7	S158
8	ENT30
9	ENT36
10	ENT41
11	CONTROL
12	MARKER

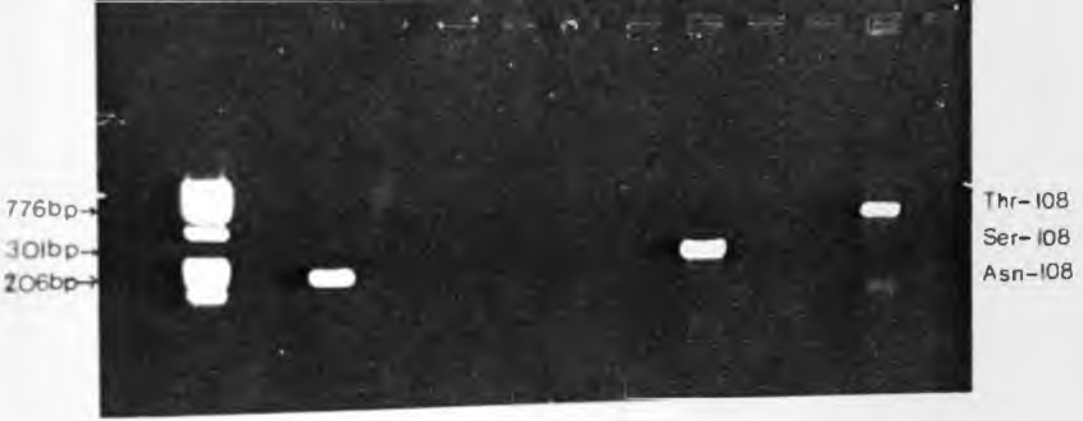
A

Thr-108

B

FIGURE 18

Phi X DNA digested with Hae III was used as the standard marker in lane 1. The reference isolate, FCB, was used as the positive control and a mastermix with no DNA was used as the negative control. FCB DNA amplified with SP1/ P2 mutant primer pair in lane 12 indicating that this isolate had the Thr AA residue at codon 108 in the DHFR gene. S158 on the otherhand showed that it had the AA residue Asn at codon 108, in lane 3 and finally ENT22 showed to have the AA Ser at codon 108 in the DHFR gene, lane 9.



PRIMERS: RP4/DIA13 SP2/P3 AND SP1/P2

ANNEALING TEMP: 54 deg C. 1min

Figure 18 Results of MS-PCR for codon-108

MARKER

CONTROL

SI58

FCB

ENT22

RP4 / D1A13

CONTROL

K39

FCB

ENT22

SP2 / P3

CONTROL

SI58

FCB

ENT22

SP1 / P2

1 2 3 4 5 6 7 8 9 10 11 12 13

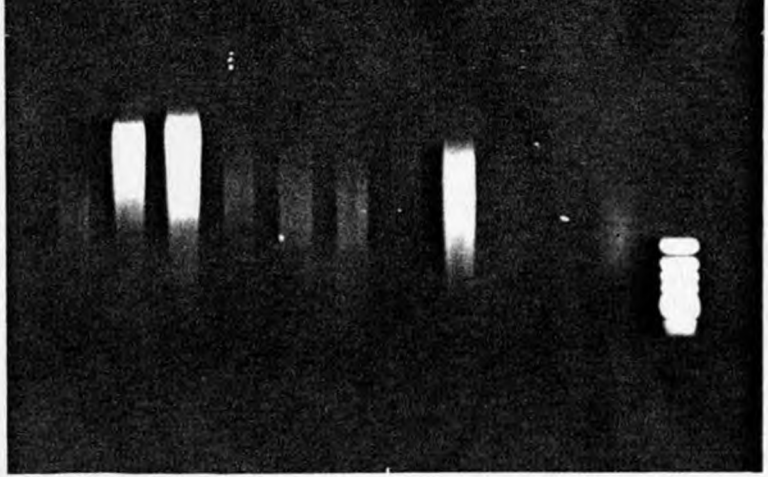
CODON-108

3.2.4 CODON 164

Fig. 19 panel A shows PCR products of 505 bp in all of the 10 isolates except in the negative control. These are amplification products obtained when DIA 14/SP11 wild type primer pair was used. This set of primers amplified DNA adjacent to the AA Ile-164. Panel B shows no specific PCR products when the mutant DIA 13/ SP14 primer pair was used. FCB reference strain amplified with the wild type primer pair whereas the Vietnamese VI/S strain amplified with the mutant or diagnostic primer pair.

