Genome Comparison of Progressively Drug Resistant
*Plasmodium falciparum* Lines Derived from Drug Sensitive Clone

Ravi Toteja, Lathika Nair, VK Bhasin

Department of Zoology, University of Delhi, Delhi  110007, India

Chloroquine has been the mainstay of malaria chemotherapy for the past five decades, but resistance is now widespread. Pyrimethamine or proguanil form an important component of some alternate drug combinations being used for treatment of uncomplicated *Plasmodium falciparum* infections in areas of chloroquine resistance. Both pyrimethamine and proguanil are dihydrofolate reductase (DHFR) inhibitors, the proguanil acting primarily through its major metabolite cycloguanil. Resistance to these drugs arises due to specific point mutations in the *dhfr* gene. Cross resistance between cycloguanil and pyrimethamine is not absolute. It is, therefore, important to investigate mutation rates in *P. falciparum* for pyrimethamine and proguanil so that DHFR inhibitor with less mutation rate is favored in drug combinations. Hence, we have compared mutation rates in *P. falciparum* genome for pyrimethamine and cycloguanil. Using erythrocytic stages of *P. falciparum* cultures, progressively drug resistant lines were selected in vitro and comparing their RFLP profile with a repeat sequence. Our finding suggests that pyrimethamine has higher mutation rate compared to cycloguanil. It enhances the degree of genomic polymorphism leading to diversity of natural parasite population which in turn is predisposes the parasites for faster selection of resistance to some other antimalarial drugs.

Key words: *Plasmodium falciparum* - malaria - drug resistance - sequencing-RFLP - dihydrofolate reductase

New malaria cases are annually increasing. This increase can be attributed partly to the development of resistance by *Plasmodium falciparum* to the most commonly used, inexpensive, synthetic antimalarial drug, chloroquine. Chloroquine has been the mainstay of malaria chemotherapy for the past five decades, but resistance to it is now widespread. Pyrimethamine or proguanil form an important component of some alternate drug combinations being used for treatment of uncomplicated *P. falciparum* infections in areas of chloroquine resistance. Pyrimethamine-sulfadoxine combination is usually deployed as a successor to chloroquine. Proguanil-atovaquone, a new antimalarial combination, first registered in the United Kingdom in 1996 for treatment of multidrug-resistant falciparum malaria and has subsequently been registered for this indication in 32 other countries worldwide (Kremsner et al. 1999, Bustos et al. 1999). Both pyrimethamine and proguanil are dihydrofolate reductase (DHFR) inhibitors (Cowan & Foote 1990), the proguanil acting primarily through its major metabolite cycloguanil (Armstrong & Smith 1974). In *Plasmodium* DHFR is formed as DHFR-TS (thymidylate synthetase) a bifunctional protein, the enzymes acting sequentially and genes for these enzymes (*dhfr-ts*) are colinear (Garrett et al. 1984). Resistance to pyrimethamine and proguanil arises due to specific point mutations in the *dhfr* gene (Peterson et al. 1988). Cross resistance between proguanil and pyrimethamine is not absolute, resistance to the two drugs being controlled by different point mutations in *dhfr* gene (Peterson et al. 1990, Foote et al. 1990).

It is, therefore, important to investigate the rate at which *P. falciparum* parasites develop resistance to both these drugs so that DHFR inhibitor with less mutation rate is favored in a drug combination to prolong the effectiveness of such combinations. Hence, we have compared point mutations in *dhfr* gene of progressively drug resistant *P. falciparum* lines to pyrimethamine and cycloguanil that were selected in vitro from a sensitive clone, and RFLP profile of whole genome of the selected lines with a known falciparum repeat sequence was also carried out. The other parameter studied include the predisposition, if any, for faster selec-
tion of resistance to sulfadoxine in the parasite line highly resistant to pyrimethamine.

**MATERIALS AND METHODS**

**Parasites** - The *P. falciparum* isolate FCD-3 and FCD-4 obtained locally from infected patients with consent and a clone F-56 derived from FCD-4 were the parasite lines used in the present study (Mehra & Bhasin 1996). The clone was obtained by the limiting dilution method from the erythrocytic stages of the isolate cultivated continuously in vitro by the candle-jar procedure of Trager and Jensen (1976).

