Evaluation of PCR To Monitor Plasmodium falciparum Treatment Efficacy in a Nonendemicity Setting

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ABSTRACT Adequate clinical and parasitological response (ACPR) after malaria treatment remains challenging to assess in settings of malaria nonendemicity. Biological evaluation of parasitological clearance relies on microscopic investigation of thick blood smears, which is a specific technique that not all diagnosis laboratories are able to perform. Rapid diagnosis tests (RDTs) and molecular biology techniques are proposed as alternatives to microscope conventional techniques; however, their performance for treatment efficacy evaluation is controversial. We present here a retrospective comparative study for RDT and PCR (nested and high-resolution-melting quantitative PCR [HRM-qPCR]) evaluation of ACPR in a nonendemicity context. Blood samples from 133 patients presenting a Plasmodium falciparum monoinfection were included. Samples obtained at the time of diagnosis and at 3, 7, and 28 days after diagnosis were investigated. Histidine-rich protein 2 (HRP-2)-based RDT results remained positive in 51% of cases 28 days after diagnosis and appropriate therapeutic management. Parasite DNA was detected by the two PCR techniques (nested PCR and HRM-qPCR) in 12% and 10% of samples 28 days after treatment initiation, respectively. No therapeutic failure was recorded in the studied patients. Persistence of positive signal might reflect the presence of circulating asexual parasites or persistence of HRP-2 and parasitic DNA in patient’s peripheral blood after parasitic clearance.

KEYWORDS imported malaria, HRM, PCR, RDT, HRP-2, follow-up, antimalarial efficacy, Plasmodium falciparum

Plasmodium falciparum is the main agent of malaria in human and is endemic in 91 countries worldwide (1). In metropolitan France, no transmission occurs and reported cases are mostly imported. Patients presenting malaria are migrants, travelers, or military staff returning from areas of endemicity, mostly sub-Saharan Africa. In 2017, 4,690 cases were estimated to have been found in France on the basis of reports to the notification network of the French National Malaria Reference Center (FNMRC) (2, 3). In accordance with World Health Organization (WHO) guidelines (4), the French Society of Infectious Diseases has recommended whole-blood microscopic observation, in addition to rapid diagnostic tests (RDTs), for malaria diagnosis. To monitor therapeutic efficacy, clinical examination and microscopic observations of sampled blood at day 3 (D3), D7, and D28 after initiation of treatment are recommended (5, 6). Microscopic diagnosis is based on detection of parasites on Giemsa-stained thin and thick blood films. Thin blood film examination provides information about the infecting species and parasitemia, helping clinicians to manage malaria access and choose the appropriate antimalarial therapy. The thick blood smear method is more sensitive and is the recommended technique to assess whether the patient is cured or not. Since this technique requires regular training and accurate skills for Plasmodium identification and species discrimination, only a few laboratories in nonendemicity settings are able...
to practice it. As a consequence, other methods such as RDTs, which detect parasitic proteins, and PCR, which detects parasite DNA, have been developed for malaria diagnosis. However, their performance with respect to evaluation of parasite clearance in areas of nonendemicity has been evaluated only partially (7–10).

Classical patient follow-up involves checking 72 h after initiation of treatment for early treatment failure and between 4 and 28 days after initiation of treatment for late treatment failure (4). Treatment success (adequate clinical and parasitological response [ACPR]) is defined by the WHO as “the absence of parasitemia at day 28 (or 42) irrespective of axillary temperature, in patients who did not previously meet any of the criteria of treatment failure, late clinical failure and late parasitological failure.” ACPR evaluation is crucial to detect therapeutic failure, to offer appropriate treatment for the relapse, and to monitor resistance to antimalarials. However, to address these issues, it is necessary to differentiate sexual and asexual Plasmodium forms.

Currently, RDTs represent the most widely used malaria diagnosis tools in areas of endemicity and nonendemicity. For follow-up, RDTs have been proposed as an option since they can accurately detect parasite proteins. Plasmodium lactate dehydrogenase (pLDH) RDTs have proven their accuracy (11), with a time of pLDH clearance of 7 days after treatment (7). Histidine-rich protein 2 (HRP-2) has been used with high accuracy for diagnosis (11–13), but its use in follow-up is not recommended due to its persistent detection after successful treatment (12–15).