FIGURE 19

Phi X DNA digested with Hae III was used as the standard marker in lane 12. A mastermix with no DNA was used as the negative control in lane 11. Panel A shows the PCR products when the wild type primer pairs, DIA13/ SP14 was used. The expected PCR product size was 505 bp. Panel A shows PCR product of 505 bp size resolved in all the Kenyan isolates indicated, lanes 1-10. Panel B shows smears under optimal PCR conditions for the mutant primer pair, DIA14/ SP11, stating that none of the isolates shown had the AA residue Leu at codon 164 in the DHFR gene.



PRIMERS: DIA 13/sp 14 AND DIA 14/sp11

ANNEALING TEMP: 56 deg C, 1 min

Figure 19 Results of MS-PCR for codon 164

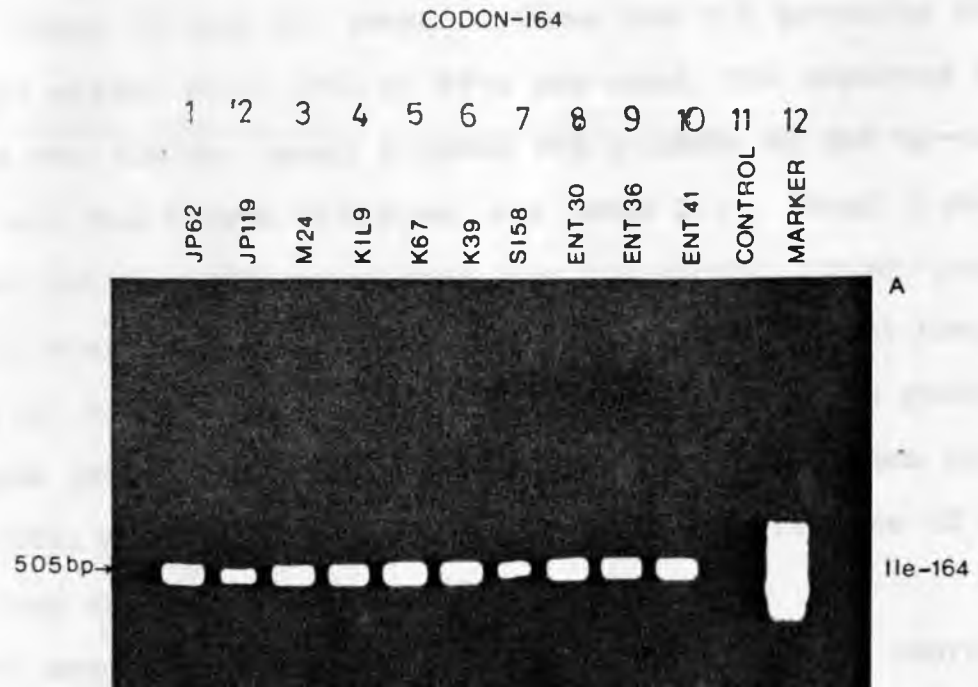
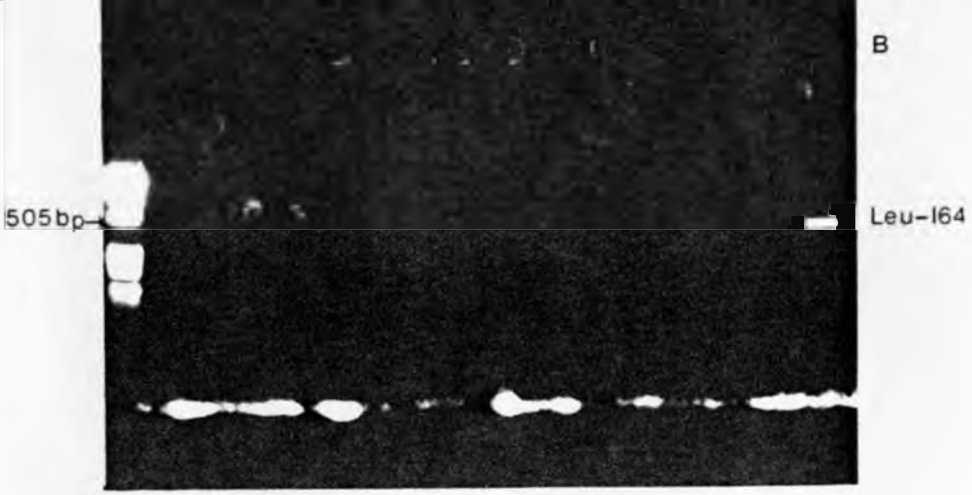

CODON-164
1
2
3
4
5
6
7
8
9
10
11
12
JP62
JP119
M24
KIL9
K67
K39
S158
ENT 30
ENT 36
ENT 41
CONTROL
MARKER
A
505bp
Ile-164

FIGURE 20

Phi X DNA digested with Hae III was used as the standard marker in lane 12. Two reference strains, VI/S and FCB, were used as positive controls in lanes 13 and 14. Panel A shows the PCR products when the wild type primer pair, DIA13/ SP14 was used. The expected PCR product size was 505 bp. Panel A shows PCR product of 505 bp size resolved in all the Kenyan isolates, see lanes 1-11. Panel B shows smears under optimal PCR conditions for the mutant primer pair, DIA14/ SP11, stating that none of the isolates shown had the AA residue Leu at codon 164 in the DHFR gene. However, a PCR product of 505 bp was resolved with the reference strain VI/S when using the DIA14/ SP11 mutant primer pair indicating the presence of the AA residue Leu at codon 164 in the DHFR gene.

