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 efficacy 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 population 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 vivo* 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. Later 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 vivo* 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 probably 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. Alternatively, 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 patients 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 DURAISINGH *et al.* (1997).

In the study presented here we typed recrudescent *P. falciparum* parasites in a drug efficacy trial carried out in Gabon (LELL *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 (1000–100000 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/sulfadoxine (Fansimef®). Full details of the study, its aims and rationale, are given by LELL *et al.* (in press). The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital.

A finger-prick sample of 2 drops of blood (approximately 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 3M, 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-

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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 patients who failed to clear their parasites during the first 6–7 d were defined as non-responders (RII/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 samples was avoided by treating the scalpels, forceps and glass plates used with 5M hydrochloric acid followed by 5M sodium hydroxide, which prevents carry-over of DNA from one sample to another.

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 tandemly repeated amino acids, which have been shown to vary in both number and sequence amongst different alleles (TANABE *et al.*, 1987; BORRE *et al.*, 1991; SMYTHE *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 1×PCR buffer (10 mM Tris–HCl, pH 8·8, 1·5 mM $MgCl_2$, 50 mM KCl, 0·1% Triton-X100®), 150 μM of dATP, dTTP, dGTP and dCTP, 100 nM 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 1. Primer sequences and cycling conditions for the amplification reactions

Locus and type	Primer names and sequences	Cycle conditions
MSP 1		
Outer	O1: CACATGAAAGTTATCAAGAACTTGTC	94°C/25s: 50°C/35s:
	O2: GTACGTCTAATTCATTTGCACG	68°C/150s: 30cycles
Nested	N1: GCAGTATTGACAGGTTATGG	94°C/25s: 50°C/35s:
	N2: GATTGAAAGGTATTTGAC	68°C/150s: 30 cycles
MSP 2		
Outer	S3: GAAGGTAATTAAAACATTGTC	94°C/25s: 42°C/60s:
	S2: GAGGGATGTTGCTGCTCCACAG	65°C/120s: 30 cycles
Nested	S1: GAGTATAAGGAGAAGTATG	94°C/25s: 50°C/60s:
	S4: CTAGAACCATGCATATGTCC	70°C/120s: 30 cycles
GLURP		
Outer	G4: ACATGCAAGTGTTGATCC	94°C/25s: 45°C/60s:
	G5: GATGGTTTGGGAGTAACG	68°C/120s: 30 cycles
Nested	G1: TGAATTCGAAGATGTTCACACTGAAC	94°C/60s: 55°C/120s:
	G3: TGTAGGTACCACGGGTTCTTGTGG	70°C/120s: 30 cycles

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 recrudescent (see Discussion).

Results

Drug efficacy

Full results of the drug trial have been presented elsewhere (LELL *et al.*, in press); 115 of the 231 children who completed the trial cleared their parasites within a week of treatment and 116 had suspected RI or RII/III parasites. Mefloquine at the low dose given was relatively ineffective at clearing parasites (13% cleared) compared 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 products 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 allele of one or more genes, and were therefore mixed infections 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 RII/RIII 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 exactly the same parasites in their respective O and P samples, and thus had type A recrudescences.

Type B recrudescences were seen in the remaining 30 children; some, but not all, of the O parasites reappeared 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 Discussion).

Considering the RI and RII/RIII resistance cases separately, 22 of the 41 RI group were type A and 19 were type B. Among the 36 RII/RIII cases, 25 were type A, of which 20 were mixed clone infections, and the remaining 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 remaining children, the O and P samples differed at 2 loci,