PCR (both real time and conventional) is the most sensitive and specific technique for Plasmodium detection and species identification and is being widely proposed as the gold standard. However, conventional PCR techniques are not yet appropriate for routine diagnosis as it frequently requires more than 2 h to produce results, which is not in accordance with WHO guidelines (4). Currently, the development of rapid loop-mediated isothermal amplification assay (LAMP) is an alternative for parasite DNA detection in 45 min and allows the identification of Plasmodium-positive samples. However, PCR could be an appropriate tool for patient’s follow-up and ACPR evaluation, since there is no emergency if there is no suspicion of therapeutic failure. Limited reports have been published concerning the use of PCR as a tool for follow-up and treatment efficacy assessment in settings of nonendemicity (8–10). The first of those reports presented results from a study that included 36 patients, among whom 3 presented with severe malaria (8), and pooled samples from day 3 to day 9, day 10 to day 19, and day 20 to day 21. The second included a majority of Caucasian patients working in areas of malaria endemicity (10). Both the first and second studies reported unexpected PCR positivity at day 28 in patients with no treatment failure who had not returned to areas of malaria endemicity. The third included 24 individuals and highlighted persistence of RDT and quantitative PCR (qPCR) positivity within a 1-month period (9).

Here, we present a retrospective comparative study on samples from treated P. falciparum malaria patients followed up in a country in which malaria is not endemic. Our aim was to evaluate the accuracy of alternative methods to microscopic investigation, by HRP-2 detection (RDT) and PCR to assess parasite clearance.

**MATERIALS AND METHODS**

**Sample collection.** In patients diagnosed in Bichat’s Hospital between 2012 and 2013, EDTA whole-blood samples were collected in the course of classical care for malaria diagnosis and follow-up. For this study, we selected samples only from patients with falciparum malaria whose diagnosis sample and both D3 and D28 or D7 and D28 samples were available. We collected microscopic and RDT results obtained at the sampling, and we performed a retrospective PCR study on DNA extracts. For each sample, Giemsa-stained thick blood smears were performed daily in the Parasitology unit. Thick blood smears were considered positive if asexual forms were visualized and negative if no parasites were detected after examining 1,000 white blood cells. Parasitic density was estimated and expressed as the number of parasites per microliter of blood, assuming 8,000 leucocytes/μl of blood. The parasite species was determined, and the presence P. falciparum gametocytes was reported. DNA extraction and RDT were routinely performed on all samples from patients with diagnosis of Plasmodium infection reported between 2012 and 2013. FNMRC collected epidemiological and biological data. Clinical and therapeutic data were issued from patient medical files.
**Rapid diagnosis test.** Core Malaria Pf rapid diagnosis tests (Core Diagnostics, Birmingham, United Kingdom) were performed on patient fresh blood samples. Tests were performed according to the manufacturer’s instructions. Results were recorded after 20 min, and control line positivity was checked as an indicator of test quality.

**PCR.** DNA extraction from blood was performed on MagnaPure with LC DNA Isolation kit 1 (catalog no. 0303990001; Roche Diagnosis, Basel, Switzerland), and 100 µl of elution buffer was used for elution, following the manufacturer’s instructions. DNA extracts were frozen at –20°C until use.

Species-specific nested PCR targeting the 185 RNA gene from *P. falciparum* (GenBank accession no. M19172) was performed as described previously (16) using rPLU1 (5’-TCA-AAG-ATT-AAG-CCA-TGC-AAG-TGA-3’) and rPLUS (5’-CTT-GTT-GCC-TTA-AAC-TTC-3’) for the *Plasmodium* genus-specific reaction and rFAL1 (5’-TTA-AAC-TGG-TT-GGG-AAACC-AAA-TAT-ATT-3’) and rFAL2 (5’-CA-AAC-TAG-CTGA-A-TCA-CCA-TAAG-3’) for the *P. falciparum* species-specific reaction. Reaction positivity was assessed with migration of the second PCR products at 206 bp in 3% agarose gel with 1/20,000 SyberSafe (Life Technology, Carlsbad, CA, USA). An AmpliSize ladder (Bio-Rad, Marnes-la-Coquette, France) (50 bp to 2,000 bp) was used to assess fragment size.

High-resolution melting (HRM) detection based on qPCR was developed in our laboratory (17) and was performed to quantify *P. falciparum* DNA in samples using a calibration range obtained from serial dilution of DNA in free water (equivalent of 40,000, 4,000, 400, 40, 20, 8, and 4 parasites/µl). The amplified sequence melting temperature (Tm) was recorded to ensure reaction specificity. A 5-µl volume of extracted DNA was used with primers Plasmo1F (5’-GTT-AAG-GGA-GTG-AAG-AGC-ATC-AGA-3’) and Plasmo2R (5’-AAC-CCA-AAG-ATT-TTC-ATT-TCT-CAA-3’), specific for *Plasmodium* (18), at a final concentration of 1 µM in MelDooR HRM master mix (Life Technologies, Courtaboeuf, France).