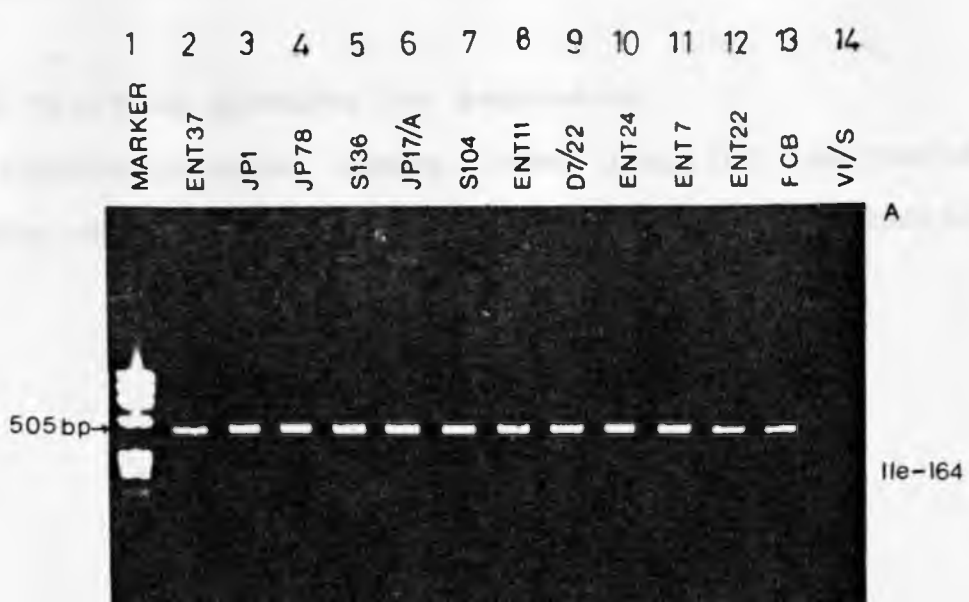
The artifact seen at the bottom of the gel is a result of improper gel preparation.



PRIMERS: DIA13/SP14 AND DIA14/SP11

ANNEALING TEMP: 56 deg C. 1 min

Figure 20 Results of MS-PCR for codon 164


CODON-164
1 2 3 4 5 6 7 8 9 10 11 12 13 14
MARKER
ENT37
JP1
JP78
S136
JP17/A
S104
ENT11
D7/22
ENT24
ENT7
ENT22
FCB
V1/S
A
505bp
Ile-164

3.2.5 Direct Sequencing

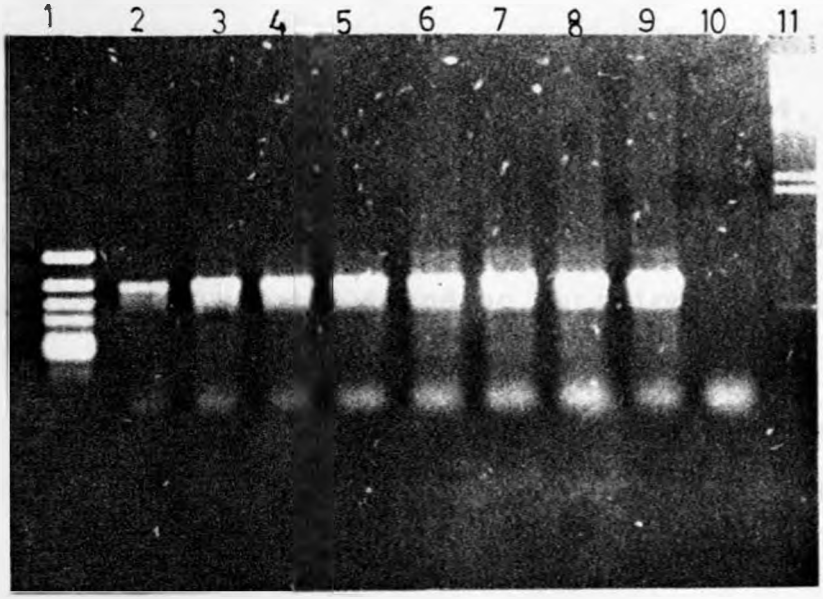
DNA sequencing of the DHFR gene was carried out on 21 Kenyan *P. falciparum* isolates in order to confirm point mutations detected by MSPCR and to check for any other base pair changes in the DHFR gene sequence.

