POLYMERASE CHAIN REACTION AND MOLECULAR GENOTYPING TO MONITOR PARASITOLOGICAL RESPONSE TO ANTI-MALARIAL CHEMOTHERAPY IN THE PERUVIAN AMAZON

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Abstract. Over the past decade, anti-malarial drug resistance has rapidly become a major public health problem in the Peruvian Amazon. This study compared polymerase chain reaction (PCR) to light microscopy for diagnosing and monitoring the parasitological response of malaria patients to anti-malarial chemotherapy in the Peruvian Amazon region of Iquitos. Typing of *P. falciparum* using MSPI, MSP2, and glutaminen-rich protein distinguished among infecting parasites. Most (73%) *P. falciparum* patients were parasitologically resistant to sulfadoxine-pyrimethamine (RI = 10, RII = 1). Sensitivity of microscopy was lower than PCR (69% for *P. vivax* and 78% for *P. falciparum*), but parasite clearance times were comparable between microscopy and PCR. PCR sensitively and specifically detected mixed infections and low-level parasitemia indicative of drug resistance, making this approach of practical use for the control of malaria at the public health level. Genotyping malaria parasites will be useful to distinguish drug failure from new infections in clinical trials of anti-malarial drugs in the Peruvian Amazon region.

INTRODUCTION

Malaria is an important and continuing public health problem in Peru. In Loreto, the northeastern department of Peru in the Amazon basin, malaria transmission occurs year long, seasonally ranging from 4,000 to 10,000 monthly cases. The ratio of *Plasmodium vivax* to *P. falciparum* malaria ranges from 2:1 to 4:1, depending on the season and sporadic epidemics of *P. falciparum*. Concomitant with the reported first appearance of *Anopheles darlingi* in the Peruvian Amazon in the early 1990s, the emergence of chloroquine- and pyrimethamine/sulfadoxine-resistant *P. falciparum* has led to major and expensive changes in the public health management of *P. falciparum* cases.†

Examination of Giemsa-stained blood smears by light microscopy remains the gold standard for diagnosis and parasitologic follow-up of malaria patients in most of the developing world, yet it is labor intensive and time consuming even for the most expert microscopists. Accurate species identification may be difficult, especially in patients with low levels of parasitemia and in those with mixed infections. Therefore, there is a need for applying better diagnostic and epidemiologic tools for malaria, particularly in light of the global emergence of multi-drug-resistant *P. falciparum*. Alternative diagnostic techniques such as the OPTIMAL,§ the polymerase chain reaction (PCR) assay, and the genotypic diversity was sufficient to be able to distinguish drug failure from reinfection in a region where *P. falciparum* diversity remains unknown.

PCR has important potential advantages for being highly sensitive and specific in detecting malaria parasitemia, being able to detect mixed infections missed by microscopy, and being amenable to analyzing large numbers of samples. Low parasitemias are readily detected by PCR, suggesting the use of this test in monitoring therapeutic responses to anti-malarial chemotherapy and early detection of drug resistance.

This study therefore had two aims: 1) to compare microscopy and PCR for the diagnosis of malaria and detection of parasitemia in a prospective cohort of *P. vivax* and *P. falciparum* infected patients after the administration of anti-malarial drugs in a rural village health post in the Peruvian Amazon and 2) to determine whether *P. falciparum* genotypic diversity was sufficient to be able to distinguish drug failure from reinfection in a region where *P. falciparum* diversity remains unknown.

MATERIALS AND METHODS

Subjects. The study population enrolled 70 consecutive patients diagnosed with *P. vivax* or *P. falciparum* malaria by light microscopy presenting to the health post of Varillal, an area endemic for malaria in the Loreto region of the Peruvian Amazon between February and March 1999. Entry criteria were absence of clinically identifiable febrile conditions other than malaria, absence of signs of severe and complicated malaria, and willingness to adhere to follow-up protocol. This study was approved by the Committee on Human Research of the Johns Hopkins Bloomberg School of Public Health and the Ethical Committees of A.B. Prisma, Lima, Peru, and the Universidad Peruana Cayetano Heredia, Lima, Peru. All treatment of malaria was provided by licensed Peruvian physicians in accordance with guidelines provided by the Peruvian Ministry of Health.