Amplification and qPCR measurements were performed using an Applied Biosystems Viia 7 real-time PCR system (Life Technologies). qPCR conditions were as follows: 10 min initial denaturation at 95°C; 40 cycles of 10 s at 95°C and 1 min at 60°C; 10 s at 95°C followed by 1 min at 60°C before the HRM phase. The HRM phase consisted of a 0.025°C/s temperature increase. HRM detection was considered positive for *P. falciparum* when the results showed a specific melting temperature of 75.432°C (standard deviation [SD] = 0.12) or of 72.830°C (SD = 0.11) or both and a threshold cycle (Ct) value of less than 38.5 (4 parasites/µl). A sample was considered negative despite the Ct result if no *P. falciparum*-specific Tm was observed or if no amplification occurred.

**Statistical analysis.** GraphPad (Prism v5) was used for statistical analysis and graphic representation. Proportions were compared using the chi-square test. The Mann-Whitney test was used to compare quantitative values between time points (parasite density estimated with PCR), and correlations between parasite quantification results were assessed using PCR and microscopy and the Spearman correlation coefficient. To compare parasitemia results between groups, unilateral t tests were performed. We considered a P value of <0.05 as significant.

**Ethical considerations.** No specific consent was required because, in coordination with the Santé Publique France organization for the care and surveillance of malaria, the human clinical, epidemiological, and biological data as well as parasitic biomarkers (parasitemia, PfHRP-2, RDT, and PCR results) were collected in the CNRP database and analyzed in accordance with the common public health mission of all French National Reference Centers [https://www.legifrance.gouv.fr/affichTexte.do?idTexte=JORFTEXT0000008010056&dateTexte=&categorieLien=id]. The study of biological samples obtained in the medical care context was considered noninterventional research (article L1221-1-1 of the French public health code) requiring only the noninterventional use of the patient during sampling (article L1221-2 of the French public health code). All data collected were anonymized before analysis.

**RESULTS**

**Sample inclusion and description.** Among the 288 patients diagnosed for *P. falciparum* malaria in Bichat’s Hospital between January 2012 and December 2013, 133 patients were included in the study since they presented pure *P. falciparum* infection, and we disposed of the corresponding follow-up samples. Average patient age was 42 (±12) years, and the sex ratio was 1.9 (male/female). Most (88.7%) of the patients were of African origin; however, 97.7% of the patients were living outside the areas of malaria endemicity (in metropolitan France) (Table 1). The distributions of ethnic groups, place of birth, and place of residence of the patients included in the study did not differ significantly from those recorded for the patients diagnosed in Bichat’s Hospital during the same period (P = 0.26, P = 0.26, and P = 0.06, respectively, using the chi-square test). We were able to perform 395 RDTs (121 at D0, 92 at D3, 101 at D7, and 81 at D28), 463 nested PCRs (125 at D0, 104 at D3, 115 at D7, and 119 at D28), and 434 HRM-qPCRs (108 at D0, 104 at D3, 111 at D7, and 111 at D28) on the corresponding samples (see Table S1 in the supplemental material).

**Biological and parasitological characteristics at diagnosis.** The mean hemoglobin level was 128.0 g/dl (±19.4). The average erythrocyte count was 4.5 T/liter (±0.6), the average leukocyte count was 5.3 G/liter (±2.4), and the average platelet count was 90.1 G/liter (±53.9). All patients presented pure *P. falciparum* infection, and the
geometric mean level of parasitemia evaluated with blood smear was $14,061 \text{ p/\mu l}$ (range, 30 to 1,170,000). These characteristics did not differ from the average levels measured for the malaria patients in Bichat’s Hospital during the same period (Table S2).

Parasite sensitivity was tested for the antimalarial drugs used for treatment, and no resistant phenotype was observed (internal data from FNMRC).

**Treatment regimen.** All patients were treated in accordance with the French recommendations provided at the time of the study. Treatment choice relied on the clinician’s interpretation of conditions. Overall, 59.4% of patients were treated with atovaquone-proguanil, 14.3% with artemether-lumefantrine, 13.6% with intravenous quinine, and 12.0% with intravenous artesunate augmented by artemether-lumefantrine. Among the included patients, no therapeutic failure was recorded.

**Detection of parasites by microscopy.** All included samples were positive for thick blood smears at diagnosis, 43% (46/107) of the samples were positive at D3, and all samples were negative by D7 and D28 (Table S3). The ranges of parasitemia levels were 30 to 1,170,000 \text{ p/\mu l} at D0 and 1 to 477,000 \text{ p/\mu l} at D3. The percentages of positive blood smears were significantly different between D0 and D3 using the chi-square test ($P < 0.0001$).

