Plasmodium falciparum Clonal Population Dynamics during Malaria Treatment

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We have developed a new fragment-analysis method to enumerate the clones and to quantify their proportions within Plasmodium falciparum isolates. We prospectively enrolled 20 adult patients with uncomplicated malaria who were returning to France from various sub-Saharan countries, from January 2000 through July 2001. The analysis of clonal populations was performed on blood samples obtained at 10 times: 1 before treatment with oral quinine and 9 during the first 96 h of the treatment. The resistance genotypes pfcr and dhfr were determined for chloroquine and antifolinics. Multiple P. falciparum genotypes were detected in 19 (95%) of 20 patients: 2, 3, 4, and 5 genotypes were found in 4, 9, 4, and 2 patients, respectively. Disappearance and reappearance of some clones within a few hours was observed. Individual clones represented 0.4%–99.4% of total parasitemia. Surprisingly, in 10 of 15 subjects tested, resistance genotypes varied according to the time of blood collection. These findings may have important implications with regard to the interpretations of resistance studies.

Malaria-associated morbidity and mortality, which occur mainly in young children, have increased in sub-Saharan Africa because of the recent spread of Plasmodium falciparum drug resistance [1]. Drug-resistant malaria is now a major health care concern in countries where malaria is endemic. Reliable data on P. falciparum drug susceptibility are thus required for effective prevention and treatment policies. Observed therapeutic efficacy still forms the basis for policy decisions on antimalarial drugs [2, 3]. In vitro tests provide additional data but are difficult to standardize. The limitations of available in vivo and in vitro methods for monitoring resistance have promoted the development of molecular assays, which are available at reasonable costs [4, 5]. The interpretation of classic and molecular efficacy testing is hindered by parasite polyclonality [6], which results in individual patients often harboring a mixture of genetically distinct parasites [7, 8]. In asymptomatic children living in areas where malaria is endemic, parasite genotypes have even been found to vary from one day to the next [9]. These mixtures change over time because of various factors, including host-parasite interactions, treatments, and/or reinfections [10, 11]. A minority resistant clone may go undetected by standard molecular methods early after infection, becoming apparent only when selected by treatment. Alternatively, such a resistant clone may be eliminated by the immune response [4]. Thus, results obtained on clinical isolates are sometimes confusing, and apparent discrepancies could prevent the effective use of molecular data to guide malaria treatment policies [5]. Classic genotyping methods can enumerate the different clones of an isolate within the limitation of their sensitivity but are unable to quantify the proportions of clones between each clone [12–14]. Since minority clones may not be clinically relevant, such a quantification appears to be essential. We have developed a new method for monitoring clonal population dynamics during the early phase of antimalarial treatment. This method may have major applications in the
areas of physiopathology, antimalarial treatments, and vaccines.

**PATIENTS, MATERIALS, AND METHODS**

**Enrollment of patients.** Twenty patients were prospectively recruited from January 2000 through July 2001 from the Department of Infectious and Tropical Diseases of a teaching hospital in Paris, France. Patients were eligible for the study if they met the following criteria: (1) *P. falciparum* infection with no gravity sign [15]; (2) residence in France and of European or sub-Saharan African origin; (3) infection during a short stay (<3 months), with the exception of patient 20, in sub-Saharan Africa; and (4) ≥15 years old. *P. falciparum* malaria attack was defined by fever, clinical signs of malaria, and asexual *P. falciparum* blood stages on thin and thick blood smears. Complete information was collected from each enrolled patient by use of standardized questionnaires. Patients signed consent forms after receiving simple, standard information about the study. The study was approved by the ethics committee of Bichat-Claude Bernard Hospital.

**Treatment and follow-up.** Each patient was seen by one of the authors of the present study, who collected standardized medical and biological data before and after treatment. Treatment consisted of oral quinine administered at 8 mg base/kg 3 times/day for 7 days. Clinical follow-up included a full clinical examination every day and temperature measurement twice a day. Parasitemia and clonal populations were studied in venous blood samples obtained at time of diagnosis and at 2, 4, 12, 24, 36, 48, 60, 72, and 96 h after initiation of treatment.

