MOLECULAR ANALYSIS OF PLASMODIUM FALCIPARUM RECRUDESCENT MALARIA INFECTIONS IN CHILDREN TREATED WITH CHLOROQUINE IN NIGERIA


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Abstract. Parasite genotyping by a polymerase chain reaction was used to distinguish recrudescent from newly acquired Plasmodium falciparum infections in 50 of 160 Nigerian children taking part in a chloroquine efficacy study in Ibadan, Nigeria. A finger prick blood sample was taken from each child before and after treatment to identify recrudescent parasites. By investigating allelic variation in three polymorphic antigen loci, merozoite surface protein-1 (MSP-1), MSP-2, and glutamate-rich protein (GLURP), we determined parasite diversity in the population and in the infected host. DNA from pretreatment and post-treatment samples from 47 of the 50 patients who failed therapy was successfully amplified by the PCR. The MSP-1, MSP-2, and GLURP genotypes in all samples showed extensive diversity, indicating polyclonal infections. The average number of clones per infection in pre-treatment sample was 2.5 with MSP-1, 4.9 with MSP-2, and 2 with GLURP. The extent of multiplicity decreased significantly (P = 0.016) in post-treatment samples. Multiplicity of infection and initial parasite density were not age dependent. Comparison of the variant alleles in pretreatment and post-treatment samples of each patient indicates that 26 of the 47 children had genuinely recrudescent disease. Conversely, post-treatment samples from five children showed completely new genotypes, indicating either a previously sequestered population of parasites or a newly acquired infection. Overall, this study has shown the diversity and complexity of P. falciparum population in Ibadan, Nigeria. The study has also shown the dynamics of P. falciparum infections in this population before and after chloroquine treatment in an area of high malaria transmission.

INTRODUCTION

Plasmodium falciparum resistance to antimalarial drugs remains a major problem for treatment of malaria infections in most endemic areas. In particular, resistance to chloroquine, the most widely used, safest and affordable antimalarial drug, has contributed to increased mortality and morbidity caused by P. falciparum infections. Resistance to antimalarial drugs can be assessed in vitro by the degree of parasite growth inhibition exerted by particular antimalarial drugs. In vitro detection of parasite drug resistance is useful for assessing innate resistance, as well as geographic or temporal patterns of resistance. However, levels of resistance reported by in vitro findings could overestimate the degree of resistance in vivo. The in vivo response of P. falciparum to antimalarial drugs is modulated by a number of factors. These include the pharmacokinetic properties of antimalarial drugs, innate and acquired immunity in the patient, as well as the complexity of infections in high transmission areas. Several of these factors may contribute to the range of permutations in the clinical expression of chloroquine resistance.

In vivo evaluation of the efficacy of antimalarial drugs is often based on clinical and parasitologic responses following treatment. Reoccurrence of parasitemia within 28 days of aminoquinoline treatment is often considered as recrudescent infections in most malaria-endemic areas. However, infections recurring between day 14 and 28 in areas of high transmission could be due to newly acquired infections. Therefore, in vivo clinical efficacy studies of antimalarial drugs should not only include clinical and parasitologic responses, but also use genetic polymorphism analysis to distinguish between recrudescent infections and reinfections.

Detection of genetic diversity in antigenic loci has enabled confirmation of recrudescent infections in paired samples taken from patients prior to and after chloroquine treatment. Restriction fragment length polymorphism and polymerase chain reaction (PCR) genotyping using three gene loci (merozoite surface protein-1 [MSP-1], MSP-2, and glutamate-rich protein [GLURP]) have also been used to distinguish treatment failures from newly acquired infections. Despite the obvious utility of PCR technology in detecting drug-resistant infections during clinical efficacy studies, it has not been widely used in malaria endemic areas.

In this report, polymorphic markers in P. falciparum isolates were used to examine genetic diversity and complexity of parasite populations in children with acute malaria infections in Ibadan, Nigeria. Population genetic approach was also used to assess the incidence of recrudescent infections in the children following treatment with standard dosages of chloroquine.

METHODS

Patient selection criteria and methods. The study was conducted at the Malaria Clinic of the Malaria Research Laboratories, College of Medicine, University of Ibadan (Ibadan, Nigeria). Malaria is hyperendemic in Ibadan, with transmission occurring year round. The study protocol was reviewed and approved by the Joint University of Ibadan/University College Hospital Ethics committee. Informed consent was obtained from parents or legal guardians of children enrolled into the study.