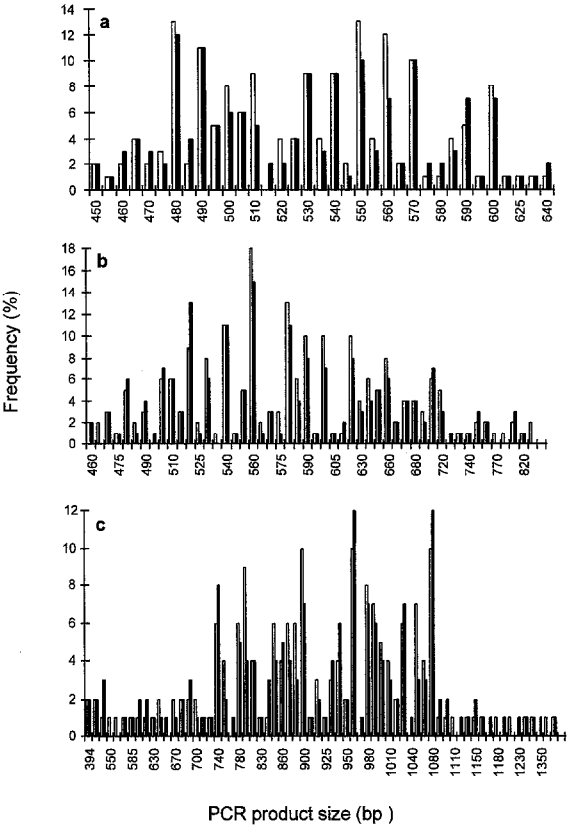

Fig. 1. Prevalence of different sized alleles of (a) *MSP-1* (b) *MSP-2* and (c) *GLURP* in the samples analysed. The white bars represent alleles found in the original samples and the black bars those found in the post-treatment samples.

Table 2. Summary of results of polymerase chain reaction typing of clinical recrudescences of *Plasmodium falciparum* infection

Drug treatment	Recrudescences Clinical definition	No.	Genotypes of parasites present in post-treatment sample None novel[a]	All novel	Novel and original
Mefloquine	RI	31	20	5	6
	RII/RIII	33	30	2	1
	Total	64	50	7	7
Pyrimethamine/ sulfadoxine	RI	17	9	3	5
	RII/RIII	6	2	1	3
	Total	23	11	4	8
Mefloquine/ pyrimethamine/ sulfadoxine	RI	17	12	3	2
	RII/RIII	4	4	0	0
	Total	21	16	3	2
All drug regimens	RI	65	41	11	13
	RII/RIII	43	36	3	4
	Total	108	77	14	17

[a]Genuine recrudescences.

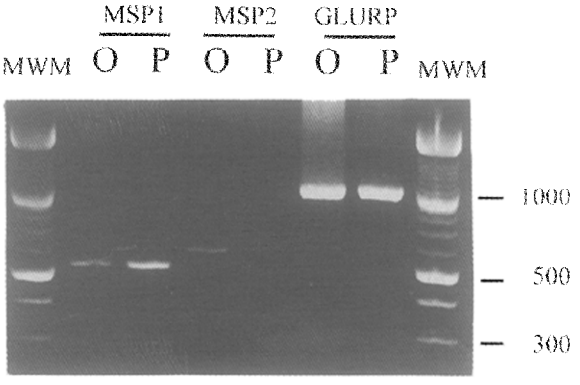

Fig. 2. Genuine RI recrudescence of resistant parasites, type A. A single identical PCR product was produced from each gene in the original (O) and post-treatment (P) samples of this child. The *MSP-2* product obtained from the post-treatment sample is faint. MWM, molecular size ['weight'] marker; numbers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

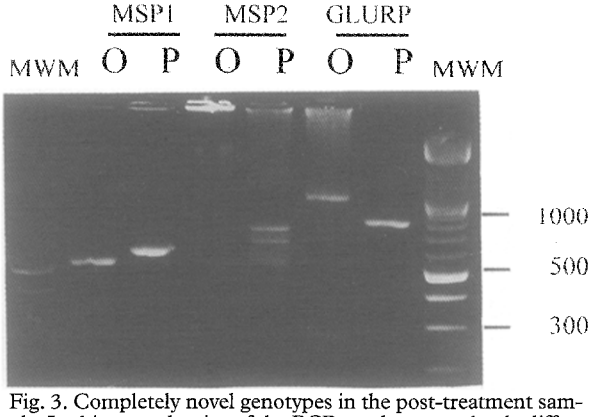

Fig. 3. Completely novel genotypes in the post-treatment sample. In this case, the size of the PCR product was clearly different for each locus in the original (O) and post-treatment (P) samples, although the *MSP-2* PCR product in the original sample was faint. MWM, molecular size ['weight'] marker; numbers indicate base pairs.