3.2.5.1 PCR Purified products for sequencing

This step removes primers, dimers, other reaction components and primer-dimers which can adversely affect the sequence reaction.

FIGURE 21

Whereas panel A of figure 21 shows the unpurified DHFR gene comprising of the primer-dimers, dNTPs as well as the non-specifically amplified products evident from the smears observed, panel B shows the DHFR gene after the purification.



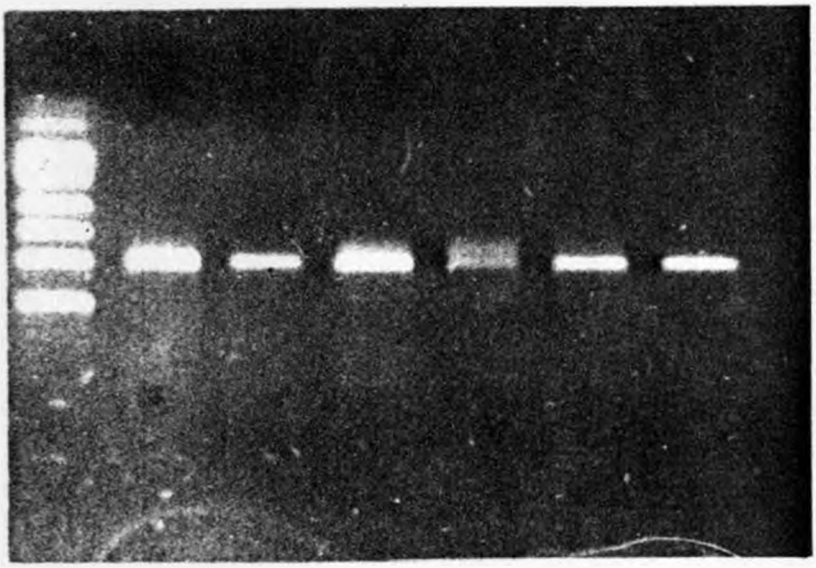


Figure 21 : PCR products obtained by amplifying the DHFR gene of P.falciparum using DIA12 and SP10 primers (A) unpurified, (B) purified.

3.2.6 Direct sequencing results

Fig. 22 is a section of a sequencing gel autoradiograph illustrating some of the point mutations detected in the DHFR gene of two Kenyan *P.falciparum* isolates, namely M24 and S104. Figure 22 a and b are showing the wild type AA at positions 108 and 164, namely Ser and Ileu respectively. Figure 22 c and d are showing the mutations at positions 51 and 108 and no mutation was detected at position 59.

Table 5 is an example of the results obtained from direct sequencing. It shows the results of the sequences upstream and downstream of point mutations of M24 and S104. The table indicates the AA substitutions as a result of the point mutations.

3.2.6 Direct sequencing results

Fig. 22 is a section of a sequencing gel autoradiograph illustrating some of the point mutations detected in the DHFR gene of two Kenyan *P.falciparum* isolates, namely M24 and S104. Figure 22 a and b are showing the wild type AA at positions 108 and 164, namely Ser and Ileu respectively. Figure 22 c and d are showing the mutations at positions 51 and 108 and no mutation was detected at position 59.

Table 5 is an example of the results obtained from direct sequencing. It shows the results of the sequences upstream and downstream of point mutations of M24 and S104. The table indicates the AA substitutions as a result of the point mutations.

TABLE 5 Sequences upstream and downstream of point mutations of M24 and S104

ISOLATE	DRUG RESPONSE	POSITION ON THE DHFR GENE	AMINO ACID	SEQUENCE UPSTREAM AND DOWNSTREAM OF CODON
M24	$Pyr^{s}Cyc^{s}$	51	Asn	5'AAATGT**AAT**TCCCTAGAT 3'
S104	$Pyr^{r}Cyc^{s}$	"	Ile	5'TGGAAATGT**ATT**TCCCTA 3'
M24	$Pyr^{s}Cyc^{s}$	59	Cys	5'AAATATTTT**TGT**GCAGTT 3'
S104	$Pyr^{r}Cyc^{s}$	"	Cys	5'AAATATTTT**TGT**GCAGTT 3'
M24	$Pyr^{s}Cyc^{s}$	108	Ser	5'AGAACA**AGC**TGGGAAAGC 3'
S104	$Pyr^{r}Cyc^{s}$	"	Asn	5'AGAACA**AAC**TGGGAAAGC 3'

