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PLASMODIUM FALCIPARUM GENOTYPING BY MICROSATELLITES AS A METHOD TO DISTINGUISH BETWEEN RECRUDESCENT AND NEW INFECTIONS

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Abstract. *In vivo* tests for susceptibility to antimalarial drugs require molecular methods to distinguish recrudescence from new infection. The most commonly used DNA markers (merozoite surface proteins [MSPs]) are under immune selective pressure, which might lead to misclassification. We evaluated immunologically neutral microsatellite markers in blood samples collected during a drug efficacy trial in Rwanda. Fifty percent of the infections classified as recrudescent by MSP were classified as new by microsatellite markers. Reciprocally, 23.3% of infections classified as recrudescent by microsatellite markers were identified as new by MSP. In drug efficacy studies, microsatellite markers should complement MSP genotyping to distinguish a recrudescence from a new infection.

INTRODUCTION

Anti-malarial drug efficacy testing relies on *in vivo* tests which monitor clinical and parasitologic failure after treatment. However, when the follow up is beyond 14 days, parasites have to be genotyped before treatment and at the moment of therapeutic failure to distinguish recrudescence from new infection.[1] The observation of identical genotypes in paired samples is interpreted as recrudescence, possibly due to drug resistance. Targeted genes must be polymorphic and the most commonly used for *Plasmodium falciparum* are those coding for merozoite surface proteins (MSP-1 and MSP-2) and glutamate-rich protein (GLURP).[2] However, results of clinical trials were found to vary considerably depending on the choice of each of the respective genes for analysis and the interpretation of genotyping results.[3] Furthermore, these genes encode antigens under immune selective pressure.[4,5] This might bias the interpretation of dissimilar parasites in paired blood samples.

Alternative markers that are easy to interpret and immunologically neutral are therefore required. Microsatellite markers, which are simple sequence repeats, are abundant in the genome of *P. falciparum*, occurring every 2–3 kb in both coding and non-coding sequences.[6] These markers demonstrated a high degree of allelic variation and were used in studies of parasite population structure.[7] However, they have never been applied in the context of a drug efficacy study for distinguishing recrudescence from new infection. We selected one polymorphic microsatellite of *P. falciparum,* a trinucleotide repeat (TAA)n located in the housekeeping gene polymerase alpha (Poly-α)[7] and simplified its analysis through the use of capillary electrophoresis. The method was then applied to paired blood samples collected during a drug efficacy study and results were compared with those obtained by MSP typing.

MATERIALS AND METHODS

The details of the drug efficacy study have been described elsewhere.[8] Briefly, children between 6 and 59 months of age attending three health centers (Kicukiro, urban/peri-urban; Rukara and Mashesha, both rural) in Rwanda with a confirmed diagnosis of uncomplicated malaria were randomized to receive either amodiaquine (AQ) or amodiaquine plus artesunate (AQ plus AS). Informed consent was obtained from parents or legal guardians of the children, and the study was reviewed and approved by the review board of the Prince Leopold Institute of Tropical Medicine (Antwerp, Belgium) and the Rwandan Ministry of Health. Patients were followed-up for 28 days after treatment. A blood slide for parasitemia was collected at days 0 (before treatment), 3, 7, 14, 21, and 28. Filter paper blood blots were collected for molecular analysis on days 0, 14, 21, and 28 or on any day of recurrent parasitemia after day 14. Children who experienced treatment failure were treated with a full course of quinine. If the child had a second episode of parasitemia after day 14, blood samples on filter paper from the first and second episodes were used to type parasite strains. The DNA was extracted from samples on filter papers as described elsewhere.[9] For all samples analyzed, results from MSP typing were available from a previous study.[8] For MSP, a recrudescent infection was defined as one that matched in size at least one allele for both MSP-1 and MSP-2 genes between the first and second samples.

The amplification of the microsatellite (Polyα) was done using the heminested polymerase chain reaction (PCR) conditions reported elsewhere.[7] Primers PolyαF, PolyαR, and Polyα-3(IR) were used,[7] but none of them was end-labeled. Analysis of the PCR products was performed by capillary electrophoresis (Agilent 2100 Bioanalyser System; Agilent Technologies, Karlsruhe, Germany) in a microchip device (DNA 500, Labchip; Caliper Technologies, Mountain View, CA).

For PCR optimization and validation of the electrophoretic resolution, well-characterized sequence variants were necessary. Therefore, Polyα PCR products of different size were identified in a polyclonal field sample and cloned using the TOPO cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA was extracted using a plasmid extraction kit (Wizard Plus minipreps; Promega, Madison, WI). Six clones showing size variation were sequenced using the dideoxy chain termination technique (GenBank accession numbers AJ851233, cl10; AJ851234, cl2; AJ851235, cl4; AJ851236, cl5; AJ851237, cl8; and AJ851238, cl9) to determine the precise size and sequence of the amplified Polyα DNA fragment.