Field methods. A laboratory technician working at the health post of Varillal identified *Plasmodium* parasites by examining 100 fields of Giemsa-stained thick blood slides in patients with history of fever, following the laboratory guidelines issued by the Peruvian Malaria Control Program. After
written consent, infected patients were enrolled in the study. Finger-prick blood samples were taken for parasite counts and spotted in triplicate onto filter paper (Scheicher & Schuell, S&S 903, Keene, NH), which were dried and used for subsequent PCR analysis.

After being started on anti-malarials, patients were asked to return to the health post for 4 consecutive days and at days 7, 15, 21, and 30 after treatment. At each visit, information regarding symptoms of malaria and medication side effects was recorded. Two sets of thick and薄 blood smears were collected for parasite density counts, and triplicate finger-prick blood samples were taken for PCR analysis.

Patients were provided with the following anti-malarial regimens, in accordance with the current Peruvian Malaria Control Program (total doses in parentheses): chloroquine (1.5 g base) plus primaquine (210 mg) for P. vivax malaria and sulfadoxine-pyrimethamine (1.5 g to 75 mg) plus primaquine (45 mg) for P. falciparum malaria. Treatment failures were treated with quinine sulfate (600 mg three times a day for 7 days) and tetracycline (500 mg twice a day for 7 days) for adults and with quinine sulfate (30 mg/kg for 7 days) and clindamycin (20 mg/kg for 5 days) for children. Response to anti-malarial treatment was reported according to criteria of the World Health Organization.14

**Specimen processing.** For quality control, all Giemsa-stained blood smears were sent to the Parasitology Department of the Universidad Peruana Cayetano Heredia, Lima, Peru, where an experienced microscope blinded to clinical presentation. Parasite density expressed as parasitized red blood cells (PRBCs)/μL was calculated by determining the number of parasites per 200 white blood cells in a thick film smear and multiplying by 8,000. Blood smears were declared negative if no parasites were seen in 100 oil immersion fields (×1,000) on a thick film. In the case of discrepancy in the first two readings, the slide was re-read by a third microscopist. For a slide to be considered positive, a minimum of two concordant readings by two different microscopists was necessary.

After air drying, filter paper samples were wrapped individually in plastic bags and transported to the Infectious Diseases Laboratory in the Department of Pathology of the Universidad Peruana Cayetano Heredia. Finger-prick blood samples (two spots of ~50 μL each) were cut out and placed in a microcentrifuge tube with 200 μL of a 5% (wt/vol) Chelex-100 (Bio-Rad, Richmond, CA) solution, according to a published method.15 Malaria DNA was extracted from the filter paper sample by vortexing (30 s) and boiling for 10 min. The samples were centrifuged at 12,000g for 1.5 min, and the supernatant was removed and centrifuged again. Soluble extract (3.0 μL) was amplified by PCR reactions (total volume, 25 μL) using oligonucleotides based on the ssrRNA genes.16,17 Two genus-specific primers rPLU 5 (5′-CTTGTGTTGCTTAAACTC-3′) and rPLU 6 (5′-TTAAAAATTGTGCAGTTAAAACG-3′) were used for the first cycle of amplification. Parasite species were detected separately using species-specific primers rFAL 1 (5′-TAAAACCTGGTTGGGAAAAACCAATATATT-3′) and rFAL 2 (5′-ACACAAATGACTATACTCACTACCCGTC-3′) for P. falciparum and rVIV 1 (5′-CGCTTCTAGCTTAAATCCACATTCTGATAC-3′) and rVIV 2 (5′-ACTTCCAGGCGAAGGAGAAATGTCCTT-3′) for P. vivax. Amplification products were detected by running 12 μL of the PCR reaction mixture on a 2% ethidium bromide-stained electrophoretic agarose gel.