One hundred sixty patients 1–12 years old presenting with acute symptoms of P. falciparum malaria were enrolled in the
study. The criteria for inclusion in the study were fever or a history of fever in the 24–48 hours preceding presentation; pure *P. falciparum* parasitemia with more than 2,000 asexual forms per microliter of blood confirmed by microscopy; no history of antimalarial drug ingestion in the two weeks preceding presentation; negative urine test result for 4-aminoquinoline (DiGlazko); and absence of concomitant illness and consent from parent or guardians. A child was withdrawn from the study if a concomitant illness developed during the follow-up period, if the parents or guardian desired it or if there was failure to comply with the study protocol. Children with sickle cell anemia were excluded from the study.

A careful history was obtained from an accompanying parent or guardian and a physical examination was performed before enrollment of each patient. Body weight, height, and axillary temperature were recorded and Giemsa-stained thick and thin blood films were prepared from finger prick blood for quantification of parasitemia and species identification. Two drops of blood was also blotted onto 3MM filter paper (Whatman International, Ltd., Maidstone, United Kingdom) for extraction and analysis of parasite DNA.

Each child was treated with chloroquine base (25 mg/kg of body weight) over a three-day period. The first dose of chloroquine was administered in the hospital with close monitoring for a period of at least one hour. Subsequent doses were given to parents or the guardian to administer to the child. Clinical observations were recorded daily for the first eight days (days 0–7) and during follow-up on days 14, 21, and 28. During the follow-up, thick blood films were prepared from each patient to monitor parasitologic response to treatment. Blood samples were also collected on filter paper at the same time as thick blood films for parasite DNA analysis.

Giemsa-stained blood films obtained from the patients were examined by light microscopy under an oil-immersion objective. Parasitemia in thick films was estimated by counting the number of asexual forms of *P. falciparum* corresponding to 200 leukocytes. The parasite density was calculated by assuming a leukocyte count of 8,000/μL of blood. Classification of responses to chloroquine was based on World Health Organization criteria. All children who failed to respond to chloroquine were cured with sulfadoxine-pyrimethamine or mefloquine.

**Extraction of DNA from samples collected on filter paper.** Parasite genomic DNA was extracted from blood samples collected on filter paper using the methanol fixation and heat extraction method as described by Plowe and others.

**PCR determination of parasite population in patient isolates of *P. falciparum*.** Genotypes of the parasite population in each sample collected from patients with microscopically confirmed *P. falciparum* infections at enrollment, and during follow-up when patients showed symptoms of malaria were determined using PCR techniques. Analysis of genetic polymorphisms was performed on paired primary and post-treatment parasites samples obtained from patients who reported to the hospital with symptoms of malaria during follow-up visits 14, 21, or 28 days after treatment or whenever symptoms occurred. Paired primary and post-treatment parasites sites were analyzed using parasite loci that exhibit repeat numbers of polymorphisms to distinguish true treatment failures from new infections. Briefly, block 2 of MSP-1, block 3 of MSP-2, and region II of GLURP were amplified by two rounds of a PCR using primers and amplification conditions previously described by Snounou and others. Ten microliters of the PCR products were resolved by electrophoresis on a 2% agarose gel and sized against a 100-basepair (bp) molecular weight marker (New England Biolabs, Beverly, MA). The banding pattern of the post-treatment parasites was compared with matched primary parasites. Post-treatment and primary infection parasites showing identical bands were considered a true treatment failure, while non-identity indicated a new infection.

Point mutations in the *P. falciparum* chloroquine resistance transporter (*pfCRT*) gene and the *P. falciparum* multidrug resistance 1 (*pfMDR1*) gene were analyzed in pretreatment and post-treatment isolates obtained from a subset of patients whose infections failed to respond to treatment with standard regimen of chloroquine and had clinical symptoms of malaria. Analysis of parasites *pfCRT* and *pfMDR1* genes was performed as described by Hapip and others.

