Molecular analysis of recrudescent parasites in a Plasmodium falciparum drug efficacy trial in Gabon

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Abstract
Recrudescent Plasmodium falciparum parasites were sampled from 108 children taking part in a drug ef- ficiency trial in Gabon. A finger-prick blood sample was taken from each child before treatment, and a post- treatment sample taken of the recrudescent parasites. Sample deoxyribonucleic acid was amplified by the polymerase chain reaction using primers specific to the P. falciparum antigen genes MSP-1, MSP-2 and GLURP. Seventy-seven children had identical parasites in their pre- and post-treatment samples, indicating genuine recrudescences of resistant parasites. Fourteen children had completely different parasites in their pre- and post-treatment samples, indicating either a fresh infection from a mosquito or growth of a popu- lation of parasites not detected in the pre-treatment sample, perhaps due to sequestration. The remaining 17 children had a mixture of pre-treatment and new parasites in their post treatment samples. This study demonstrated the use of polymorphic markers to confirm whether parasites in patients with clinical recrudescences after drug treatment are genuinely resistant.

Keywords: malaria, Plasmodium falciparum, drug resistance, recrudescence, genotypes, polymerase chain reaction

Introduction
The response in vitro of malaria parasites exposed to chloroquine, and subsequently also other chemotherapeutic agents, is classified into 4 grades, sensitive (S) and resistant at RI, RII and RIII levels, according to World Health Organization guidelines (PAYNE, 1982, WHO, 1986). If the parasites are cleared within a week of treatment, and there is no subsequent reappearance of parasites within 28 d, the parasites are judged to have been sensitive to the drug. RII and RIII responses indicate that the parasitaemia is not cleared within the first week, and alternative therapy is usually prescribed. An RI response involves the reduction of parasitaemia below the detection threshold for at least 2 d within the first week after treatment, followed by the reappearance of parasites. In early recrudescence, these parasites reappear before day 14 after treatment; in delayed recrudescence they appear between days 15 and 28 of the follow-up period.

A difficulty in using this clinical definition is that the recrudescent population of parasites, particularly in the delayed recrudescences, may not be resistant parasites persisting from the original infection, but parasites from a new infection of the patient. This apparent recrudescence may therefore consist of parasites sensitive to the drug. Late recrudescences are more likely to be new infections in areas of high transmission than in areas of less intense transmission. Estimates of the frequency of drug resistance based on uncontrolled tests in vitro in highly endemic areas may therefore be exaggerated.

Parasites can be identified as genuinely recrudescent by genotyping those in the original and subsequent parasitaemias (BABIKER et al., 1994). The technique involves amplification by the polymerase chain reaction (PCR) of regions of 3 highly polymorphic parasite genes. The likelihood of a patient being reinfected with a parasite containing exactly the same alleles at each of 3 loci is very small. If, therefore, the parasites in the post-treatment samples carry identical alleles to those of the original infection at all 3 loci, they most proba- bly represent a genuine recrudescence of resistant forms. However, if the parasites in the recrudescent samples carry different alleles from those in the original sample, the infection is likely to be a new one. Alterna- tively, it could represent a population of parasites which was not detected in the original sample, perhaps due to sequestration in capillaries leading to their absence from the circulating blood.

This technique has been used previously to show that the RI chloroquine response of P. falciparum in 6 pa- tients in a Sudanese village was indeed due to recrudescence of resistant parasites (BABIKER et al., 1994). Subsequently, essentially similar techniques have been used to examine recrudescent parasites by, for example, VIRIYAKOSOL et al. (1995), KAIN et al. (1996), AL- YAMAN et al. (1997) and DURASINGH et al. (1997).

In the study presented here we typed recrudescent P. falciparum parasites in a drug efficacy trial carried out in Gabon (LELI et al., in press). We analysed 108 pre- and post-treatment sample pairs to investigate the incidence of genuinely resistant parasites, using a simple method of sample collection and storage.

Subjects, Materials and Methods
Study area and sample collection
The drug efficacy trial was carried out at the Albert Schweitzer Hospital in Lambaréné, Gabon between January 1995 and January 1996, in an area of hyperendemic malaria where the predominant parasite species is P. falciparum, followed by P. malariae and P. ovale (see WILDLING et al., 1995).