**Stock solutions of drugs** - Pyrimethamine (Sigma) 10⁻² M stock solution was prepared in 0.05% lactic acid and 10⁻² M stock of cycloguanil 70% in ethanol. Further dilutions were made in RPMI-1640 medium prior to use. Sulfadoxine 10⁻¹ M stock solution was made in DMSO and diluted further in Waymouth MB752/1 medium, which is similar to RPMI-1640 except it does not contain para-aminobenzoic acid. Cycloguanil was a gift from ICI pharmaceuticals, UK and sulfadoxine from Lupin, India.

**Susceptibility test method** - Susceptibility of parasites to DHFR inhibitors and other antimalarials was determined by exposing parasites in triplicate in 24-well tissue culture plates to graded concentrations of each drug by the modified 48 h test method (Nguyen-Dinh & Payne 1980). The parasite material for experiments was obtained from stock cultures and subjected to sorbitol lysis to get synchronized ring stages (Lambros & Vanderberg 1979). The parasitemia was adjusted to less than 1% by uninfected fresh erythrocytes and this material was made to 50% cell suspension with appropriate medium. Aliquots of 20 µl of the above suspension were added into series of wells of the test plate, each holding 480 µl of complete medium with or without a drug, yielding final cell suspension of 2%. Loaded test plates were incubated at 37°C in a candle-jar for 96 h with daily changes of medium. The drug was included in experimental wells for the first two days only. Blood smears were made at the end of 48 and 96 h, stained with Giemsa. A minimum of 5,000 erythrocytes was enumerated to determine parasitemia in each well. Percentage reduction of parasitemia in relation to control was calculated from percentage inhibition of growth. Fifty percent, 95% and minimum complete inhibitory concentrations (IC₅₀, IC₉₅ and MIC, respectively) were extrapolated from the semilog plot of various concentrations of the drug against percent inhibition of growth obtained from averages of triplicate wells.

**In vitro selection of drug resistance** - The cloned line F-56 and a highly pyrimethamine resistant line selected in vitro were separately subjected to increasing sulfadoxine pressure in Waymouth MB752/1 medium to select a series of progressively sulfadoxine resistant lines. The parasites were subjected to their IC₉₅ concentration of the drug for 48 h. After 48 h drug was removed and the surviving parasites were allowed to multiply in drug free medium. The susceptibility of these parasites to sulfadoxine was determined. If there was no change in the sensitivity of these parasites to sulfadoxine, they were again exposed to the same IC₉₅ concentration. The procedure was repeated till there was an increase in the IC₅₀ to the parasites. The surviving more resistant parasites were subjected to their new IC₉₅ concentration of sulfadoxine to select more resistant line. This protocol for selection of progressively sulfadoxine resistant parasites was repeated for each of the subsequent rounds. Selection procedure for increased DHFR inhibitor resistance has been described by Bhasin and Nair (1996).

**Extraction of parasite DNA** - The frozen parasite samples (~10¹⁰ parasites) were thawed, mixed with two volumes of 1% cold acetic acid and centrifuged at 6,000 rpm for 20 min at 4°C. Supernatant, containing most of the haemoglobin, was removed and the pellet was resuspended in two volumes of 1% Triton X-100. It was again spun at 6,000 rpm for 20 min at 4°C and supernatant discarded. The resulting pellet was washed twice with 0.85% NaCl (pH 7.4). The washed parasite pellet was resuspended in DNA extraction buffer (10 mM Tris pH 8.0; 0.1 M EDTA pH 8.0; 20 µg/ml RNAase A; 0.5% SDS) in a volume equal to that of the original frozen sample and incubated at 37°C for 1 h with the intermittent shaking. Proteinase K was added at concentration of 100 µg/ml to the above mixture, mixed gently and incubated overnight at 37°C. DNA was extracted with an equal volume of phenol once, phenol:chloroform (1:1) twice, chloroform: isooamyl alcohol (24:1) once and chloroform twice. To the aqueous phase of the final extraction step was added 0.2 volume of 10 M ammonium acetate and 2.5 vol. of cold absolute alcohol. This was left overnight at -20°C for DNA to precipitate. DNA was pelleted by centrifugation at 12,000 rpm for 30 min at 4°C and supernatant carefully removed. The pellet was washed with cold 70% ethanol and dissolved in 200 µl of TE (10 mM Tris Cl pH 7.5; 1 mM EDTA pH 8.0).