**Determination of *P. falciparum* clonal profile in recrudescent infections.** Each *P. falciparum* infection was characterized on the basis of the fragment size of the PCR products for each locus and determining the size of the alleles of MSP-1, MSP-2, and GLURP genes. Infections were defined as clonal if parasites in matched primary and post-treatment samples from the same patient showed more than one allele from one or more genes. If an isolate had one allele at each of the three loci, the clone number was taken to be one. Treatment failure or a resistant infection was defined as the occurrence of the same or a subset of the alleles at all three loci in the primary and post-treatment samples. A lack of allelic identity in all three loci in matched primary and post-treatment samples indicated a newly acquired infection. The complexity of infection was calculated for each typing reaction (MSP-1, MSP-2, and GLURP) independently as the average number of distinct fragments per PCR-positive sample. The complexity of infection was analyzed with respect to age and parasite density.

**Data analysis.** One-way analysis of variances was used to compare the geometric mean parasites density in patients from different age groups. Values are expressed as the mean ± SD. The Student’s *t*-test was used to compare mean number of alleles of *P. falciparum* MSP-1, MSP-2, and GLURP. The Pearson moment correlation test was performed to assess associations between multiplicity of infection, parasite density, and age. The paired *t*-test was used to compare complexity of infections in pretreatment and post-treatment isolates. *P* values < 0.05 was considered significant for all statistical analysis.

**RESULTS**

**Patients and treatment outcome.** One hundred sixty patients were enrolled and followed for 28 days after treatment with a standard regimen of chloroquine. The geometric mean parasite density in the patients was 13,343 parasites/μL of blood on the day of enrollment. Parasitemia in the patients ranged from 2,340 to 150,360 parasites/μL of blood. The mean ± SD parasite clearance time in patients cured of the infection with chloroquine was 3.22 ± 1.19 days (n = 110). Infections in 110 of the 160 patients (68.75%) cleared with a standard dosage of chloroquine, while 50 (31.25%) patients failed treatment with standard regimen of chloroquine. R1 resistance was observed in 33 (20.6%) of the 50 patients who
failed chloroquine treatment. Parasitemia in 11 (6.9%) of the 50 patients who failed chloroquine treatment was reduced significantly by day three, but did not clear and were classified as resistant at the RII level. Six (3.75%) of the 50 patients who failed treatment showed no significant reduction and even an increase in parasitemia on day three occurred after initiation of treatment and were classified as resistant at the RIII level.

**Reflection of population diversity of by polymorphisms of**

Parasites genotypes based on polymerase chain reaction (PCR) on MSP-1, MSP-2, and GLURP. Matched sample pairs collected before and after treatment from 47 patients who failed chloroquine treatment were successfully analyzed at all three loci (MSP-1, MSP-2, and GLURP). Genotyping of these samples showed that the allelic families of MSP-1 and MSP-2 were often represented in parasite DNA derived from a single patient, indicating a polyclonal infection. Specifically, three allelic families of MSP-1 were assessed. Alleles were classified according to the size of PCR fragments. The distribution of the allelic families of MSP-1, MSP-2, and GLURP in these parasites is shown on Table 1. Amplification products of the MSP-1 allelic family K1 was positive in 37 (79%) of 47 samples, and yielded four different fragments (90–800 bp). The MSP-1 allelic family MAD20 was detected in 15 (32%) of 47 samples and produced two major bands (200–250 bp). The MSP-1 RO33 allele was detected in 38% (18 of 47) of the samples, and produced amplification products of four different sizes with a predominant 190-bp fragment. Forty samples (85%) were positive for the MSP-2 IC1 and/or FC27 alleles and produced up to 12 fragment sizes (IC1 = 400–950 bp, FC27 = 200–800 bp). The region II of GLURP was present in 33 (70%) of 47 samples and produced five different fragments (225–900 bp) (Table 1). Comparison of the prevalence between the allelic families of MSP-1 and MSP-2 showed a difference indicating a very diverse *Plasmodium* population per infection. The number of different alleles per infection detected with MSP-2 was higher than that obtained for MSP-1 or GLURP or the combination of both markers. All patients showed multiple alleles of MSP-1, MSP-2, and GLURP (Table 1).