**Clonal analysis.** The method described here, derived from that developed by Denamur et al. for the detection and quantification of a 9-bp insert in Wilms’ tumor suppressor gene [16], was based on the polymorphism of the gene encoding merozoite surface protein 2, which is an abundant surface component on the erythrocyte-invading stage of *P. falciparum* [17]. A highly polymorphic region showing considerable variations in size and sequence has been described in block 2 of the msp-2 gene [18]. Polymerase chain reaction (PCR) amplification with a fluorescent primer, followed by capillary gel electrophoresis, was used to discriminate alleles of different sizes. A preliminary study of artificial DNA mixtures of *P. falciparum* clones 3D7 and HB3 was conducted to validate the method.

**Preparation of artificial mixtures.** DNA was extracted, as described elsewhere [19], from 100-μL aliquots of fresh cultures of *P. falciparum* clones 3D7 and HB3. Samples were diluted in Tris-EDTA buffer (10 mmol/L Tris per 1 mmol/L EDTA) to obtain a final DNA concentration of 50 ng/μL and then were mixed at the following ratios: 99:1, 98:2, 90:10, 75:25, 50:50, 25:75, 10:90, 2:98, and 1:99. The method was also tested on artificial mixtures of the clones; cultures of the clones 3D7 and HB3 with a parasitemia of 1% were mixed at the following 3D7:HB3 ratios: 999:1, 99:1, 98:2, 95:5, 50:50, 5:95, 2:98, 1:99, and 1:999. The same ratios were tested on artificial mixtures of clones 3D7 and HB3 with a parasitemia of 0.1%. The fragment-analysis method was also used to study clinical isolates. The detection limit was determined by testing serial 10-fold dilutions of a *P. falciparum* culture (1000, 100, 10, 1, and 0.1 parasites/μL).

**Parasite DNA preparation.** DNA was extracted from artificial mixtures and clinical isolates, as described elsewhere [19].

**Fluorescent PCR.** Block 2 of the msp-2 domain was amplified as follows: the reaction mixture (final volume, 50 μL) consisted of 100–200 ng of genomic DNA, 15 pmol of each primer—fluorescein-5′-GAAGGTAACTAAAAACATTGTC-3′ (sense, 5′ labeled with fluorescein) (Genset SA Europe) and 5′-GACACCTCGTC-GTTGATGGAG-3′ (anti-sense) [20]—buffer (15 mmol/L Tris-HCl [pH 8.0], 50 mmol/L KCl, and 6 mmol/L MgCl₂), 200 μmol/L dNTP, and 1.25 U of AmpliTaq Gold DNA (Perkin Elmer Applied Biosystems). The thermal cycling conditions were as follows: 7 min at 95°C, followed by 40 cycles for 30 s at 94°C, 30 s at 42°C, and 30 s at 72°C. All PCR assays were performed in triplicate.

**Fragment-analysis method.** Amplification products were then processed in an ABI Prism 3100 Genetic Analyzer (Perkin Elmer Applied Biosystems) to enumerate and quantify fluorescent fragments. Mixtures containing 18.5 μL of deionized formamide, 0.5 μL of an internal lane standard GENESCAN-2500 carboxy-tetramethylrhodamine (TAMRA; Perkin Elmer Applied Biosystems), and 1 μL of PCR fluorescein-labeled PCR products were heated for 2 min at 95°C and then were placed in ice. After denaturation, the products were distributed in 96-well Costar microplates and run on an ABI Prism 3100 Genetic Analyzer. The results were analyzed using GeneScan software (version 3.7; Applied Biosystems). The internal lane standard was labeled with TAMRA, and the 5′-primer used for the PCR was labeled with fluorescein to distinguish PCR products from the standards. The internal lane standard consisted of 28 bands ranging in size from 37 to 14,079 bp. Each clone was characterized by the size and the area under the curve of the peak corresponding to its msp-2 PCR products. As reported elsewhere, the area under the curve was proportional to the quantity of PCR products for each clone [16]. The ratio of PCR products was calculated as the ratio of the area under the curve for each clone. Relative parasitemia was calculated as follows: Parasitemia \( C_o \) (number of parasites per microliter) = total parasitemia \( x \times 100 \), where \( x \) is the ratio of the clone on the basis of the area under the curve.