**Polymerase chain reaction** - In vitro amplification of 1.1 Kb domain of the dhfr-ts gene from the template, parasite DNA, was carried out by PCR (Mullis & Faloona 1987) using the following oligonucleotide primer pairs: 5'-ATG ATG GAA CAA GTC TGC GAC-3' (sense) and 5'-CAC CTA CTC GTG TCG-3' (antisense). These synthetic
oligonucleotides were customarily designed from the complete DNA sequence of the gene. PCR reaction mixture consisted of approximately 70 ng of template DNA, 15 pmol of each primer, 200 µM dNTPs in a final volume of 100 µl containing reaction buffer. The reaction mixture was overlaid with a drop of mineral oil, heated for 5 min at 94°C to denature the template DNA completely, while the reaction was still at 94°C, 0.5 µl (5 units/µl) of Taq polymerase (Genei, India) was added. Amplification was carried out under standard conditions for 25 cycles.

Cloning PCR products and sequencing - The amplified PCR products were polished by incubating with the Klenow fragment in presence of dNTPs (Frohman 1994). The polished amplicon was purified/extracted from electrophoresed sample on 0.7% low melting agarose gel for insertion into Sma I digested pUC-19 by ligation (Sambrook et al. 1989). The ligation mix was used to transform competent JM 101 Escherichia coli cells. Recombinant white colonies were picked up from agar plates containing antibiotic, X-gal and IPTG. Plasmids from the white colony were harvested using one step plasmid isolation method and DNA analysed. Appropriate recombinant plasmid preparation was purified by CsCl gradient centrifugation for sequencing. DNA sequencing reactions were carried out manually by dideoxy chain termination method of Sanger et al. (1977) using Sequenase™ Version 2.0 DNA sequencing kit from USB™, as per their protocol.

RFLP by Southern hybridisation - Parasite DNA digested with Hind III, Alu I or Taq I restriction endonuclease was size fractionated on 0.8% agarose gel by electrophoresis and transferred to nylon membrane (Southern 1975). The membrane was probed with synthetic 21-oligomer, 5' end labelled with 32P using T4 polynucleotide kinase. The probe used for Southern hybridization had the following sequence: 5'-AGGTCTTAACTTGACTAACAT-3'. This oligonucleotide is known to be repeat sequence in P. falciparum genome (Aslund et al. 1985). The hybridized membrane was exposed to X-ray film for autoradiogram.

RESULTS

Sensitivity of parasites to antimalarials - Susceptibility of the P. falciparum parasites to cycloguanil, sulfadoxine and pyrimethamine are presented in Table I. Data shows that all parasites are clinically sensitive to sulfadoxine. Clone F-56 and FCD-4 isolate are sensitive to pyrimethamine and cycloguanil as well. FCD-3 is a naturally occurring pyrimethamine resistant isolate. PR-5, a line derived from F-56 clone by in vitro selection procedure, is highly resistant to pyrimethamine.

Selection of drug resistant parasites - Progressively drug resistant falciparum lines to cycloguanil, sulfadoxine and pyrimethamine were derived from the cloned F-56 line by continuously cultivating the parasites in vitro with or without respective drug pressure for number of days (Table II). The IC50 to cycloguanil in CY-1 line was 2 X 10^-9 M, a decrease in sensitivity by about 75-fold in 351 days. Only about 35-fold decrease in sensitivity to sulfadoxine was observed at the end of 555 days of in vitro selection in SD-1 line. From susceptibility data both CY-1 and SD-1 can be regarded clinically sensitive lines to cycloguanil and sulfadoxine, respectively. Susceptibility to pyrimethamine progressively increased in various PR lines, registering an increase in 2.5 fold in 24 days (PR-1) to 2400 fold rise in IC50 value in 348 days (PR-5) of cultivation/selection, derived from F-56 sensitive clone. Susceptibility profile of PR-5 to various drugs in vitro is presented in Table I.