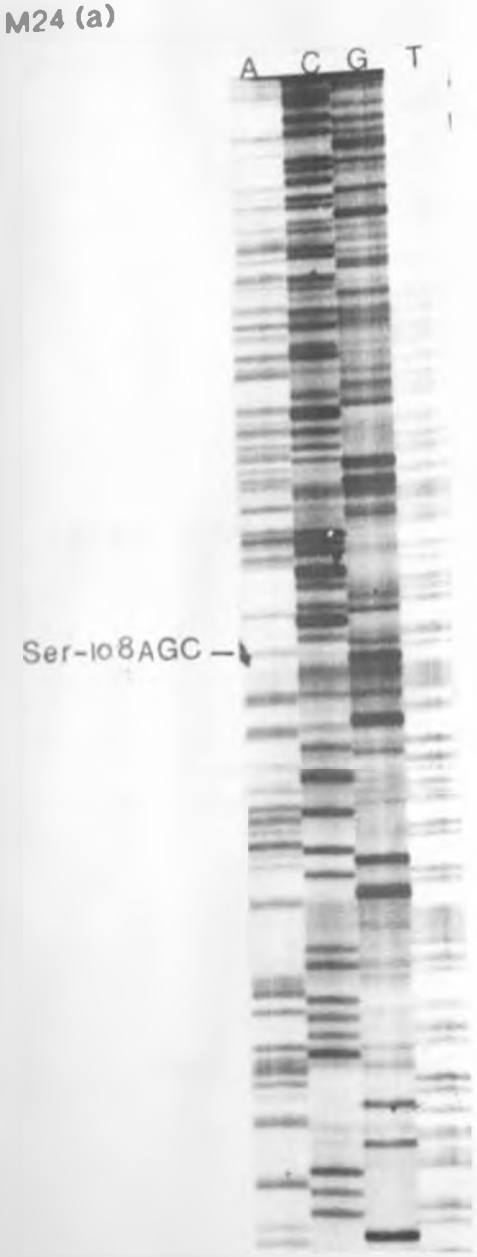
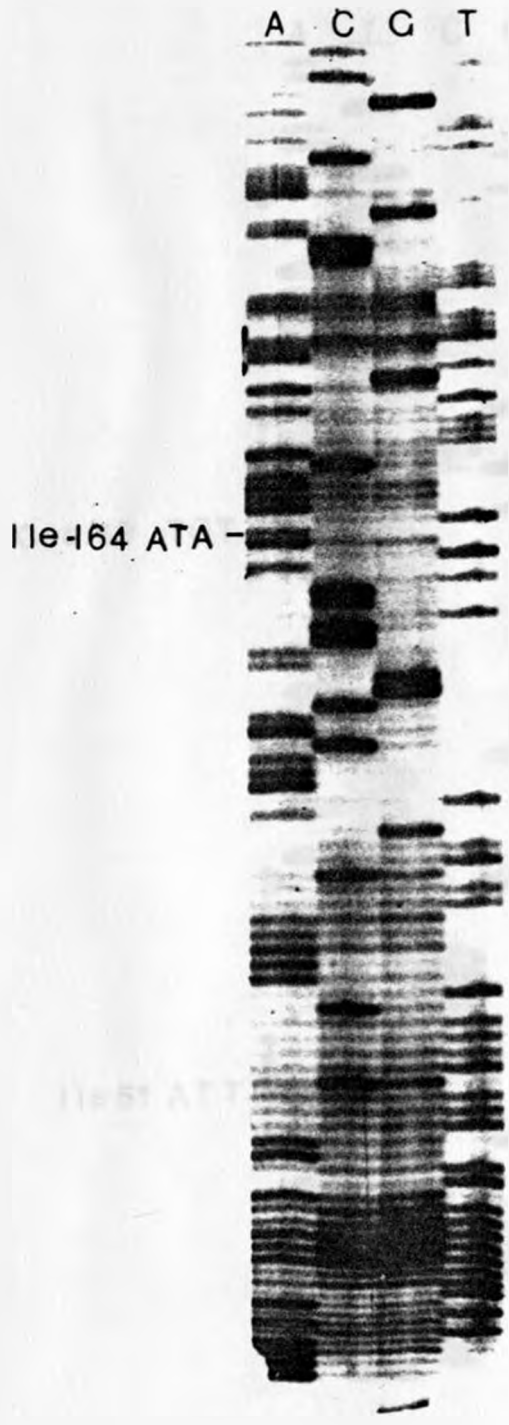
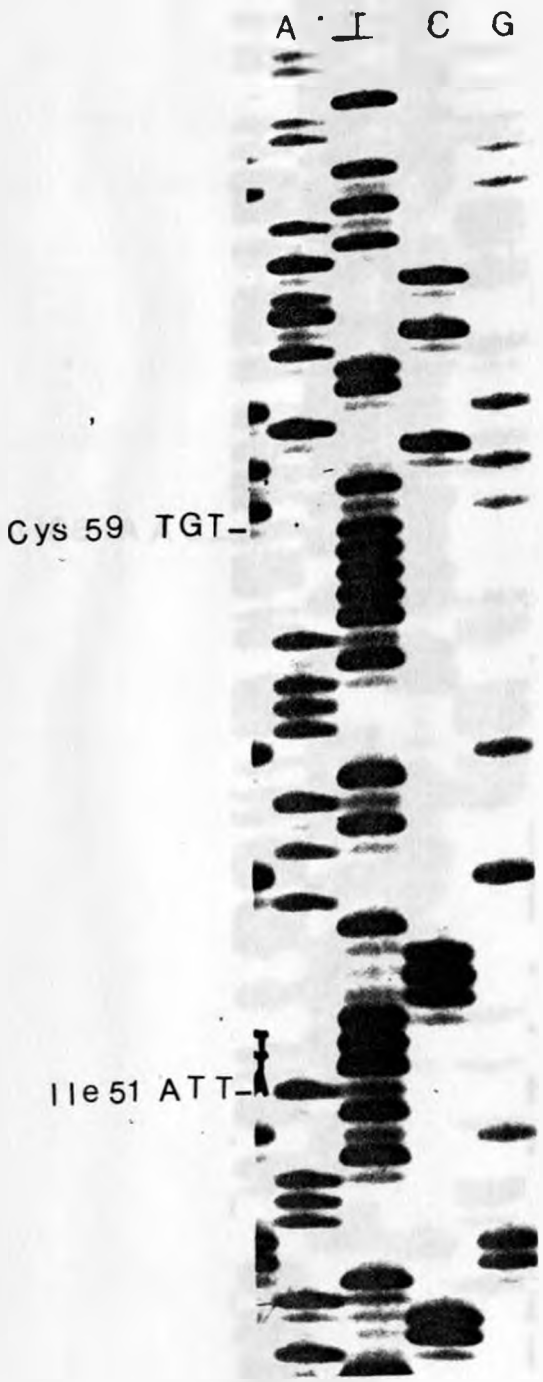