**Study of resistance markers pfldhfr and pfcr.** We also studied the relationship between clonal population dynamics during treatment and the determination of resistance-marker genotypes. In previous studies, resistance markers pfldhfr108 and pfcrf76 correlated with *P. falciparum* resistance to antifolinics.
and to chloroquine, respectively [21–24]. These genotypes were
determined in samples from 15 of the 20 patients by use of
molecular beacons, as described elsewhere [14, 25]. Because of
some problems with preservation of DNA, the determination
of resistance-marker genotype was not performed for 5 patients.

RESULTS

Artificial mixtures of HB3 and 3D7 clones. Ratios calculated
by the fragment-analysis method were very close to the theo-
retical ratios of artificial mixtures of clones 3D7 and HB3 (table
1). Electrophoregrams of artificial mixtures of clones HB3 and
3D7 clearly showed peaks corresponding to PCR products of
different sizes (figure 1). HB3 PCR products were 646 bp long,
and 3D7 PCR products were 680 bp long, as controlled by
DNA sequencing (data not shown). Reproducible results were
obtained in 3 runs. In the 3 trials, minor alleles of mixtures
equal to 2% were always detected (table 1), whereas those equal
to 1% showed ambiguous detection, and those equal to 0.1% were
never detected. The sensitivity of the method was as high
for artificial mixtures containing 0.1% parasitemia (5000 tro-
phozoites/µL) as it was for samples containing 1% parasitemia.
In clinical isolates, minority clones representing 0.4% of total
parasitemia were sometimes detected (patient 10).

Overall sensitivity. The method consistently detected as
few as 1 parasite/µL in 3 independent serial dilutions of P. falci-
parum HB3 cultures (1000, 100, 10, 1, and 0.1 parasite/
µL). The detection limit for clinical isolates was thus taken to
be 1 parasite/µL.

Clinical isolates. All the patients had stayed exclusively in
urban areas or had made short stays in rural areas. After 28
days of treatment, all patients presented with no fever and tested
negative for parasitemia by thin and thick smears. On day 4
(96 h), all but 2 of the patients tested negative for parasitemia.
Patients 6 and 10 tested negative by thick smears, whereas the
fragment-analysis method showed detectable parasitemia. No
gametocytes were detected at any time in any patient. The
fragment-analysis method was applied to isolates obtained from
all the patients, from time of diagnosis to 96 h after initiation
of treatment (table 2). Figure 2 shows the clonal population
dynamics of P. falciparum in 3 representative patients.

Between 1 and 5 clones were detected in each patient. Multiple
P. falciparum genotypes were detected in 19 (95%) of 20 patients:
2, 3, 4, and 5 genotypes were found in 4, 9, 4, and 2 patients,
respectively; 1 genotype was found in 1 patient. Twenty-seven
different msp-2 genotypes were detected. All 19 patients in
whom >1 genotype was detected had a dominant clone (i.e.,
representing >50% of the total parasitemia) detected at some
point during follow-up. We observed as many as 3 different
dominant clones in a given patient (patients 4, 6, 7, 8, 9, 10,
14, 16, 19, and 20). Some clones fluctuated during treatment,
whereas others were highly stable. Some clones appeared to
disappear and reappear within periods as short as 2 h.

Fourteen patients harbored clones with different behaviors:
some clones showed major fluctuations, whereas others showed
gradual decline. In patients 7, 13, 14, 17, and 18, all the clones
showed almost no fluctuation except a slow decline toward the
final clearance of parasitemia (table 2). Individual clones rep-
resented 0.4%–99.4% of total parasitemia.

pfcrt and pfldhfr genotypes. The pfldhfr and pfcrt resistance
markers were studied in 15 of the 20 patients. Ten patients had
resistance profiles that fluctuated according to the sampling time
(mutated genotype, wild-type genotype, or mixtures of mutated
and wild-type genotypes) (table 3). For example, patient 12
showed the wild-type genotype pfldhfr (S108) in the initial isolate
and a mixture of wild-type and mutated genotypes in the sample
collected 4 h after initiation of treatment (table 3).