**Molecular genotyping of P. falciparum.** Three genes were used as molecular markers of genetic diversity in P. falciparum: MSP1, glutamate-rich protein (GLURP), and MSP2. Briefly, MSP1 was typed in a nested PCR assay using the M1-OF/OR primer set for the first PCR reaction and three different primer sets for the second PCR reaction for allelic typing (for MAD20 family alleles, M1-KF/KR primers; for K1 family alleles M1-MF/MR primers; and for R033 family alleles, M1-RF/RR primers) according to published protocols.18 MSP2 allelic type (Fc27A versus 3D7) was determined using a nested PCR assay, with the first PCR performed with MS-OF/OR primers and the second reaction with M2-FCF/R (FC27 family-specific primers) and M2-ICF/R (3D7 family-specific primers) according to published protocols.18 MSP2 restriction fragment length polymorphism determination was done using restriction enzymes RsI and HinII on the nested PCR product, as published.19 Genotyping using the GLURP gene was done using hemi-nested PCR using G-OF/NF primers for the first PCR reaction and using G-OR as the second primer with G-OF/NF primers in a second reaction.18 GLURP genotype detection is based on size polymorphisms involving tandem repeats in the highly polymorphic RII region of the molecule.18,20

**Definitions.** PCR was used as the gold standard in all comparisons. The sensitivity of microscopy for each species was evaluated both at baseline, including all study subjects, and during follow-up, only in single infection cases reported concordantly by microscopy and PCR. Parasite clearance was measured in days, defined by the occurrence of two consecutive parasite-free results, and assumed to occur the day after the last positive sample. Drug resistance was classified in three levels, RI, RII, and RIII, according to published criteria.2,21 RI is defined by parasite clearance before day 7 but recrudescence appears within the 28-day follow-up. RII is defined by parasitemia reduction > 75% but fails to clear within 7 days. Finally, RIII is defined if the patient has < 75% parasitemia reduction in the first 3 days of treatment.

**Statistical analysis.** Differences in clearance time were assessed using a time-to-event approach with the Kaplan-Meier method. The statistical significance was determined using the log-rank test.22 The confidence intervals for the median clearance time were calculated based on the method described by Klein.23 Log10 transformations were applied to parasite counts both for statistical comparisons as well as for graphical display. A longitudinal generalized estimating equations model was used to determine the rate of change in the geometric mean of parasite counts during treatment as well as differences in parasitemia between plasmodium species and drug-resistant strains. Student’s t test was used to determine whether, within each species, the PCR results were associated to differences in parasitic counts. Differences in the sensitivity of microscopy measured on baseline and follow-up specimens were assessed with χ2 tests. Stata 8.0 (Stata Corp., College Station, TX) was used for all analyses.

**RESULTS**

Use of the PCR in diagnosing malaria in the Peruvian Amazon field setting. Seventy patients were diagnosed with malaria by microscopy among patients that went to the Va-
rillal health post with a malaria-compatible clinical presentation during the study period. Fifty-one individuals were diagnosed with *P. vivax* and 19 with *P. falciparum*. The PCR assay results concurred with microscopy in 58 (83%) of all samples. The positive predictive value of microscopy versus PCR was 80.4% (41/51) for *P. vivax* and 89.5% (17/19) for *P. falciparum*. Microscopy reported two PCR mixed infections as single infections, two *P. falciparum* samples as *P. vivax*, and eight PCR-negative results as *P. vivax* (7) and *P. falciparum* (1), respectively.

The 12 discordant diagnoses were masked and re-examined blindly both by microscopy and PCR. The PCR results were 100% reproducible, but nine (75%) microscopy results changed (Figure 1). Two samples initially reported as *P. vivax* by microscopy but *P. falciparum* by PCR were reported as *P. falciparum* after re-examination. Seven of eight samples initially diagnosed as PCR-negative but positive by microscopy (6 *P. vivax* and 1 *P. falciparum*) were found to be microscopy false positives. Three PCR-microscopy discrepancies remained unchanged despite repeated microscopic examination. Two samples reported as mixed infections by PCR were repeatedly reported by microscopy as simple infection with *P. vivax* and *P. falciparum*, respectively, and the remaining PCR-negative patient was confirmed as *P. vivax*. After the re-examination of discordant results, the positive predictive value of microscopy was 97.7% (42/43) for *P. vivax* and 95.0% (19/20) for *P. falciparum* (Table 2).