Selection of sulfadoxine resistance from a pyrimethamine resistant line - In pyrimethamine sensitive cloned F-56 line there was only 35-fold decrease in sensitivity to sulfadoxine at the end of 555 days (Table II) of selection protocol. Highly pyrimethamine resistant the PR-5 line, derived from F-56, when subjected to selection pressure of sulfadoxine, the IC50 dose in vitro rose from 4 µM to 2900 µM, thus a 725-fold change in susceptibility was recorded only in 363 days of selection procedure.

PCR and sequencing dhfr gene - Sufficient DNA from cryopreserved parasites of the F-56 clone, CY-1, PR-1 to PR-5 lines and FCD-3 iso-

<table>
<thead>
<tr>
<th>Parasites</th>
<th>Pyrimethamine (nM)</th>
<th>Cycloguanil (nM)</th>
<th>Sulfadoxine (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50</td>
<td>IC90</td>
<td>IC50</td>
</tr>
<tr>
<td>Clone F-56</td>
<td>1.5</td>
<td>3.6</td>
<td>0.072</td>
</tr>
<tr>
<td>Isolate FCD-4</td>
<td>1.5</td>
<td>4.6</td>
<td>0.084</td>
</tr>
<tr>
<td>Isolate FCD-3</td>
<td>320</td>
<td>2700</td>
<td>-</td>
</tr>
<tr>
<td>Line PR-5</td>
<td>3600</td>
<td>8000</td>
<td>74</td>
</tr>
</tbody>
</table>

Table I

In vitro susceptibility of erythrocytic stages of Plasmodium falciparum parasites to antimalarial drugs, determined by 48-h test. The starting parasitemia was less than 1% and hematocrit 2%.
Comparison of Resistant \textit{falciparum} Lines • Ravi Toteja et al.

The 1.1 Kb domain spanning \textit{dhfr-ts} genes from each of the DNA samples amplified using sense and antisense primers by PCR. The polished amplicon was cloned into pUC-19 for sequencing. The nucleotide codons obtained by sequencing at position 51, 59, 108 and 164 amino acid residues of the DHFR enzyme are presented in Table III. The nucleotide codons at the above positions were found to be similar in amplified DNA samples of F-56, CY-1, PR-1 and PR-2 lines. From in vitro susceptibility data all these were clinically pyrimethamine sensitive lines. The clinically pyrimethamine resistant parasites, the PR-3 to PR-5 in vitro selected lines and the natural isolate FCD-3, all showed point mutations leading to alterations in the codon 59 and 108 (Table III). The codon 59 had changed to CGT and 108 to AAC. In naturally occurring pyrimethamine resistance in the field isolate FCD-3, an additional mutation was found in codon 51 (AAT to ATT).

\textit{Restriction fragment length polymorphism (RFLP) analysis} - DNA from the parent cloned F-56 line, CY-1 and PR-1 to PR-5 lines, was each digested separately with \textit{Hind} III, \textit{Alu} I or \textit{Taq} I restriction enzyme, size fractionated on an agarose gel and probed with a synthetic oligomer, known to be a repeat sequence in \textit{P. falciparum} genome, by Southern hybridization. No difference was seen in the band profile of F-56, CY-1, PR-1, PR-2 or PR-3 lines (data not shown), whereas RFLP analysis clearly depicts changes in the band profile of \textit{Taq} I digested DNA of PR-4 and PR-5 lines compared to F-56 (Figure). The prominent changes in band profile were detected simultaneously with the appearance of high level of pyrimethamine resistance in PR-4 and PR-5. Similar changes were also observed in an autoradiogram of \textit{Alu} I digested Southern blot (data not shown).