**Complexity of infections in pre and post-treatment samples.** Analysis of MSP-1, MSP-2, and GLURP showed a great extent of polymorphism within the 47-paired isolates analyzed. Twenty-nine (62%) of 47, 42 (89%) of 47, and 25 (53%) of 47 isolates were polymorphic for MSP-1, MSP-2, and GLURP, respectively, suggesting polyclonal infections with at least two parasite genotypes. Up to 12 different fragments were found in the allelic families of MSP-2. The estimated mean ± SD number of genetically distinct parasite population in pre-treatment isolates was 2.5 ± 1.91 with MSP-1, 4.9 ± 0.66 with MSP-2, and 2 ± 1.38 with GLURP. There was a higher mean multiplicity of infection with MSP-2 compared with MSP-1 and GLURP.

The influence of age on the complexity of *P. falciparum* infection showed that younger children less than two years of age had the highest mean ± SD number of different clones in their isolates: 3.2 ± 1.78 with MSP-1, 6.8 ± 1.9 with MSP-2, and 1.5 ± 1 with GLURP. Although the average number of clones identified by MSP-1 or MSP-2 per isolate was slightly lower in the older age groups, the difference was not significant (Table 2).

Four of six children with high-grade chloroquine resistance (RII/RIII) were young children less than five years old. Isolates from all these patients were shown to have a minimum of four *P. falciparum* genotypes per infection when the MSP-2 locus was used as an estimate. The relationship between geometric mean parasite density and multiplicity of infection was analyzed using the Pearson moment correlation test. No correlation was found between parasite density and the number of MSP-1 (P = 0.332, r = 0.151), MSP-2 (P = 0.057, r = -0.086), or GLURP (P = 0.243, r = -0.182) alleles.

To assess the effect of the drug administration on multiplicity of infections, the average number of clones per isolate was compared in enrollment and post-treatment isolates obtained from patients who returned to the clinic with symptoms of malaria infections. Parasites genotypes in the pretreatment and post-treatment isolates obtained from 25 of the 47 children (53%) were identical (Table 3). Three of the 25 children with parasites harboring identical genotypes in pre-treatment and post-treatment isolates had infections with a single clone identified by single alleles in the MSP-1, MSP-2, and GLURP loci. Analysis of genotypes in post-treatment isolates obtained from 22 of 47 children (47%) showed parasites similar to pre-treatment isolates, but also contained new parasite populations with different genotypes. Four of the 22 children had high clinical drug resistant infections (RII/RIII), and showed a mixture of pre-treatment and novel genotypes in post-treatment isolates (Table 3). In all cases, the PCR products were identical at all loci, but also contained new genotypes in post-treatment isolates obtained from 22 of 47 children (47%) showed parasites similar to pre-treatment isolates, but also contained new parasite populations with different genotypes. Four of the 22 children had high clinical drug resistant infections (RII/RIII), and showed a mixture of pre-treatment and novel genotypes in post-treatment isolates (Table 3). In all cases, the PCR products were identical at all loci, but also contained new genotypes in post-treatment isolates obtained from 22 of 47 children (47%) showed parasites similar to pre-treatment isolates, but also contained new parasite populations with different genotypes. Four of the 22 children had high clinical drug resistant infections (RII/RIII), and showed a mixture of pre-treatment and novel genotypes in post-treatment isolates (Table 3). In all cases, the PCR products were identical at all loci, but also contained new genotypes in post-treatment isolates obtained from 22 of 47 children (47%) showed parasites similar to pre-treatment isolates, but also contained new parasite populations with different genotypes.

### Table 1

<table>
<thead>
<tr>
<th>Loci</th>
<th>No. positive by PCR (%)</th>
<th>No. of distinct alleles</th>
<th>Sizes of alleles (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>37 (79)</td>
<td>4</td>
<td>90–290</td>
</tr>
<tr>
<td>MAD20</td>
<td>15 (32)</td>
<td>2</td>
<td>200–250</td>
</tr>
<tr>
<td>RO33</td>
<td>18 (38)</td>
<td>2</td>
<td>190 (predominant)</td>
</tr>
<tr>
<td>MSP-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC1/3D7</td>
<td>40 (85.1)</td>
<td>4</td>
<td>400–950</td>
</tr>
<tr>
<td>FC27</td>
<td>40 (85.1)</td>
<td>9</td>
<td>200–800</td>
</tr>
<tr>
<td>GLURP</td>
<td>33 (70)</td>
<td>5</td>
<td>225–900</td>
</tr>
</tbody>
</table>

* bp = basepairs; MSP = merozoite surface protein; GLURP = glutamate-rich protein.