A total of 252 children under 15 years of age presenting with mild P. falciparum malaria (1000–100 000 parasites/μL blood) was enrolled in the trial and allocated at random to receive one of 3 low dose drug regimens: (i) mefloquine (Lariam®), (ii) pyrimethamine/sulfadoxine (Fansidar®), and (iii) mefloquine/pyrimethamine/sulf-adoxine (Fansimal®). Full details of the study, its aims and rationale, are given by LELI et al. (in press). The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hos- pital.

A finger-prick sample of 2 drops of blood (approxi- mately 100 μL) was taken for PCR analysis from each patient on admission before the appropriate drug(s) was (were) administered. The blood was collected on a filter paper circle (Whatman 3 M, 5 cm diameter) and allowed to air-dry on a clean surface. Each filter paper was sealed in a separate plastic bag, and stored at 4°C or at room temperature. These samples were denoted the original (O) samples.

Following the single dose drug treatment on day 0, the patients were monitored clinically and parasitologi-
cally every 24 h until they were free of symptoms and parasites, and then routinely on days 7, 14, 21 and 28 after treatment. Patients who cleared their parasites in the first week of treatment and whose blood films remained aparasitaemic until day 28 were considered cured (parasites fully sensitive to the drug(s) given). Any patient who failed to clear their parasites during the first 6–7 days were deemed non-responders (RI/RIII). A second filter paper blood sample was taken from these patients before rescue therapy. RI cases showed an initial clearance of parasites and symptoms during the first week, followed by the reappearance of parasitaemia during follow-up on days 14, 21 or 28. A second finger-prick sample was also taken from these patients before rescue therapy. The second sample taken from each patient was denoted the post-treatment (P) sample.

**Extraction of deoxyribonucleic acid from filter paper samples**

The filter paper samples were transported at ambient temperature to Edinburgh, UK. Deoxyribonucleic acid (DNA) was extracted using the method of PLOWE and colleagues (1995). Briefly the blood spot was excised from the filter paper and solubilized overnight in a mild detergent (0.5% saponin). It was then incubated with Chelex-100® resin (Bio-Rad Laboratories, USA) to remove contaminants inhibitory to the amplification process, and the DNA was stored at -20°C or -70°C. Cross-contamination of DNA from one sample to another was prevented by using disposable scalpels, forceps and glass plates used with 5M hydrochloric acid followed by 5M sodium hydroxide, which prevents carry-over of DNA. Deoxyribonucleic acid was stored at -20°C or -70°C. Cross-contamination of DNA from one sample to another was prevented by using disposable scalpels, forceps and glass plates used with 5M hydrochloric acid followed by 5M sodium hydroxide, which prevents carry-over of DNA. Deoxyribonucleic acid was stored at -20°C or -70°C.

**DNA amplification**

The genes chosen for this study were those encoding 3 highly polymorphic *P. falciparum* antigens, the merozoite surface proteins MSP-1 and MSP-2 and the glutamate rich protein GLURP (HOLDER & FREEMAN, 1982; STANLEY et al., 1985; BORRE et al., 1991). Each gene exists as a single copy in the parasite genome, and they are unlinked. The genes contain regions encoding randomly repeated amino acids, which have been shown to vary in both number and sequence amongst different alleles (TANABE et al., 1987; BOW et al., 1991; SMYTHE et al., 1985; BORRE et al., 1991). Variations in the lengths of these regions can be identified as size differences of PCR products following electrophoresis. Over 20 different PCR products can be distinguished for each of these 3 single copy genes. Since the malaria parasite in the human host is haploid, the presence in a single blood sample of 2 or more alleles of any one gene denotes a mixed infection with parasites of different genotype.

The PCR analysis of each gene involved 2 rounds of amplification, using nested primers in the second round. This approach increases the sensitivity of PCR detection and allows DNA extracted from samples with low parasitaemia to be amplified successfully. Using artificial mixtures of parasites cultured in *vitro*, the techniques used here were able to detect 2 alleles clearly if the minority genotype was present at a level equivalent to at least 1% of the majority genotype, and the lowest parasitaemia detectable was 0.001% or 50 parasites per μL of whole blood (data not shown).