Of 60 patients with a single infection reported both by microscopy and PCR, 49 (81.7%) completed the follow-up period (34 *P. vivax* and 15 *P. falciparum*). All *P. vivax* infections responded to chloroquine/primaquine treatment. Four patients with *P. falciparum* malaria were cured (26.7%), and 11 (73.3%) exhibited evidence of drug resistance (RI = 10, RII = 1; Table 1). Two resistant cases did not return to the health post, and nine cases received second line anti-malarial drugs. Six patients completed follow-up after the administration of treatment, whereas three were followed only for 13 – 17 days. Only one patient did not clear parasitemia after receiving second-line drugs. The median parasite clearance time both for *P. vivax* and *P. falciparum* was comparable using either PCR or microscopy. Using either method, the median clearance time in drug-resistant cases was 4 days longer than the 2 days found in drug-susceptible *P. falciparum* cases.

We compared the positive predictive value of microscopy to PCR in 49 pre-treatment and 68 post-treatment samples from the 49 patients with concurrent initial diagnosis and complete follow-up. The positive predictive value of microscopy for *P. vivax* decreased significantly between baseline and follow-up (97.7% to 22/28 = 78.6%, *P* = 0.014), but remained virtually unchanged for *P. falciparum* (95.0% to 37/40 = 92.5%, *P* = 1.000). Consistently, parasite counts in microscopy-positive samples were higher in *P. vivax* PCR-positive specimens compared with PCR-negative specimens (*P* = 0.001, Figure 2), but only marginally increased for *P. falciparum* samples that were microscopy positive versus negative (*P* = 0.079).

The geometric mean of the parasite count declined in the first 3 days after treatment, as would be expected (Figure 3; *P* < 0.001, *P* < 0.001, and *P* = 0.037, respectively). The parasite count in *P. falciparum* malaria cases was higher in patients treated with first-line anti-malarials compared with subjects treated with second-line drugs or non-resistant strains.
Patients with *P. vivax* infections had the lowest parasite counts in average (*P* < 0.001). Genetic diversity of *P. falciparum*. The 19 patient samples containing *P. falciparum* were chosen to determine whether it would be feasible to distinguish drug-resistant *P. falciparum* parasites from *P. falciparum* re-infection in the study region. There is a paucity of data regarding the genetic diversity of *P. falciparum* in the Peruvian Amazon region. Genotyping was performed using a combination of *MSP-1*, *MSP2*, and *GLURP* alleles, including restriction fragment length polymorphism analysis of the *MSP2* PCR fragments (Table 3). Of 21 patients with confirmed *P. falciparum* infection, 20 patients had MSP-1 genotypes determined on initial samples; 19 had the K1A allele, and 1 the MAD20 allele; the R033 allele was not detected in this patient population. In one of these patients (E044) who initially presented with the K1A allele, the MAD20 allele appeared at day 30, indicating reinfection or recrudescence of a previously undetectable parasite strain. Of the 21 *P. falciparum* confirmed patients, 13 had detectable *GLURP* alleles on initial samples; 12 had allele A, and 1 allele B. One patient (E060) with a *GLURP* allele A had allele B detected on days 15 and 30, suggesting the appearance of a drug-resistant strain either as a reinfection or a recrudescence. In *MSP2* genotyping, 6/21 patients had the Fc27A genotype and 12/21 had the 3D7 genotype; of the 12 patients with 3D7 genotypes of the *MSP2* marker, 8 were allele A and 4 allele B. Overall, of 19 patients for whom complete genotyping data were available, 12 had distinguishable overall haplotypes (combinations of individual genotypes reflecting individually segregating alleles, similar to the term “multilocus genotypes”24; Table 3). The overall haplotype was determined by combining unique patterns of independently segregating alleles. Haplotype 3 was most frequent (4 of 21 patients; Table 3). The R033 allele of *MSP1* seen in Africa was not observed. Only two sizes of the *GLURP* PCR product were seen (Table 3). Of note, eight patients with molecularly and microscopi-
cally confirmed *P. falciparum* did not have parasites amplifiable with the GLURP primers or with other internal GLURP-specific primers (data not shown). This observation could suggest the presence of novel alleles of GLURP in this study population, or alternatively, that PCR amplification of GLURP in these particular samples was inefficient under the conditions used.