### Table 2

<table>
<thead>
<tr>
<th>Age groups, years (n)</th>
<th>Parasite density*</th>
<th>Number of clones identified by <em>P. falciparum</em> locus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSP-1</td>
<td>MSP-2</td>
</tr>
<tr>
<td>&lt;2 (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5–5 (10)</td>
<td>22,297</td>
<td>2.5 ± 1.28, 4.5 ± 1.98, 4.2 ± 1.96</td>
</tr>
<tr>
<td>5.1–12 (31)</td>
<td>10,998</td>
<td>2.0 ± 1.35, 2.0 ± 1.25, 1.72 ± 1.51</td>
</tr>
<tr>
<td>P values</td>
<td>0.270</td>
<td>0.351</td>
</tr>
</tbody>
</table>

* n = number of patients. For definitions of other abbreviations, see Table 1.
† Geometric mean parasite density/microliter of blood.
§ Student t-test.
Polymerase chain reaction genotyping of isolates obtained from recrudescent *Plasmodium falciparum* infections after the children were treated with standard regimen of chloroquine*.

<table>
<thead>
<tr>
<th>Treatment outcome</th>
<th>Number of patients</th>
<th>Pre- and post-treatment parasites genotypes</th>
<th>Pre-treatment plus new</th>
<th>New</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI</td>
<td>30</td>
<td>Identical 12 15 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RII/RIII</td>
<td>17</td>
<td>Identical 13 4 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>Identical 25 17 5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Identical = identical alleles at all three loci, either for single or multiple clone infections present in pre-treatment and post-treatment parasites; Pre-treatment plus new = post-treatment parasites showed alleles matching those in pre-treatment parasite genotypes as well as parasites with no similar allelic identity (new) at all three loci of MSP-1, MSP-2, and GLURP; New = patients with post-treatment isolates showing no identical alleles of all three loci (MSP-1, MSP-1, and GLURP) with the pre-treatment isolates; RI = initial clearance of parasitemia followed by recurrences of parasites between days 7 and 21 post-treatment; RII = reduction of the parasitemia to 25% of its original density three days after commencement of treatment; RIII = no reduction in parasitemia or reduction of the parasitemia to 75% of its original density by day three after commencement of treatment.

Comparison of the multiplicity of infections between paired pretreatment and post-treatment isolates obtained from patients who failed treatment with chloroquine showed a significant reduction in the number of *MSP-2* alleles in patients who failed treatment compared with post-treatment isolates when infections recrudesced (*P* = 0.016). However, the extent of multiplicity of infections was not significantly different between the number of *MSP-1* (*P* = 0.14) and GLURP (*P* = 0.453) alleles in enrollment and post-treatment isolates (Table 5).

**DISCUSSION**

Decades after the official formulation of the definition of clinical drug resistance in malaria by the World Health Organization, there was no adequate tool to assess whether treatment failure is due to the same or new parasite populations especially in areas of intense malaria transmission such as Ibadan, Nigeria. Recently, malaria researchers have overcome this limitation of the in vivo test by taking advantage of current advances in *Plasmodium* biology and PCR technology to determine parasite genotypes in pretreatment and post-treatment samples of patients clinically classified as treatment failures. This approach is based on the most frequently used markers for genotyping. In this study, we demonstrated the usefulness of the PCR typing method to confirm the presence of parasites resistant to chloroquine in infections failing to respond to standard regimen of chloroquine in an area of intense malaria transmission in Nigeria. This study showed similarities in the parasite genotypes from primary and post-treatment infections in 89% (42 of 47) of children who failed chloroquine treatment. In 59% (25 of 42) of these children, identical parasite genotypes were present before and after treatment, suggesting that all these parasites were resistant to the curative dose of chloroquine. Point mutations in *P. falciparum* *pfcrt* and *pfmdr1* genes associated with chloroquine resistance was also confirmed in 23 isolates and have been previously reported. In the children in whom only a subset of the original parasites present in the pre-treatment isolates were detected in the post-treatment samples, it could be concluded that proportion of the original parasites populations sensitive to chloroquine were cleared, while those remaining represented resistant populations. In sum, 53% of all patients who failed to respond to treatment with chloroquine had identical parasites in the pretreatment and post-treatment isolates. In the remaining patients, recurrence of disease could be attributed to a new infection. An alternative