The primers and conditions used for the amplification are listed in Table 1. Further details for the genes MSP-1 and MSP-2 are given by RANFORD-CARTWRIGHT et al. (1993) and for GLURP by PAUL et al. (1995). For each sample, 50 μL reaction mixtures were prepared containing 1x PCR buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton-X100®), 150 μM of dATP, dTTP, dGTP and dCTP, 100 μM of each appropriate primer, and 1 unit of thermostable DNA polymerase. 0–7 μL of sample DNA was used as template for the outer PCR of MSP-1 and MSP-2. One μL of sample DNA was used for the GLURP outer PCR. For all 3 nested amplification reactions, 2–5 μL of the outer PCR product was transferred as template. Following nested primer amplification, 5–8 μL of the PCR product were loaded on to 1.8% (MSP-1 and MSP-2) or 1.5% (GLURP) agarose gels. The original and post-treatment samples from each patient were run in adjacent lanes for ease of comparison. Following electrophoresis and ethidium bromide staining, the sizes of the PCR products were compared between the sample pairs. Sizes (in terms of numbers of base pairs [bp]) were calculated according to their mobility relative to molecular size standards run on the gel.

If an amplification reaction was unsuccessful it was attempted again with more, or less, starting sample DNA. If a DNA sample failed to be amplified on 3 occasions it was recorded as 'fail'.

**Determination of recrudescence**

Genuine recrudescence of parasites resistant to the drug(s) given was defined as the presence of identical PCR products in the O and P samples. Such recrudescences were classified further into 2 types. In type A, the parasites in the O and P samples possessed exactly the same alleles at the 3 loci; this represented recrudescence of single or multiple clone infections. In type B recrudescence, only a subset of the alleles found in the O sample was found in the P sample. This result could be explained by the original parasites being a mixture of sensitive and resistant parasites, with drug selection removing the sensitive forms before the P samples were taken.

If the parasites in the O and P samples differed at all 3 loci, they clearly represented a new population of circulating blood forms. If they differed at only 2 loci, the frequency of the common allele at the third locus was determined in the parasite population of all the patients sampled. If it was high, there could have been reinfection with parasites which by chance carried the same al

<table>
<thead>
<tr>
<th>Locus and type</th>
<th>Primer names and sequences</th>
<th>Cycle conditions</th>
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<tr>
<td><strong>MSP 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer</td>
<td>O1: CACATGAAAAGCTATCAGAGACCTTGTC</td>
<td>94°C/25s: 50°C/35s:</td>
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<tr>
<td></td>
<td>O2: CACATGCTATATCAGGAGGAC</td>
<td>68°C/150s: 30cycles</td>
</tr>
<tr>
<td>Nested</td>
<td>N1: GCCGTATGCGAGGTTATGG</td>
<td>94°C/25s: 50°C/35s:</td>
</tr>
<tr>
<td></td>
<td>N2: GATTGAAAGATTTATG</td>
<td>68°C/150s: 30cycles</td>
</tr>
<tr>
<td><strong>MSP 2</strong></td>
<td></td>
<td></td>
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<tr>
<td>Outer</td>
<td>S1: CAGAGTGAATTTAACAATTGTC</td>
<td>94°C/25s: 42°C/60s:</td>
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<tr>
<td></td>
<td>S2: CACGATGTTGGCTGTCAGCAGAG</td>
<td>65°C/120s: 30cycles</td>
</tr>
<tr>
<td>Nested</td>
<td>S1: GAGGATAGGAGAGATGAG</td>
<td>94°C/25s: 50°C/60s:</td>
</tr>
<tr>
<td></td>
<td>S4: CTAGAAGCAGATCTAGTCC</td>
<td>70°C/120s: 30cycles</td>
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<tr>
<td><strong>GLURP</strong></td>
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<td></td>
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<td>Outer</td>
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<td>94°C/25s: 45°C/60s:</td>
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<tr>
<td></td>
<td>G2: GATTCGAAATGTTGACCC</td>
<td>68°C/120s: 30cycles</td>
</tr>
<tr>
<td>Nested</td>
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<td>94°C/60s: 55°C/120s:</td>
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<tr>
<td></td>
<td>G3: GATTCGAAATGTTGACCC</td>
<td>70°C/120s: 30cycles</td>
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lele as the original infection at this locus only. If the O
and P samples were identical at 2 loci, but differed at
one, the parasites in the P sample were classified as re-
crudescent (see Discussion).