Despite the small sample size, the data presented here suggest that sufficient *P. falciparum* genetic diversity is present in the Iquitos region to make it possible to distinguish *P. falciparum* drug-resistant parasites from reinfection with the three marker genes used here. Here, we found that three patients had a new genotype on resampling (patients E044, E060, and E069; Table 3), showing the use and discriminatory power of the combination of the three molecular markers we used in this study.

### DISCUSSION

This study had two major findings. First, in a region where *P. falciparum* and *P. vivax* are co-endemic, PCR was useful both for diagnosis and for monitoring response to antimalarial therapy in the field setting. Second, *P. falciparum* genetic diversity is higher than previously reported, for example, compared with Colombia or French Guyana where the population of parasites has recently been reported as being homogeneous or of low complexity. Molecular genotyping therefore will prove to be an important tool for epidemiologic and clinical trials in the Peruvian Amazon region. Notably, in 5 of 19 patients in whom molecular genotyping of *P. falciparum* was performed (confirming *P. falciparum* infection), *P. vivax* emerged on day 15 or 30 after primary treatment of *P. falciparum*. Whether this appearance of *P. vivax* was caused by drug failure or new infection cannot be determined from our data, although vivax malaria is 4-10 times more frequent than malaria caused by *P. falciparum* in the sites we studies (data not shown). Two possibilities for the appearance of *P. vivax* in these patients include an inadequate dosing regimen of primaquine or failure to take the primaquine as prescribed. Because we did not obtain serum drug levels post-treatment, it is difficult to distinguish these possibilities, although recent data suggest that total dosing of primaquine as causal prophylaxis against *P. vivax* needs to be higher than provided under Ministry of Health policies.

After examination of discordances, the positive predictive value of microscopy for the diagnosis of both *P. vivax* and *P. falciparum* infections was >95% only, consistent with results from previous studies. PCR results were used to diagnose two cases (2.9%) of mixed infections that were repeatedly reported as simple infections after microscopy re-examination. It has been proposed that, in mixed infections, one species may predominate. Thereafter, it is likely that microscopy fails to detect low parasitemias in cases of dual infection, where one species may suppress parasitemia of the other. The limitation of microscopy for identifying mixed infections may lead to an overestimation of the true prevalence of such infections. It should also be noted that seven samples initially reported as microscopy positive (10% of all patients in the study) were confirmed as microscopy negative in subsequent analysis. The low specificity of microscopy examination has not been well described but reflects the reality of the field situation in malaria diagnosis, where highly expert malaria microscopists in rural villages are likely not to be immediately available. In severe or moderately anemic patients such as those in our study population, the presence of artifacts such as Howell-Jolly bodies can be easily mistaken for malaria parasites by inexperienced microscopists. PCR can be used for quality control purposes to assess and improve microscopy accuracy. In some areas, PCR should be amenable to field use where reasonable electricity sources are available.

The genetic markers used to determine parasite diversity, *MSP1*, *GLURP*, and *MSP2*, have been well described in Asian and African study populations, but as a group have not been extensively used together for determining genetic diversity in Peruvian or other Latin American populations, except as noted above for Colombia and French Guiana, and in Brazil, where primarily single markers of diversity, such as *MSP1* have been studied. New markers in *P. falciparum* have recently been developed based on microsatellite polymorphisms, as well as single nucleotide polymorphisms in *P. vivax*, both of which have yet to be validated in the Peruvian Amazon region. Genetic linkage analysis has shown the use of groups (haplotypes) of markers in distinguishing parasite strains, as we have performed in this study. Future studies of genetic diversity of all malaria parasites using *MSP1*, *GLURP*, and *MSP2*, as well as microsatellite markers and single nucleotide polymorphisms, will be useful as new clinical trials of drugs and possible vaccine candidates are performed in the Amazon region.