DISCUSSION

Mosquitoes are known to inject genetically diverse P. falci-
parum inocula, thereby increasing the chances of parasite survival in
genetically diverse hosts [6]. This diversity is thought to present
a major challenge to immune protection, drug efficacy, and
malaria control. In areas of high transmission, subjects who
survive repeated malaria infections during childhood rarely pre-
sent severe clinical effects, due to the development of a semi-
immunity, which has a marked strain-specific component [9–
Detecting polyclonal infections is crucial for molecular epidemiology and for studies dealing with drug resistance and pathogenicity. A significant finding of the present study is that, in most patients with polyclonal infections, some clones showed major fluctuations that were apparently unrelated to drug exposure, whereas other clones declined gradually during treatment. The phenomenon of clones disappearing and then reappearing during short periods of time has been described elsewhere [9]. Such variations during the follow-up of treated patients were shown here for the first time. The differential dynamics of clones within an isolate could be related to the various abilities of clone populations to be rapidly sequestered on capillaries of deep organs. Differential adhesion capacities in genetically diverse hosts, associated with differential expression of var genes, have already been shown in *P. falciparum* [26]. It has been postulated that these variations may play a role in parasite virulence. There is also speculation that the virulence of multiple infections by genetically diverse clones is higher than that by monoclonal infections, because more tissue ligands could be recognized by parasites [27]. Another striking feature of the present study was the successive disappearance of clones from the peripheral circulation, as was clearly observed to have occurred in patient 13 (figure 2) (7 similar examples not shown). This apparently shifted efficacy of quinine, with regard to various subpopulations, could originate from differences in parasite hepatic development and release.

Figure 1. Electrophoregrams showing peaks of the fluorescein-labeled polymerase chain reaction (PCR) products in artificial mixtures of *Plasmodium falciparum* clones 3D7 and HB3. Carboxy-tetramethylrhodamine–labeled DNA fragments of the Genescan 2500 internal lane standard (37–14,079 bp) are shown in each electrophoregram. Peaks near 50 bp correspond to primers. HB3:3D7 ratios, 98:2 (A), 2:98 (B), 90:10 (C), and 10:90 (D). Clone HB3, 646 bp; clone 3D7, 680 bp.
Table 2. Characteristics of the patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Previous malaria</th>
<th>Country of infection</th>
<th>Length of stay, days</th>
<th>Time to diagnosis after first symptoms, days</th>
<th>Prophylaxis</th>
<th>No. of clones</th>
<th>No. of fluctuating clones b</th>
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<tr>
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<td>2</td>
<td>CQ</td>
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<td>3</td>
</tr>
<tr>
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<td>1</td>
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<tr>
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<td>4</td>
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<td>Yes</td>
<td>Benin</td>
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<td>PG + CQ</td>
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<td>2</td>
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<td>3</td>
<td>None</td>
<td>4</td>
<td>3</td>
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</table>

NOTE. CQ, chloroquine; MQ, mefloquine; PG, proguanil.

a Obtained by use of standardized questionnaire.
b Fluctuating clones, clones showing quantitative variations between serial blood samples.

of successive waves of preerythrocytic stages in the bloodstream [28]. The number of clones per patient in the present study is consistent with those found in previous reports [29]. Since the patients made only short stays in regions where malaria is en- demic, they probably received a very limited number of bites from infected mosquitoes. It is likely that the real number of clones harbored by the patients was higher [6]. A major finding of the present study was the detection of a dominant clone during the follow-up of each patient with a polyclonal infection. This majority clone may have a particular responsibility in the appearance and the intensity of symptoms. An estimation of the relative parasite burden of each subpopulation could be deduced from the ratios of clones, determined by the fragment-analysis method, and the parasitemia, easily determined by microscopy. However, the total parasite burden cannot be assessed in peripheral blood.