**Table 3**

<table>
<thead>
<tr>
<th>Parasites loci</th>
<th>Mean ± SD number of alleles in isolates</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP-1</td>
<td>2.51 ± 1.91</td>
<td>0.141</td>
</tr>
<tr>
<td>MSP-2</td>
<td>4.95 ± 0.66</td>
<td>0.016</td>
</tr>
<tr>
<td>GLURP</td>
<td>2.00 ± 1.38</td>
<td>0.453</td>
</tr>
</tbody>
</table>

* For definitions of abbreviations, see Table 1.
† Pair-wise t-test.
§ Significant reduction in the number of *MSP-2* alleles in pre-treatment and post-treatment isolates of *P. falciparum* obtained from patients who failed chloroquine.

**Table 4**

<table>
<thead>
<tr>
<th>Pfcr alleles</th>
<th>Day of recrudescence</th>
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<tbody>
<tr>
<td>046</td>
<td>T76</td>
</tr>
<tr>
<td>050</td>
<td>T76</td>
</tr>
<tr>
<td>055</td>
<td>K76</td>
</tr>
<tr>
<td>066</td>
<td>T76</td>
</tr>
<tr>
<td>090</td>
<td>K76 and T76</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pfmdr1 alleles</th>
<th>Day of recrudescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y86†</td>
<td>N86 and Y86</td>
</tr>
<tr>
<td>N86 and Y86</td>
<td>N86</td>
</tr>
<tr>
<td>N86‡</td>
<td>N86 and Y86</td>
</tr>
<tr>
<td>Y86</td>
<td>Y86</td>
</tr>
<tr>
<td>N86</td>
<td>N86</td>
</tr>
</tbody>
</table>

* *Pfcrt* = *P. falciparum* chloroquine resistant transporter gene; *pfmdr1* = *P. falciparum* multidrug resistance gene 1; D0 = isolates obtained before treatment; Day of recrudescence = isolates obtained after failure to respond to treatment with a standard regimen of chloroquine.
† Mutant allele of *pfcrt*.
‡ Wild-type allele of *pfcrt*.
§ Wild-type allele of *pfmdr1*.
explanation is that some of the parasites detected in post-treatment isolates were not detected in the pre-treatment samples because they may have arisen from blood-stage parasites emerging from the liver after the pre-treatment DNA isolate was collected. The limitation of the detection of some parasite population by PCR could also explain this result. This may be exacerbated by the fact that infections in these patients were polyclonal. Thus, during PCR amplification of these heterogeneous samples, abundant and rare DNA templates compete for the oligonucleotide primers, resulting in only abundant sequences being detected.

Previous studies have suggested that parasites in recrudescing infections are more likely to be genuinely resistant if they reappear early rather than late after treatment. Indeed, in children who showed a delayed recrudescence (RI), the parasites in the recrudescent isolates matched exactly only 60% of the pre-treatment isolates. This shows that delayed recrudescences are more likely to contain novel parasites than earlier ones. Isolates from five children with delayed recrudescence in this study had new parasite genotypes only. The presence of new parasite genotypes in post-treatment isolates could be due to new infections from mosquitoes, taking in consideration the fact that children involved in the study live in an area of intense malaria transmission and had continued exposure during the follow-up period. 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 different from the original one. New infections emerging from the liver are generally considered a plausible source of late treatment failures during trials of malaria chemotherapy, and these can now be identified and confirmed by current genetic typing methods. It is therefore possible that new parasites introduced by new mosquitoes bites may have been responsible for the increase in parasitemia and onset of symptom in the children who failed treatment. Alternatively, these new genotypes may represent parasite populations that were undetectable by the PCR or at a different stage of development that is not present in the peripheral blood at the time the pretreatment isolates were obtained. Analysis of pfcrt and pfmdr1 point mutations in pretreatment and post-treatment isolates obtained from these five patients showed a high prevalence of mutant alleles of both pfcrt and pfmdr1. Based on the higher prevalence of the mutant pfcrt T76 and pfmdr1 Y86 alleles that have been associated with chloroquine resistance in previous studies, it could be argued that the infections were due to parasites resistant to chloroquine, despite the appearance of new genotypes in post-treatment isolates as shown by MSP-2. However, difficulties in establishing a correlation between mutations in pfcrt or pfmdr1 associated with chloroquine resistance and clinical treatment outcome in P. falciparum in Ibadan, has been reported previously. The high prevalence of the pfcrt T76 allele observed in parasites from the five patients with new genotypes in the post-treatment isolates is consistent with earlier reports from other malaria endemic countries of Africa. The role of parasites population dynamics on the detection of either the mutant or wild-type alleles of both pfcrt and pfmdr1 and if this represents the predominant parasites populations in isolates obtained from the patients is not clear. The high prevalence of multiplicity of clones observed in patient isolates from Ibadan previously reported and further demonstrated in this study needs to be considered because it may constitute a confounding factor in interpretation and association of mutations in pfcrt or pfmdr1 genes with parasites response to chloroquine in vivo. Four children that demonstrated high-grade chloroquine resistance (RIII) had novel parasite genotypes in the post-treatment samples. It is possible that the recrudescent forms of P. falciparum in these children were derived from parasites that were sequestered when the primary samples were taken, and thus not present in pre-treatment samples. Parasites of different genotypes have been shown to appear in the blood on successive days of infection, presumably due to two or more cohorts of parasites undergoing alternate 24 hours period of sequestration. Alternatively, both populations of parasites may have been present but in different proportion and the minority clone was not amplified successfully by the PCR technique. It is also possible that amplification reactions at all three loci were less sensitive at detecting minority genotypes in multiple infections.