This study has reported a very high rate of resistance to sulfadoxine-pyrimethamine in the region (73.3%). In Santa Clara, a malaria endemic village located near our study location, 75.6% resistance has been reported. Because our patients were not isolated during the follow-up period, reinfection could be responsible for parasite reappearance. In these settings, PCR techniques can be used to monitor the emergence of resistance, to identify specific mutations conferring resistance, and to study the epidemiology of different *Plasmodium* strains.

Previous studies have reported that clearance time determined using the PCR assay could be significantly higher when compared with microscopy. Our results suggest non-significant differences in median clearance time between mi-

### Table 2

Sensitivity of microscopy in PCR-positive specimens by day of follow-up

<table>
<thead>
<tr>
<th>Day</th>
<th>Sensitivity (%) M/PCR*</th>
<th>Sensitivity (%) M/PCR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 34/34</td>
<td>100 15/15</td>
</tr>
<tr>
<td>1</td>
<td>69   20/29</td>
<td>77 10/13</td>
</tr>
<tr>
<td>2</td>
<td>67   2/3</td>
<td>86 6/7</td>
</tr>
<tr>
<td>3</td>
<td>83   5/6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>67   2/5</td>
<td></td>
</tr>
<tr>
<td>5–7</td>
<td>67   2/5</td>
<td></td>
</tr>
<tr>
<td>8–14</td>
<td>100  7/7</td>
<td></td>
</tr>
<tr>
<td>15–21</td>
<td>100  1/1</td>
<td></td>
</tr>
<tr>
<td>22–30</td>
<td>100  4/4</td>
<td></td>
</tr>
<tr>
<td>31+</td>
<td>69   22/32</td>
<td>78 25/32</td>
</tr>
<tr>
<td>8+</td>
<td>100  12/12</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>85     56/66</td>
<td>88 52/59</td>
</tr>
</tbody>
</table>

* Microscopy and PCR-positive samples.
### Table 3

Molecular genotyping of *Plasmodium falciparum* using MSP1, GLURP, and MSP2 as markers

<table>
<thead>
<tr>
<th>Patient</th>
<th>MSP1</th>
<th>GLURP A/B</th>
<th>MSP2</th>
<th>Overall genotype</th>
</tr>
</thead>
</table>

*1, allele present; 0, allele absent.*

† Restriction fragment length polymorphism genotype for MSP2; initial diagnosis only.

neg, no *P. falciparum* parasites detected by PCR for that gene that day. – indicates no sample available that day because of lack of patient availability for follow-up sample.
crosscopy and PCR for \textit{P. falciparum} infections. Of importance and consistent with previous reports, we observed a trend for longer clearance time in drug-resistant \textit{P. falciparum} cases compared with the drug-sensitive group (4 versus 2 days). Median clearance time was used instead of mean clearance in the analyses to avoid biases introduced by subjects with prolonged parasitemias as well as patients that did not achieve clearance.

Despite the similarities between microscopy and PCR for evaluating the parasite clearance time, during the first days of follow-up, we observed a higher rate of microscopy negative slides that were PCR positive. These results are consistent with the lower specificity of PCR observed in resistant \textit{P. falciparum} cases during the follow-up. Because all patients in the study had accurate malaria diagnosis, it is unlikely that these results were PCR false positives. Therefore, microscopy may have failed to detect the presence of low levels of parasitemia during those days, which are most crucial in evaluating anti-malarial drug efficacy. Defining clearance time by the presence of two consecutive negative results improves the validity of microscopy for assessing parasite clearance, because it decreases variability. Because PCR is more sensitive than microscopy for the detection of low levels of parasitemia, it may be a useful tool for more precise individual follow-up particularly in the first days after treatment.

This study showed that PCR is a consistent and reliable technique with logistical applicability to the field situation in the Peruvian Amazon, where the intensity of transmission and malarial disease patterns differ from other locales where molecular diagnosis has previously been evaluated. PCR diagnosis and molecular genotyping will be particularly useful in field studies where distinguishing mixed infections, drug resistance, and re-infection are critical parameters of success.

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