Table 3. Details of the recrudescences of *Plasmodium falciparum* malaria confirmed by polymerase chain reaction typing

Clinical definition	No. confirmed	Recrudescences Type A[a] Single clone	Multiple clones	Total	Type B[b]
RI	41	6	16	22	19
RII/RIII	36	5	20	25	11
Total	77	11	36	47	30

[a]Identical genotypes present in original and post-treatment samples.
[b]Some of the genotypes present in the original sample were no longer present in the post-treatment sample.

but shared a common allele at the third; in most cases this allele was common in the population as a whole. Eleven of the 14 children in this group had infections which had been classified as RI, and 3 as RII/RIII. It could be concluded that in these children the parasites found in the O samples were sensitive to the drug used. The possible origins of the parasites in the P sample are discussed below.

In addition to the 108 children discussed above, samples were collected from 4 children who developed a new parasitaemia after 28 d, although the parasites in these children had been defined as sensitive to the drug. The P sample from all 4 of these children contained parasites of novel genotype.

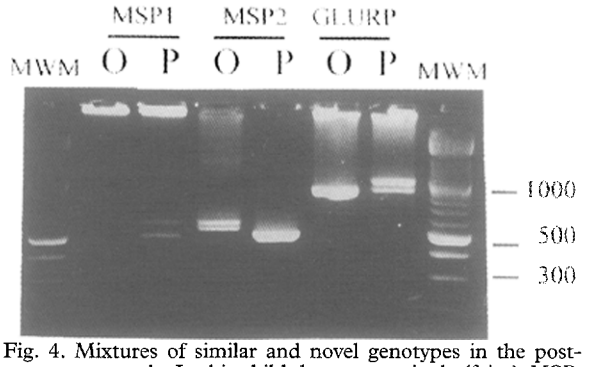

Fig. 4. Mixtures of similar and novel genotypes in the post-treatment sample. In this child there was a single (faint) *MSP-1* PCR product in the original (O) sample, which was also found in the post-treatment (P) sample, together with a smaller, new PCR product. Two *MSP-2* PCR products were obtained from the original sample, and a different product was obtained from the post-treatment sample. The *GLURP* PCR product from the original sample was also found in the post-treatment sample, and again a new, different PCR product was observed in the post-treatment sample. MWM, molecular size ['weight'] marker; numbers indicate base pairs.

(iii) Mixtures of similar and novel parasites in the recrudescences. With 17 children, there was a mixture of original and novel genotypes in the P samples (Table 2 and Fig. 4).

Discussion

This study demonstrated the usefulness of the PCR typing method to confirm the presence of parasites resistant to a given drug in a recrudescent infection. The technique allows a comparison of the genes of the parasites in the primary infection with those of the recrudescence. We have shown that in 77 children the same genotypes of parasites were found in the original and recrudescent infections. These parasites should be resistant to the drugs used at the doses given; 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-