Statistical analysis was done using SPSS version 10.0.05

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(SPSS, Inc., Chicago, IL). Children were considered not to be parasitological or clinical failures if their parasitemia between days 14 and 28 was classified as a new rather than recrudescent infection. Outcomes were defined according to the new World Health Organization classification[10]: clinical failure was defined as the sum of early treatment failure (ETF) and late clinical failure (LCF). Total treatment failure (TTF) includes parasitologic and clinical failure (ETF plus LCF plus late parasitologic failure). Comparison of outcomes between the two treatment groups was done using chi-square analysis.

RESULTS

The PCR was conducted in two steps, giving a secondary PCR product ranging in size from 118 to 217 basepairs. Comparison of the sizes of the sequenced clones with the one evaluated by the bioanalyser demonstrated the high-resolution power of this method. In addition, size variants differing by three nucleotides (one microsatellite repeat) also showed a three-nucleotide difference by bioanalyser (clones 10 and 4: 159 and 162 basepairs by sequencing and 163 and 166 basepairs by capillary electrophoresis). The stability of microsatellites was demonstrated by observing identical patterns with DNA from a *P. falciparum* strain cultivated for 11.7 months. The microsatellite detection threshold was compared with that of MSP markers by application on serial dilutions of DNA extracted from culture parasites, and found to be similar to MSP-1 and 10 times lower than MSP-2.

For the interpretation of the microsatellite typing, two criteria were defined. First, to avoid counting an allele twice (as a result of the presence of primary PCR products), only fragments within a range of 49 basepairs above the smaller amplicon of a given sample were considered recrudescent (49 nucleotides is the difference between the position of the two reverse primers used in the hemi-nested reaction). Second, fragments with a size difference of less than three basepairs between paired samples were considered identical (MICRO-3 criterion). In most cases, several alleles were observed in each sample; if at least one allele was shared in the paired samples, this was interpreted as a recrudescence. A less stringent threshold was also evaluated: fragments with a size difference of less than four basepairs were considered identical (MICRO-4 criterion).

These criteria were applied to compare the degree of genetic diversity in the three sites. A higher number of microsatellite allelic types was found in Rukara (23 versus 19 in Kicukiro and 21 in Mashesha). For each marker and each setting, the ratio between the average number of allelic types observed in an individual at day 0 and the total number of alleles observed in all patients was computed. This provided for each marker an estimate of the probability of being re-infected with a specific allele. For Mashesha, the following results were observed: microsatellite markers (1.9/21 = 0.09), MSP-1 (3.1/14 = 0.22), and MSP-2 (4.1/20 = 0.20).

Of 308 children included in the drug efficacy study, 75 were parasitemic at either days 21 or 28 of follow-up.[8] Sixty-nine paired samples could be analyzed by both the MSP (-1 and -2) and microsatellite markers. The latter (using both MICRO-3 and MICRO-4 criteria) showed more new infections (39/69 = 56.5% and 33/69 = 47.8%, respectively) than MSP (23/69 = 33.3%). Of 46 samples classified as recrudescence by MSP, 23 (50%) were classified as new infections by microsatellite markers. However, among the 30 samples classified as recrudescent by microsatellites markers, 7 (23.3%) were classified as new infections by MSP. No ETF was observed. Late clinical failure was higher in the AQ group than in the AQ plus AS group, and the difference became highly significant when corrected by the MSP PCR results.[8] Similar results were obtained using MICRO-3 or MICRO-4. Total treatment failure was lower when corrected by the microsatellite markers than by MSP (Figure 1). However, the relative risk for failure between AQ and AQ plus AS did not change substantially.

In more than half (65.1%) of the 23 patients classified as recrudescence by MSP and as new infections by the microsatellite markers, the total number of shared MSP alleles was between two and four (Table 1). The majority (74%) of the other 23 patients classified as recrudescence by both MSP and microsatellite markers had more than five MSP alleles. Therefore, a more stringent MSP-based definition of recrudescence (at least one common allele in both MSP-1 and MSP-2 and at least five identical alleles in each pair instead of two) would decrease the discrepancy between MSP and microsatellite markers.

DISCUSSION

We have demonstrated the use of a single microsatellite marker (Poly-α) in distinguishing *P. falciparum* recrudescence from re-infection. The microsatellite technique has several advantages. The microsatellite PCR primers used are specific for *P. falciparum* and do not co-amplify other malaria species such as *P. vivax* and *P. malariae*.[7] Our method of analysis is simpler than the one reported for population structure analysis where fluorescent end-labeled primers were used and the PCR product was analyzed with a sequencer.[7] We used conventional, unlabeled primers and capillary electrophoresis for resolution of PCR products. Microsatellite genotyping is also faster to conduct than MSP typing (two versus six hours, respectively).