The population structure of P. falciparum from infections in the children analyzed with the polymorphic markers MSP-1, MSP-2, and GLURP showed extensive diversity in parasite populations in Ibadan. The MSP-1, MSP-2, and GLURP markers showed 10, 12, and 5 allelic families, respectively. This diversity of the P. falciparum population in Ibadan, Nigeria is reflected in the complexity of parasite populations in patient samples. A catalog of genetically distinct parasite populations co-infecting Nigerian children, based on PCR amplification of the GLURP, MSP-1, and MSP-2 markers, showed that multiplicity of infection was very common. However, MSP-2 was shown to be the best marker of multiplicity in both pretreatment and post-treatment isolates because it showed more clones than other markers. Multiclonality of infections has been shown to be a common feature in most malaria-endemic areas. Epidemiologic data from various study sites in Africa suggest that the multiplicity of P. falciparum infection may be directly related to the intensity of transmission.

This multiplicity of infections may also have important implications for the epidemiology of drug-resistant P. falciparum malaria and the outcome of treatment in patients. The initial presence of several parasite populations with different drug response profiles would result in elimination of drug-sensitive populations and selection of resistant parasites. This was confirmed in the present study by the decrease of multiplicity between isolates obtained on the day-of-enrollment (an average of 4.9 distinct populations with MSP-2 as the marker) and isolates obtained after treatment failure (3.08 distinct populations). Similar analysis of samples collected from children with malaria treated with chloroquine or sulfadoxine-pyrimethamine in Uganda showed a decrease in the average number of distinct P. falciparum populations from 4.2 before treatment to 3.4 on day 7 after treatment.

Analysis of the influence of age on the complexity of P. falciparum infection showed that children less than five years of age had the highest average multiplicity of infection when MSP-2 was used as the marker. This average was especially high, varying between 4 and 6.8 in all cases of high-grade chloroquine resistant infections (RII/RIII). However, there was no significant difference between multiplicity of infection with regard to age or parasitemia, confirming observations by other investigators in Uganda, Tanzania, and Kenya.
of infection correlates negatively with age but positively with parasitemia.

The definition of clinical resistance as described by the World Health Organization is sufficiently precise for operational research. However, in areas of intense malaria transmission, infections due to resistant parasites or newly inoculated infections occurring between day 14 and day 28 cannot be easily distinguished by clinical response to chloroquine and microscopic analysis. Therefore, it is necessary to use more sensitive techniques including biologic markers to define parasite genotypes in pretreatment and post-treatment samples. Findings in this preliminary study confirms the value of using polymorphic markers to distinguish reinfections from true drug-resistant infections following treatment with chloroquine.

Overall, this study has shown the diversity and complexity of P. falciparum populations in Ibadan, Nigeria. The study has also shown the dynamics of P. falciparum infections in this population before and after treatment with chloroquine, the first-line treatment of malaria in the country. The MSP-2 marker was shown to be the most reliable genetic marker to evaluate diversity and complexity of P. falciparum infections in Ibadan, Nigeria. The approach used in this study could be used in addition to other molecular methods as part of the surveillance program for monitoring drug-resistant malaria infections.