The major finding with regard to the fragment-analysis method was its ability to detect and quantify minority clones within an isolate with a high sensitivity, compared with that of previously available molecular methods. In the present study, this approach consistently detected minor alleles (up to 2% in artificial mixtures) in samples containing >5000 trophozoites/μL. In clinical isolates containing ≥2 clones, the method was able to detect minority clones with a higher sensitivity. This could be due to a weaker competition on PCR reagents of the dominant clone of multiclonal isolates, compared with bclonal artificial mixtures where 1 clone represented nearly all the para-sitemia [30]. In comparison, drug resistance alleles <10% are not usually detected by PCR restriction fragment–length polymorphism, standard mutation–specific PCR, or molecular beacons [14, 31]. Comparable sensitivity has been obtained in studies using 32P-labeled primers or probes, but these techniques are time-consuming, have all the drawbacks of radioisotopic methods, and are unsuitable for epidemiological studies [31, 32]. Our fragment-analysis method was much simpler and showed a higher accuracy in estimating the proportion of alleles in artificial mixtures of the 2 cloned lines, HB3 and 3D7, compared with an in situ PCR method reported elsewhere by Ranford-Cartwright and Walliker [33].

Our demonstration of rapid dynamic fluctuations of circulating clones within a patient, by use of the fragment-analysis method, relies on the polymorphism of the msp-2 gene, which has been established in previous studies [17, 18, 20]. Use of the msp-2 gene proved to be more powerful than use of the other genes often used for genotyping, such as msp-1 or glutathione-rich protein gene [9, 34]. Since >80 alleles have been
Table 3. Fluctuations of the merozoite surface protein–2, pfcrt, and pfdhfr108 alleles in peripheral blood of patients 10, 12, and 13 during treatment with quinine.

<table>
<thead>
<tr>
<th>Patient, sampling time</th>
<th>Parcites/μL</th>
<th>Clone(s) ( % ± SD )</th>
<th>DHFR108 allele(s)</th>
<th>CRT76 allele(s)</th>
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<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hd</td>
<td>36,498</td>
<td>a (100)</td>
<td>M</td>
<td>M</td>
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<td>H2</td>
<td>42,504</td>
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<td>H4</td>
<td>46,200</td>
<td>a (100)</td>
<td>M</td>
<td>M</td>
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<td>H12</td>
<td>62,370</td>
<td>a (100)</td>
<td>M</td>
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<tr>
<td>H24</td>
<td>64,650</td>
<td>a (95.8 ± 1.05), b (4.2 ± 1.2)</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>H36</td>
<td>35,773</td>
<td>a (43.5 ± 1.12), b (2.2 ± 0.4), c (52.2 ± 1.11), d (2.1 ± 0.15)</td>
<td>M + w</td>
<td>M + w</td>
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<tr>
<td>H48</td>
<td>296</td>
<td>a (0.9 ± 0.1), b (0.9 ± 0.1), c (39.2 ± 0.9), d (0.4 ± 0.08), e (58.5 ± 1.16)</td>
<td>M + w</td>
<td>M + w</td>
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<tr>
<td>H60</td>
<td>212</td>
<td>c (100)</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>H72</td>
<td>88</td>
<td>c (47.8 ± 2.6), e (52.2 ± 2.6)</td>
<td>M + w</td>
<td>M + w</td>
</tr>
<tr>
<td>H96</td>
<td>21</td>
<td>c (18.3 ± 1.45), e (81.6 ± 1.4)</td>
<td>W</td>
<td>W</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hd</td>
<td>1389</td>
<td>a (100)</td>
<td>W</td>
<td>W</td>
</tr>
<tr>
<td>H2</td>
<td>4167</td>
<td>a (100)</td>
<td>W</td>
<td>W</td>
</tr>
<tr>
<td>H4</td>
<td>8334</td>
<td>a (95.4 ± 1.2), b (4.6 ± 1.02)</td>
<td>M + w</td>
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<tr>
<td>H12</td>
<td>16,205</td>
<td>a (95.3 ± 1.5), b (4.7 ± 1.2)</td>
<td>M + w</td>
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<td>H24</td>
<td>926</td>
<td>a (95 ± 1.02), b (5 ± 1.06)</td>
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<td>a (96.2 ± 0.51), b (3.8 ± 0.85)</td>
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<td>H48</td>
<td>46</td>
<td>a (100)</td>
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<td>59,640</td>
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<td>M</td>
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<td>H12</td>
<td>42,245</td>
<td>b (68.7 ± 1.8), c (31.3 ± 1.8)</td>
<td>M</td>
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<td>H24</td>
<td>11,928</td>
<td>b (100)</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>H36</td>
<td>497</td>
<td>b (100)</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>H48</td>
<td>0</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>H60</td>
<td>0</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>H72</td>
<td>0</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>H96</td>
<td>0</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