Figure 22 A sequencing gel autoradiograph indicating some of the point mutations on the DHFR gene.

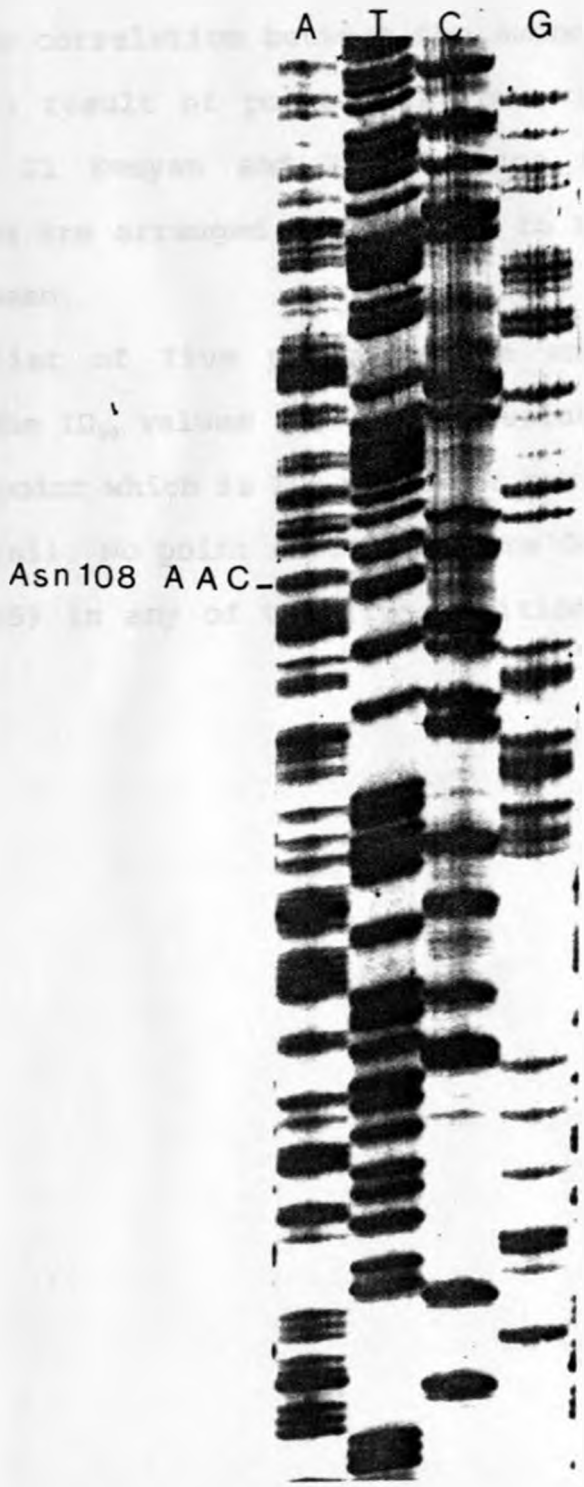
M24 (b)



S104 (c)



S104 (d)



4.1.1 Correlation between *in vitro* drug sensitivity tests and direct sequencing

Table 6 shows the correlation between the amino acid changes in the DHFR gene as a result of point mutations, and their ID_{50} values in nM/ L of 21 Kenyan and 2 reference *P. falciparum* isolates. The isolates are arranged in groups A to E depending on the point mutations seen.