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REFERENCES

19. Sutherland CJ, Allouche A, Curtis J, Drakeley CJ, Ord R, Dura-
raisingh M, Greenwood BM, Pinder M, Warhurst D, Targett
GA, 2002. Gambian children successfully treated with chloro-
quine can harbor and transmit Plasmodium falciparum gameto-
cytes carrying resistance genes. Am J Trop Med Hyg 67:
578–588.
20. Adagu IS, Warhurst DC, Ogala WN, Abdu-Aguye I, Audu LI,
of Plasmodium falciparum from Zaria, Nigeria. Trans R Soc
Pgh1 modulates sensitivity and resistance to multiple anti-
22. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM,
Ferdig MT, Ursos LM, Sidhu AB, Naude B, Dietsh KW, Su
XZ, Wootton JC, Roff PE, Wellems TE, 2000. Mutations in
the P. falciparum digestive vacuole transmembrane protein
PfCRT and evidence for their role in chloroquine resistance.
2001. Analysis of pfcr point mutations and chloroquine sus-
ceptibility isolates of Plasmodium falciparum. Mol Biochem
Parasitol 114: 95–102.
24. Djimde A, Doumbok OK, Cortese JF, Kayentao K, Doumbou
S, Diouille T, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems
TE, Ploew CV, Coulthay D, 2001. A molecular marker for
chloroquine-resistant falciparum malaria. N Engl J Med 344:
247–263.
Plasmodium falciparum in Kenya: high prevalence of drug-
resistance-associated polymorphisms in hospital admissions
with severe malaria in an endemic area. Ann Trop Med Para-
sitol 95: 661–669.
phisms in the Plasmodium falciparum pfcr and pfmdr-1 genes
and clinical response to chloroquine in Kampala, Uganda. J
Infect Dis 183: 1417–1420.
27. Thomas SM, Ndir O, Dient T, Mboup S, Wypij D, Maguire KJ,
Wirth DF, 2002. In vitro chloroquine susceptibility and PCR
analysis of pfcr and pfmdr1 polymorphisms in Plasmodium
falciparum isolates from Senegal. Am J Trop Med Hyg 66:
474–480.
H, Fandeur T, Roger B, Mercereau-Puijalon O, Druilhe P,
1996. Rapid turnover of Plasmodium falciparum populations
in asymptomatic individuals living in a high transmission area.
namics of Plasmodium falciparum subpopulations in asympto-
56: 538–547.
30. Nzouli F, Contamin H, Rogier C, Bonnefoy S, Trape JF, Mer-
creau-Puijalon O, 1995. Age-dependent carriage of multiple
Plasmodium falciparum merozoite surface antigen-2 alleles in
asymptomatic malaria infections. Am J Trop Med Hyg 52:
81–88.
31. Smith T, Beck HP, Kitua A, Mwankusye S, Felger I, Fraser-Hurt
N, Irion A, Alonso P, Teuscher T, Tanner M, 1999. Age de-
pendence of the multiplicity of Plasmodium falciparum infec-
tions and of other malariological indices in an area of high
32. Magesa SM, Mdira KY, Farnert A, Simonsen PE, Bygbjerg IC,
infection structure of recrudescent from new infec-
tions by PCR-RFLP analysis in a comparative trial of CGP 56
parasites from Kenya is not affected by antifolate drug selection.
CH, 2002. Genetic diversity of Plasmodium falciparum para-
sites from Kenya is not affected by antifolate drug selection.
34. Arnot D, 1998. Unstable malaria in Sudan: the influence of the
dry season. Clone multiplicity of Plasmodium falciparum infec-
tions in individuals exposed to variable levels of disease
Nrothdurft HD, von Sonnenburg F, Loscher T, 1999. Popula-
tion structure of recrudescent Plasmodium falciparum isolates
36. Irion A, Felger I, Abdulla S, Smith T, Mull R, Tanner M, Hatz C,
Beck HP, 1998. Distinction of recrudescent infections from new
infections by PCR-RFLP analysis in a comparative trial of CGP 56
697 and chloroquine in Tanzanian children. Trop Med Int
Health 3: 490–497.