**NOTE.** Sampling times: Hd (time of diagnosis), H2, H4, H12, H24, H36, H48, H60, H72, and H96 (numerals indicate hours after initation of treatment). m, mutant; m + w, mutant and wild type; w, wild type.
Figure 2. *Plasmodium falciparum* clonal population dynamics and resistance genotypes in patients 10, 12, and 13. m, *pfcrt* or *pfdhfr* wild-type genotypes; w, *pfcrt* or *pfdhfr* mutated genotypes; Sampling times: Hd (time of diagnosis), H2, H4, H12, H24, H36, H48, H60, H72, and H96 (numerals indicate hours after initiation of treatment).

reported for *msp-2* [12], the analysis of this gene by use of a highly specific and sensitive method could be proposed to separate therapeutic failures from new infections, in most cases. Today, genotyping studies are usually performed by classic amplification of polymorphic alleles on pretreatment and failure-day samples: paired samples with different band profiles on agarose or polyacrylamide gel are classified as reinfections, whereas a recrudescence is concluded to have occurred if the band profiles are similar [35]. Artifacts due to nonspecific PCR products and the multiplicity of bands of various intensities are sometimes misleading [12]. Because of the technical shortcomings of the usual molecular genotyping methods [30, 36], rates of antimalarial-treatment failure in areas of high transmission may have been overestimated. Fragment sizes separated by as few as 5 bp were easily distinguished in the present study. Previous studies have shown that the fragment-analysis method can discriminate between human gene PCR products differing by <10 bp [16]. Because the polymorphism in block 2 of the
msp-2 gene results from variations in repeated units [17], different alleles are unlikely to have identical molecular weights. However, this could be a limitation of the method. To increase the resolutive power of the assay, it may be possible to extend the methodology to the msp-1 gene. More than 10 alleles can be coamplified with different fluorescent primers and run under the same capillary gel electrophoresis. Such systems appeared to be sufficiently powerful, robust, and reliable to be able to be used as investigative tools in the forensic sciences [37].

Genotyping methods also have intrinsic limitations. First, in the field, a patient initially infected with a *P. falciparum* genotype may be reinfectated with the same genotype. This could occur more likely in areas where a given genotype is over-represented [38]. Second, PCR-based methods may not detect a subpatent clone submitted to variations of its circulating fraction because of the effects of synchronization or sequestration [27].

An increasing number of current reports underline the role of multiple subpopulations in *P. falciparum* infections [9, 11, 12, 29]. A major weakness in the interpretation of drug-efficacy studies may originate from the presence of a drug-resistant clone at a low level. Its detection may be hindered by the presence of other susceptible clones and, as has clearly been demonstrated in the present study, by the variations of its relative parasitemia over time. Drug-resistance genotypes sometimes varied markedly in the present study according to the time of sampling (e.g., patients 10, 12, and 13 in figure 2). These findings may have important implications with regard to the validity of interpretations of previous studies dealing with the association between specific genotypes and in vivo outcomes and should be taken into account for the design of new studies. Another difficulty identified as important is the interpretation of in vivo drug-efficacy studies in places where reinfections are frequent. According to a World Health Organization consultation about monitoring antimalarial drug resistance, which took place in Geneva, Switzerland, in December 2001, there is a need to standardize the definition of “recrudescence” and “reinfection” on the basis of molecular analysis. Considering the possible complexity of the parasite population in a single individual, it was proposed to investigate the usefulness of analyzing blood samples collected at day 0 and at day 1. This would require a modification of the follow-up schedule.

The real value of this promising method, compared with that of existing methods, should now be evaluated in a clinical setting. The method appeared to be simple to use, rapid, and practical. Our ability to differentiate recrudescence from reinfection in vivo studies may be more precise than that of other methods. Also, the association between a specific drug-resistant genotype and in vivo outcomes may be more correctly determined. In addition to its role in epidemiological studies, this method could be of interest in the exploration of the dynamics of clones during antimalarial treatment or challenge with vaccine [39].

**References**