Analysis of field sample showed that based on microsatellite markers, 50% of the samples considered recrudescent by MSP markers were new infections. The new alleles detected by the microsatellites markers are unlikely to be due to the mutations during the testing period because the markers were stable over a one-year period. This should be checked for any new microsatellite marker used for typing, especially for

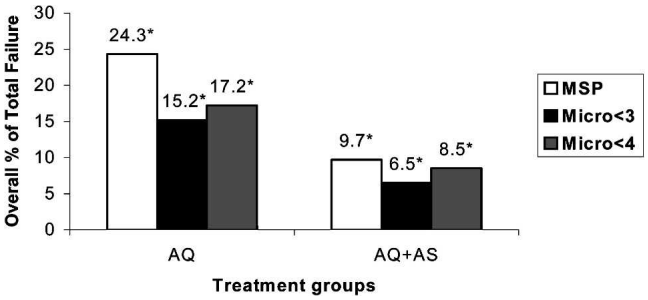

FIGURE 1. Total treatment failure defined by a merozoite surface protein polymerase chain reaction or microsatellite (Micro) by treatment. AQ = amodiaquine; AS = artesunate. *χ^2, $P < 0.05$ (comparison between AQ and AQ + AS treatment groups).

TABLE 1

Distribution (%) of merozoite surface protein 1 (MSP-1) and MSP-2 allelic types in subjects classified as recrudescence by both MSP and microsatellite markers, and recrudescence by MSP but new infection by microsatellite markers

	Total number of MSP alleles shared in paired samples			
	2*	3†	4†	≥5†
Recrudescence for MSP only	3 (13%)	7 (30.4%)	5 (21.7%)	8 (34.9%)
Recrudescence for MSP and microsatellite	1 (4.3%)	1 (4.3%)	4 (17.4%)	17 (74%)

* One shared allele for MSP-1 and 1 shared allele for MSP-2, i.e., the criterion applied in this study to define MSP recrudescence.

† One or more shared MSP-1 allele(s) and 1 or more shared MSP-2 allele(s).

those located in more unstable subtelomeric regions. The difference between microsatellite and MSP results is unlikely to be due to differences in mutation rates between the two markers. Indeed, microsatellites are considered to be rapidly evolving,[11] but MSP genes can vary with a similar mechanism, i.e., amplification/deletion of short simple repeat DNA sequences.[12] The difference could be explained by allelic diversity and the number of alleles per sample, which may influence the probability of re-infection with the same defined allele. When each marker was considered individually, this probability was smallest for the microsatellite markers (0.09 versus 0.22 and 0.20 for MSP-1 and MSP-2, respectively). However, when MSP-1 and MSP-2 were considered together, the probability, assuming complete independence between MSP-1 and MSP-2 (this happens only in a panmictic population), would be 0.044. Therefore, combining the two MSP markers should have a lower risk of misclassification. However, this is higher than expected when the two shared allele criterion (one for MSP-1 and one for MSP-2) are used. Indeed, 65.1% of discrepant cases shared 2–4 MSP alleles, while 74% of the congruent cases showed five or more shared MSP alleles (Table 1). Finally, selective pressure could also be involved in the discrepancies between MSP and microsatellite markers. The latter are considered to be selectively neutral[7] and prone to genetic drift, although possible selection by drug pressure was reported for microsatellite markers located around the drug target genes.[13,14] In contrast, MSP are antigens and their variation is subject to diversifying natural selection because it contributes to the ability of the parasite to evade the immune response of the human host.[12] Nevertheless, a fraction of infections classified as recrudescence by microsatellite were typed as new by MSP markers. Microsatellite homoplasy is considered to be rare,[12] but this phenomenon might explain the latter results. Indeed, the Poly-α region is organized into several blocks of microsatellites interrupted by few non-microsatellites bases.[15] We found in our Poly-α sequences that expansion of one block could be accompanied by contraction of another. If both variations would imply an identical number of repeats, the total length of the PCR-amplified product would remain the same. This problem could be overcome by using different microsatellites[7,15] or by sequencing.

Under our experimental conditions, microsatellite markers showed more new infections than MSP. This suggests that the latter, when used in support to *in vivo* efficacy tests of one given drug, would overestimate failures. However, this apparently does not affect the comparison between different treatments. Indeed, in present case, even if the TTF varied for each of the two regimens, both MSP and microsatellite markers would show a lower TTF with AQ plus AS than with AQ.

Recrudescence is conceptually more complicated to demonstrate because until a difference between two paired samples has not been shown, a new infection cannot be excluded with certainty. A possible solution is to use both microsatellite and MSP markers together so that the probability of misclassifying new infection would be lower. In this case, since microsatellites are generally more resolvable than MSP, samples could first be analyzed by microsatellite markers; only those classified as recrudescence would be further tested by MSP-1 and MSP-2 markers for confirmation. Our results also suggest the need for re-evaluating the criteria used for interpreting MSP data by taking into consideration the diversity and frequency of allelic types in the parasite population under study. The use of more stringent criteria (more than two shared alleles) would be advisable.

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