Results

Drug efficacy

Full results of the drug trial have been presented else-
where (LELL et al., in Dress); 115 of the 231 children
who completed the trial cleared their parasites within a
week of treatment and 116 had suspected RI or RIUIII
parasites. Mefloquine at the low dose given was relative-
ly ineffective at clearing parasites (13% cleared) com-
pared to pyrimethamine/sulfadoxine (69% cleared) and
mefloquine/pyrimethamine/sulfadoxine (67% cleared).

Number of DNA extractions and samples analysed

Complete sample pairs from 108 of the 116 children
with recrudescences were successfully analysed at all 3
loci. Six pairs were missing or incomplete, and 2 pairs
could not be analysed because one or both samples
failed to amplify at least 2 loci. Amplification success at
the 3 loci was 97% for the O samples and 99% for the P
(recrudescent) samples, over all loci.

Allelic diversity and extent of mixed infections

Thirty-nine, 34 and 66 differently sized PCR prod-
ucts were found for MSP-1, MSP-2 and GLURP,
respectively. The prevalence distributions of these alleles are
shown in Fig. 1. 90/108 (97%) of the O samples and 77/
108 (71%) of the P samples contained more than one al-
lele of one or more genes, and were therefore mixed in-
fected with more than one parasite clone.

Classification of post-treatment infections

Results from the 108 children whose paired samples
were successfully typed are shown in Table 2. Most
(65%) of the children with genuine recrudescences had
received the low dose of mefloquine, and over half of
these had RIUIII responses, as discussed by LELL and
colleagues (in press).

(i) Genuine recrudescences of resistant parasites. Table 3
lists the types of recrudescence seen in the 77 children
containing genuinely resistant parasites. Eleven had a
recrudescence of a single clone, seen as single alleles at
all 3 loci which were identical in the O and P samples
(Fig. 2); 36 children had mixed infections in which all
of the clones detected in the O sample were also present
in the P sample. In total, therefore, 47 children had ex-
actly the same parasites in their respective O and P sam-
plies, and thus had type A recrudesences.

Type B recrudesences were seen in the remaining 30
children; some, but not all, of the O parasites reap-
peared in the P sample. In 3 cases the PCR products
were identical at all loci, but an additional allele was
found at one locus in the P sample; in all instances this
was at the *MSP*-1 locus. The parasites in these patients
were classified as genuinely recrudescent (see Discus-
sion).

Considering the RI and RIUIII resistance cases sep-
arately, 22 of the 41 RI group were type A and 19 were
type B. Among the 36 RIUIII cases, 25 were type A, of
which 20 were mixed clone infections, and the remain-
ing 11 were type B (Table 3).

(ii) Other recrudescences. Fourteen of the 108 children
were found to have completely different parasites in the
O and P samples (Table 2 and Fig. 3). With 4 of these
children, the parasites differed at all 3 loci. With the re-
mainning children, the O and P samples differed at 2 loci,
The possible origins of the parasites in the P sample are considered below. In this case, the size of the PCR product was clearly different for each locus in the original (O) and post-treatment (P) samples of this child. The MSP-2 product obtained from the post-treatment sample was faint. MWM, molecular size ['weight'] marker; numbers indicate base pairs.

![Fig. 2. Genuine RI recrudescence of resistant parasites, type A. A single identical PCR product was produced from each gene in the original (O) and post-treatment (P) samples of this child. The MSP-2 product obtained from the post-treatment sample was faint. MWM, molecular size ['weight'] marker; numbers indicate base pairs.](image)

Eleven of the 14 children in this group had infections which had been classified as RI, and 3 as RIIIRIII. It could be concluded that in these children the parasites found in the O samples were sensitive to the drug used. However, most of these children had been given a very low dose of mefloquine, which appeared to be suboptimal (LELL et al., in press), and so it should not be concluded that these parasites were resistant to a normal curative dose of mefloquine. With 14 patients, genetically different parasites were seen in the recrudescences, showing that the parasites of the primary infections in these instances were probably drug-sensitive.