Group A is a list of five pyrimethamine and cycloguanil sensitive isolates. The ID_{50} values for these isolates fall below the standard cut-off point which is 100 nM /L for pyrimethamine and 80 nM /L for cycloguanil. No point mutations were detected (Figs. 16,11,14,9,20,10,19,15) in any of the five positions in the DHFR gene in the isolates.

TABLE 6

Summary of point mutations and ID_{50} values of 21 Kenyan isolates and two reference strains

	ISOLATE	AMINO ACID ON DHFR GENE			ID_{50} nM/L	
		51	59	108	Pyr	Cyc
A	M24	Asn	Cys	Ser	2.3± 1.1	1.9± 0.5
	JP62	Asn	Cys	Ser	1.7± 0.6	2.5±0.5
	K67	Asn	Cys	Ser	0.3± 0.2	0.9± 0.1
	S136	Asn	Cys	Ser	5.1± 0.4	1.4± 0.1
	ENT22	Asn	Cys	Ser	3.6	1.7
B	D722	Asn	Arg	Asn	312± 100	8.5± 0.6
	JP78	Asn	Arg	Asn	175± 45	9.9± 1.9
	JP17/A	Asn	Arg	Asn	184± 34	18.5± 3
	ENT7	Asn	Arg	Asn	532± 176	25.3±3.4
	ENT24	Asn	Arg	Asn	583± 112	17.2± 4
	ENT11	Asn	Arg	Asn	349± 150	11± 5.5
	JP1	Asn	Arg	Asn	183± 80	7.9± 1.6
	ENT37	Asn	Arg	Asn	785± 113	22.2± .6
	S104	Asn	Arg	Asn	347± 68	12.2±3.1
C	K39	Ile	Cys	Asn	581± 210	13.5±2.5
	S158	Ile	Cys	Asn	431±163	17± 8.4
	JP119	Ile	Cys	Asn	246±50	11.7±4.2
	KIL9	Ile	Cys	Asn	352±123	12.6±3.1
D	ENT36	Ile	Arg	Asn	231± 128	3.6± 2.0

B	ENT41 ENT 30	Ile:Asn Ile:Asn	Cys:Arg Cys:Arg	Ser:Asn Ser:Asn	0.3/290 300/	0.4/17

REFERENCE STRAINS **AMINO ACID IN DHFR GENE** **ID_{50} nM/L**

	16	51	59	108	164	Pyr	Cyc
FCB	Val	Asn	Cys	Thr	Ile	1.3 ±o.3	31.3± 4.5
VIS	Ala	Ile	Arg	Asn	Leu	1254 ± 209	65.8±23.9

Table 6

Pyr: Designates pyrimethamine

Cyc: Designates cycloguanil

Group B consists of nine isolates whose ID_{50} for pyrimethamine was above the 100 nM /L cut-off value. These isolates still were cycloguanil sensitive but pyrimethamine resistant. Two mutations in the DHFR gene were detected at positions 59 and 108, (Figs. 14, 16, see also table 5).

Group C consists of four isolates whose ID_{50} for pyrimethamine was high and had two AA changes at positions 51 and 108, (Figs. 15,18 11).

Group D shows one isolate, ENT36 with an ID_{50} value of 231 for pyrimethamine. Three AA substitutions at the active site of the DHFR/TS (Figs. 15, 11 and 13) enzyme occur at positions 108, 51 and 59.

Group E consists of two mixed isolates i.e., ENT 41 and ENT 30. In this case PCR amplification for positions 51, 59 and 108 occurred when using both wild type and mutant type primer pairs, (Figs. 11, 12, 13). In addition results from direct sequencing produced equivalent nucleotide intensities on the auotradiograph at positions 51, 59 and 108.