\begin{table}[ht]
\centering
\caption{Increase in susceptibility of in vitro selected lines of \textit{Plasmodium falciparum} derived from the drug sensitive parent clone, F-56 (cycloguanil IC$_{50}$ = 0.072 nM, sulfadoxine IC$_{50}$ = 22 µM, pyrimethamine IC$_{50}$ = 1.5 nM). The parasites were under continuous cultivation for several days with or without drug pressure.}
\begin{tabular}{|c|c|c|c|}
\hline
Selected line & Days of continuous cultivation/selection & Fold increase in (IC$_{50}$) & Clinical status \\
\hline
CY$^a$.-1 & 351 & 75 & CY sensitive \\
SD$^b$.-1 & 555 & 35 & SD sensitive \\
PR$^c$.-1 & 24 & 2.5 & PR sensitive \\
PR-2 & 120 & 17.3 & PR sensitive \\
PR-3 & 204 & 140 & PR resistant \\
PR-4 & 261 & 340 & PR resistant \\
PR-5 & 348 & 2400 & PR resistant \\
\hline
\end{tabular}
\end{table}

\begin{table}[ht]
\centering
\caption{Amino acid residues present in the DHFR enzyme of different parasite lines as inferred from sequencing of the \textit{dhfr} gene.}
\begin{tabular}{|c|c|c|c|c|}
\hline
Clone/Isolate & Position of Amino acids & 51 & 59 & 108 & 164 \\
\hline
F-56 & Asn & Cys & Ser & Ile \\
PR-1 & Asn & Cys & Ser & Ile \\
PR-2 & Asn & Cys & Ser & Ile \\
PR-3 & Asn & Arg & Asn & Ile \\
PR-4 & Asn & Arg & Asn & Ile \\
PR-5 & Asn & Arg & Asn & Ile \\
FCD-3 & Ile & Arg & Asn & Ile \\
\hline
\end{tabular}
\end{table}

\section*{DISCUSSION}

The exposure of parasite population to submaximal inhibitory drug concentrations provides the selective pressure to resistance. The resistant mutants can survive in the presence of these intermediate levels whereas the drug sensitive parasites are killed. Repeated and intermittent exposure of the surviving parasite population to intermediate drug levels provides the optimum conditions for the emergence of resistant parasites which are biologically fit mutants to propagate. A key point mutation or multiple gene mutations may be required for a reduction of susceptibility. These mutations occur spontaneously and rarely. As they exist infrequently in parasite populations not exposed to antimalarial drugs, these mutations must be acquired and lost at a similar rate in wild parasite populations to maintain equilibrium (Mackinnon & Hastings 1998). Thus, the chance of a resistant mutant malaria parasite being selected
by that antimalarial drug depends on several factors, such as the total number of parasites exposed to drug pressure, the mutation frequencies, the elimination profile of the drug, and the drug susceptibility and fitness of mutants (Rosario et al. 1978). Some of these factors can not be accurately controlled by employing in vivo study systems. In vitro systems, on the other hand, provide a means to study most of these parameters under controlled conditions. By using in vitro cultivated erythrocytic stages of the cloned P. falciparum drug sensitive line, it was possible for us to expose the known number of genetically homogenous population of parasites to desired drug concentrations, repeatedly for fixed duration to select resistant lines and compare the rate at which parasite resistance develops to antimalarial drugs. The progressively drug resistant lines, like PR-1 to PR-5 derived from pyrimethamine sensitive cloned line F-56, form a good material to study accompanying changes at gene or genome level of the parasites.

In the present in vitro selection of drug resistant parasites, resistance to pyrimethamine developed much faster and stronger compared to cycloguanil or sulfadoxine. In 348 days continuous cultivation under pyrimethamine selection there was 2400-fold increase in IC50 value to pyrimethamine whereas only 75-fold decrease in sensitivity to cycloguanil was registered in 351 days cultivation under cycloguanil selection pressure (Bhasin & Nair 1996). There was 35-fold decrease in sensitivity to sulfadoxine in 555 days. Emergence of strains resistant to pyrimethamine appeared in the field rapidly soon after introduction of the pyrimethamine-sulfadoxine combination as an alternate to chloroquine in all countries where this combination was deployed. The resistance to pyrimethamine-sulfadoxine combination is fast increasing (Ronn et al. 1996). Proguanil, unlike pyrimethamine, still plays a useful role in prophylaxis and treatment in combination with other drugs (Peters 1998). These clinical observations are in accordance with our findings that parasites develop resistance to cycloguanil reluctantly compared to pyrimethamine.