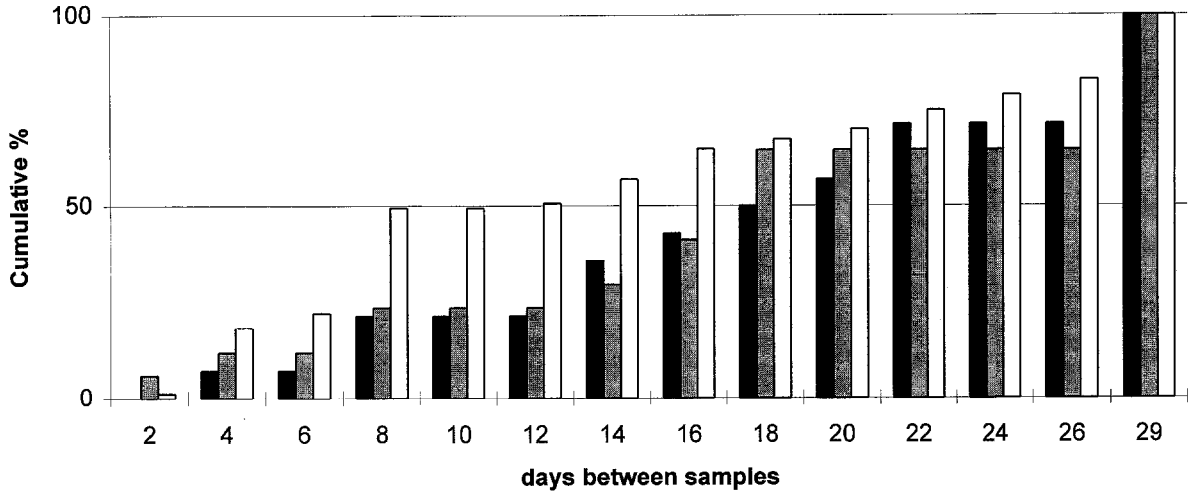

Fig. 5. A comparison of the cumulative percentages of children with genuine recrudescences (white bars), with novel genotypes in the post-treatment samples (black bars), or a combination of both (grey bars), plotted against the time between the two samples; 50% of all genuine recrudescences seen had occurred by day 8 after treatment.

curate only to within 10–15 bp, so it is likely that at least some of the alleles placed in different size classes were in fact the same size. However, the absolute size of the PCR product is unimportant since the comparisons between primary and recrudescent parasites were carried out by examining the PCR-amplified products of each run in adjacent tracks of the same gel.

In the 70% of the children with identical O and P parasites, we could conclude with confidence that these parasites were resistant to the drugs used at the doses administered, which in this trial were about one-twelfth of the normal recommended dose (LELL *et al.*, in press). In the children with type B recrudescences, in which only a subset of the original parasites reappeared, we could conclude that some of the original parasites were sensitive to the drug(s) given, and were thus cleared by it (or them). An alternative explanation could have been that some of the parasites in the O sample were not detected in the P sample because of sequestration or limitations of the detection technique.

Parasites present in recrudescences are more likely to be genuinely resistant if they reappear early rather than late after treatment, and this was found to be the case in our study (Fig. 5). Among the children who did not respond to the drug at all (RII and RIII responses), 84% had some or all parasites identical in both samples. However, in children who exhibited an RI response (recrudescence after at least 7 d), the parasites in the recrudescent sample matched some or all of those in the initial infection in only 61% of cases. The distribution of samples by time to recrudescence shown in Fig. 5 suggests that later recrudescences are more likely to contain novel parasites than earlier ones. Samples collected from 4 children whose parasitaemia reappeared a long time after 28 d contained novel genotypes only.

Novel post-treatment parasites could have originated from several sources. First, they could be due to new infections from mosquitoes. This is the likely explanation of the late recrudescences in which parasites disappeared from the blood for 2 weeks or more. These parasites may even have been resistant to the drug(s) given, if present at therapeutic levels at the time the P sample was taken. Seven children with novel parasites in the P sample presented with an RII/RIII response. These early recrudescences could also have been due to new infections, but the recrudescent forms must have been present as liver stages at the time the drugs were administered. The pre-erythrocytic cycle takes at least 5 d and the 3 drugs used in these trials are not believed to act against the pre-erythrocytic stages. In these cases, however, the drug levels would be expected to be high 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 2 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 were 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 P sample had a similar distribution to the novel parasite cases alone, with 50% of all cases arising by day 18 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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