In order to be sure whether the parasites are the same or different, the loci examined must be highly polymorphic in the local population, with no one allele predominating at any locus. In highly endemic areas like Gabon, genetic reassortment of unlinked genes is likely to be frequent, and so clonality, i.e. the over-representation in the population of parasite clones with a fixed combination of alleles at all loci, is unlikely. The 3 loci studied here, MSP-1, MSP-2 and GLURP, each had over 30 alleles in this population; the most common allele did not exceed 15% of the total alleles at that locus and there was also no evidence of one or a few three-locus genotypes predominating. There is still a chance that 2 parasites that are different from one another could have the same allele at any one locus, but examining more than one locus makes the chance of alleles being the same at both, or all 3, loci very small indeed.

In addition to the allelic size polymorphisms of the genes studied here, there are also likely to be variations in their sequences. We have not examined this variation. It is theoretically possible that certain alleles which were identical in size in the primary and recrudescent parasites could have varied in their sequences. However, in view of the allelic diversity seen at all 3 loci studied, we consider it unlikely that a parasite with PCR products of identical size at all 3 loci in the O and P samples was not the same parasite.

Estimating the size of PCR products from a gel is ac-
Molecular analysis of recrudescent malaria parasites

In the post-treatment samples (black bars), or a combination of both (grey bars), plotted against the time between the two samples;

Fig. 5. A comparison of the cumulative percentages of children with genuine recrudescences (white bars), with novel genotypes in the post-treatment samples; 50% of all genuine recrudescences seen had occurred by day 8 after treatment.

At the time of post-treatment sampling, so it is likely that the novel parasites were resistant to the drug.

Another explanation is that the recrudescent forms were derived from resistant parasites which were sequestered when the primary samples were taken, and thus not present in the circulating blood at that time. Parasites of differing genotype have been shown to appear in the blood on successive days of infection, presumably due to two cohorts of parasites undergoing alternate 24 h periods of sequestration (DauBersies et al., 1996; Färnert et al., 1997). Alternatively, both populations of parasites may have been present but in different proportions, so that the minority clone was not amplified successfully in the PCR. This would be most likely if one parasite clone was present as less than 1% of the total infection in the original sample. There is a possibility that the MSP-1 amplification reaction was less sensitive at detecting a minority genotype in mixed infections.

Half of all genuine recrudescences seen in this study occurred within 8 d of the initial treatment, whereas half of all reappearances of parasitaemia in which there were novel parasites in the P sample did not occur until about 18 d after treatment (Fig. 5). The cases in which novel and genuinely recrudescent parasites were found in the same sample had a similar distribution to the novel parasite cases alone, with 50% of all cases arising about 18 d after treatment. It has been suggested that the onset of symptoms in a chronically infected, previously asymptomatic individual may be correlated to the introduction of a new parasite, differing from that in the original infection (Contamin et al., 1996). It is therefore possible that the new parasites alone may have been responsible for the increase in parasitaemia and onset of symptoms in these children. If there had been no reinfection, the original parasites might have remained at levels undetectable in a blood film and the child might have been asymptomatic, and therefore no P sample would have been taken.

The analysis presented here has a number of improvements compared to the technique published previously (Babiker et al., 1994). The addition of a third locus makes it less likely that genuinely resistant parasites will be confused with reinfections in a recrudescence. The GLURP locus has proved to be very useful because its PCR products exhibited a much wider range of sizes than the other 2 loci. In this study the allele sizes ranged from 400 to over 1300 bp. Mixed infections were therefore very easily identified, since the PCR products often varied greatly in size. A major improvement was the use of dried blood samples collected on filter paper, which...
avoided the need for refrigeration and expensive shipment costs for frozen samples. The DNA extraction procedure published by Plowe and colleagues (1995) is simple and quick to perform, and the amplification success from this DNA was over 97%.

This work has shown the value of the use of polymorphic markers to confirm whether parasites in patients with clinical recrudescences after drug treatment are genuinely resistant. In future, it should be possible to type parasites at the loci responsible for the resistance; however, for the majority of antimalarial drugs in use, these loci have not been identified. An exception is the case of pyrimethamine resistance, where certain alleles of the parasite DHFR gene which are associated with resistance can be identified in ‘field’ samples by mutation-specific PCR techniques (Plowe et al., 1995).

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References


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