Clinical field isolates resistant to pyrimethamine show a key point mutation leading to alteration of Ser-108 or Thr-108, found in sensitive isolates, to Asn-108 (Peterson et al. 1988) in the enzyme DHFR. Our sequencing studies also demonstrate the existence of Asn-108 point mutation in pyrimethamine resistant lines, the PR-3 to PR-5 and its absence in F-56, CY-1, PR-1 and PR-2 lines, all being pyrimethamine sensitive. There was, however, no co-relation found between the number of point mutations observed in the dhfr gene and decrease in pyrimethamine sensitivity in the in vitro selected lines, beyond some point. For instance, point mutations leading to Arg-59 and Asn-108 found in PR-3 to PR-5 remained unchanged in PR-5 with no additional mutation observed in the sequenced dhfr domain whereas there was substantial rise in the resistance, increasing from 140-fold to 2400-fold. The naturally resistant FCD-3 isolate had an additional alteration isoleucine-51 whereas in all other lines sequenced had Asg-51 residue in the DHFR. Some other factors are possibly contributing to the pyrimethamine sensitivity pattern observed. These considerations prompted us to look for alterations in the whole genome of progressively resistant falciparum lines by RFLP.
No apparent differences were observed in band profile of F-56, CY-1, PR-1, PR-2 or PR-3 lines whereas clear and gross changes in the band profile of \textit{Tag} I digested DNA of PR-4 and PR-5 lines were seen. These changes obviously occurred during selection either due to loss of existing or gain of new \textit{Tag} I restriction sites, caused by pyrimethamine. The prominent changes in band profile were detected simultaneously with emergence of strong pyrimethamine resistance in the selected lines PR-4 and PR-5. The slightly resistant PR-3 line showed no change in band pattern. The RFLP also shows that pyrimethamine enhances the genomic polymorphism by random mutations in the genome of pyrimethamine resistant parasites and this may lead to even diversity of natural parasite population, which in turn might be predisposed for faster selection of resistance to other antimalarial drugs. To test this hypothesis we subjected the highly pyrimethamine resistant PR-5 line for selection to sulfadoxine resistance and compared the rate of emergence of sulfadoxine resistance with pyrimethamine sensitive F-56 line. Resistance to sulfadoxine in PR-5 line appeared much faster and stronger than F-56. In 555 days of cultivation selection there was only 35-fold rise in IC\textsubscript{50} to sulfadoxine in F-56 whereas 725-fold rise was recorded in 363 days in PR-5 line. Clearly the PR-5 line was predisposed for faster selection to sulfadoxine resistance. This predisposition might be due to randomly introduced mutations by pyrimethamine in the pyrimethamine resistant line.

This finding is supported by another field observation (Djimde et al. 1997) in Mali. An increase in resistant mutants from 10\% to 90\% of infections in 109 residents of an endemic area of Mali who received pyrimethamine alone prophylactically for five weeks. Similar, study on genes for \textit{dhfr} and \textit{dhps} (dihydropteroate synthetase), in parasites in children in Tanzania before treatment with chloroquine-dapsone or pyrimethamine-sulfadoxine and in parasites recurring three weeks after treatment, showed that both drug combinations selected for mutations in \textit{dhfr}. Pyrimethamine-sulfadoxine, but not chloroquine-dapsone, were also selected for the \textit{dhps} locus (Curtis et al. 1998). In conclusion we can tentatively infer that pyrimethamine resistant \textit{falciparum} parasites are selected faster than cycloguanil. The pyrimethamine resistant parasites are predisposed for faster selection of resistance to sulfadoxine. The pyrimethamine selection pressure enhances genomic diversity by introducing random mutations in the genome of pyrimethamine resistant parasites and finally proguanil is a better choice over pyrimethamine in antimalarial drug combinations.

**REFERENCES**


912.