4.1.2. Point mutations found in the DHFR gene of Kenyan *P. falciparum* isolates

Of the 21 *P.falciparum* Kenyan isolates studied only 5 were found to be pyrimethamine susceptible (sensitive) while the remaining 16 isolates were found to be pyrimethamine resistant. Those isolates having the wild-type AA, Ser at residue 108 were shown to be highly susceptible to pyrimethamine and furthermore,

those isolates having the mutant AA, Asn-108, were shown to be pyrimethamine resistant. We observed that all the pyrimethamine sensitive and resistant isolates were susceptible to cycloguanil. Results obtained from MSPCR indicated the absence of point mutations associated with cycloguanil resistance, i.e., no amplification occured when using the primer pairs that would amplify the target sequence adjacent to the AA residue Thr at codon 108 and Val at codon 16 in the DHFR gene implicated in cycloguanil resistance in the absence of pyrimethamine resistance, (See Figs 9, 10 and 17). We did not find isolates with Leu 164 (See Fig 19 and 20) which, if coupled with two other AA changes i.e., Cys 59 to Arg 59 and Ser 108 to Thr 108, would confer cross-resistance to pyrimethamine and cycloguanil. From the above interpretation of results it could be said that at least two *P.falciparum* isolates from each of the three provinces of Kenya are pyrimethamine sensitive and cycloguanil sensitive, and that five isolates are pyrimethamine resistant and cycloguanil sensitive.

Apart from K39, all isolates used in this study were uncloned. However, one of the objectives of the study was to find a test which could be applicable in a practical field situation and it is well known that field isolates contain several strains of *P. falciparum*.

In the case of mixed isolates sequencing results will produce equivalent nucleotide intensities on the auotradiograph for both sensitive and resistant strains whereas PCR can amplify

a minor component as effectively as the predominant one. However, in the field, evidence of resistant strains is important as these will eventually dominate during therapy.

Results obtained from one way analysis of variance (ANOVA) stated a statistical difference between group A and those of groups B, C and D. However, groups B, C and D are not statistically different from each other with respect to their ID_{50} values.

In summary, for Kenyan *P.falciparum* isolates so far investigated in the present study, only three point mutations have been detected. These are Asn-51 to Ile-51, Cys-59 to Arg-59 and Ser-108 to Asn-108. The mutations in the DHFR gene that lead to pyrimethamine resistance have already been well documented, (Zolg, *et al.*, 1988; Foote *et al.*, 1990; Peterson *et al.*, 1991). Our data on several more isolates strengthen the importance of the Asn at positions 108 being responsible for pyrimethamine resistance and that the effects of changes at 51 and 59 confer further pyrimethamine resistance.

4.1.3. Alternatives of combating drug resistance

The results of this work support clinical observations that alternative anti-DHFR agents can be effective against some strains of pyrimethamine resistant malaria. Point mutations affecting one inhibitor might be countered in some areas by alternative antifolate drugs capable of acting against the mutant enzyme. It was found that ID_{50} values for cycloguanil were lower

than for pyrimethamine, (Table 4). Therefore it is possible that cycloguanil is effective even when there is pyrimethamine resistance. The amount of available drug could also be increased by increasing the metabolization of the drug - only 10% of the biguanides are metabolized to the active antimalarials. Hence the effectiveness of the biguanides could be further increased.

Since in our case only 3 point mutations were involved, it may be possible to design primers which simultaneously differentiate between the three mutations by the difference in the sizes of the resulting PCR products or rely on the mutation at 108 since the ones at 51 and 59 only occur if the former one also does.

4.1.4 Advantages of direct sequencing versus conventional sequencing

The PCR amplification of the 774 bp encompassing DHFR gene accomplishes within three hours what might otherwise conventionally take days of biological growth and biochemical purification. In addition, conventional sequencing of the amplified product would involve multiple steps of cloning, isolation and purification. In this particular case the PCR product has been sequenced directly without these additional steps.

4.1.5 Advantages of MSPCR

Existing methods for identifying the presence and degree of antifolate resistance in communities are cumbersome. However, results obtained in this study suggest that the MSPCR has the potential to become a viable alternative to the *in vitro* testing of *P.falciparum* resistance to antifolate, even when compared to a fast micro test system (30-hr) currently in use (Wernsdorfer, 1980). Hence, it should be feasible to screen large sample numbers with MSPCR to monitor the appearance, persistence and spread of antifolate resistance of *P.falciparum* in a population.

It is evident that the use of 3'end match/mismatch primers in combination with the amplification of minute quantities of DNA (0.1-1ng) is in many aspects superior to the oligonucleotide probing technology (Wernsdorfer, 1980) and ideally suited to trace genetic changes in which as little as a single nucleotide exchange is of diagnostic interest.

4.1.6. CONCLUSION

With the development of rapid and sensitive techniques of detection, DNA probes could be used as an aide in choosing the appropriate regimen for the treatment of malaria. With increased resistance of *P. falciparum* to a large number of antimalarial drugs coupled with a paucity of new antimalarials, such a diagnostic test will become a necessity. Information on base pair changes will also enable three dimensional molecular models of the altered DHFR enzyme to be built. Study of its interactions with inhibitors may help in the design of more specific and

therefore more active drugs.

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