**Detection of parasites by RDT.** HRP-2 RDT detection was positive for 88% (107/121) of patients tested at diagnosis, 90% (83/92) at D3, and 64% (65/102) at D7. At D28, 51% (42/81) remained positive for HRP-2 RDT (Table S3). The percentages of positive blood smears were significantly different between D0 and D3 using the chi-square test ($P < 0.0001$). The percentages of positive HRP-2 RDT did not differ between D0 and D3 and D7/D28. Among the HRP-2-positive RDT results seen on D28, no influence of treatment was observed, with similar proportions ($\sim 51\%$) of positive tests for atovaquone-proguanil and quinine combined versus artemether-lumefantrine and artesunate. However, the level of parasitemia at D0 was significantly higher in patients with positive HRP-2 RDT results at D28 using a unilateral $t$ test ($P = 0.02$).

**Detection of parasite DNA by *P. falciparum*-specific PCR and HRM-qPCR.** PCR results confirmed *P. falciparum* monoinfection in all included patients with a specific HRM profile. At diagnosis, all included samples were positive in conventional PCR and HRM-qPCR. At D3, 73% (76/104) of samples were positive by nested PCR and 82%
At D7, 53% (61/115) of samples were positive by nested PCR and 55% (62/111) by HRM-qPCR. At D28, 12% (14/119) and 10% (11/111) of samples were positive by nested PCR and HRM-qPCR, respectively (Table S3). The percentages of positive PCRs were significantly different between D0/D3, D3/D7, and D7/D28 using the chi-square test ($P < 0.0001$). Among the samples that were positive at D28, no statistically significant difference was observed between the atovaquone-proguanil/quinine group and the artemisinin-based combination therapy (ACT)/artesunate group ($P = 0.175$). The initial (D0) level of parasitemia measured for patients displaying positive PCR at D28 did not differ from that seen with the D28 PCR-negative patients using a unilateral t test ($P = 0.35$).

A linear quantitation zone for parasite density was determined with a calibration range of between $22.5$ parasites/$\mu$L and $45,000$ parasites/$\mu$L ($R^2 > 0.99$). Results of Spearman tests analyzing correlations between qPCR-HRM and microscope-based parasite density estimations were statistically significant ($X = 0.778$). The parasite load estimated with HRM-qPCR was significantly lower at D3 than at D0 ($P = 0.011$) and had decreased at follow-up (Fig. 1).

**DISCUSSION**

The levels of accuracy of the different methods for diagnosing malaria are well studied. However, the evaluation of these tools for follow-up and treatment efficacy evaluation remain limited in nonendemicity settings. Here, we present data from a retrospective study conducted in Paris, France, where no malaria transmission occurs. Our goal was to evaluate the accuracy of each method at each patient’s follow-up after treatment initiation. We focused on 133 patients diagnosed between 2012 and 2013 from whom we had access to an early posttreatment sample and a sample 28 days after treatment initiation. In addition to standard microscopy, RDT detection was performed as well as *P. falciparum* DNA detection and quantification by PCR. PCR was studied in the context of malaria diagnosis and follow-up, as this method has been proposed as the new gold standard for malaria diagnosis (19). RDT and PCR, two of the more frequently employed techniques used to diagnose malaria, were compared to microscopy as a reference, displaying different analytical performances for malaria diagnosis and follow-up.

RDTs detecting *P. falciparum*-specific HRP-2 protein were used. We confirmed previous studies showing a high rate of HRP-2 persistence despite ACPR to treatment (13). Focusing on treatment regimen, HRP-2 RDT on D28 showed positivity rates
following treatment with artemisinin (artesunate or derivatives) similar to those seen with nonartemisinin treatment. The HRP-2 clearance rate after artesunate/ACT treatment is controversial, as a previous study showed that HRP-2 clearance is faster after ACT treatment (20), an observation that was not confirmed by further study (15). In addition, the nonpathogenic sexual forms of the parasite (gametocytes) have been known to secrete HRP-2 (13, 21), which could induce positive test results after clearance of asexual forms. HRP-2 protein is secreted from the erythrocyte and persists even after successful treatment. In addition, HRP-2 contained in the infected erythrocytes can be secreted after treatment with delayed hemolysis (22).

qPCR-HRM-based detection confirmed mono-infection of the selected patients for *P. falciparum*. At diagnosis, PCR displayed high sensitivity (100% for the two studied methods), as reported in the literature (23, 24).

Surprisingly, PCR parasite detection was positive at a level of 12% at D28 with the nested conventional technique and at 10% with qPCR-HRM, despite all patients presenting with an ACPR. This apparent discrepancy has been reported in nonendemicity settings by Dakić et al., Homann et al., and Phuong et al. (8–10). The topic of the persistence of *Plasmodium* DNA in blood is controversial. The authors of one study conducted on animal models reported that the parasite’s DNA is eliminated after the death of the parasite (25), while other authors suggested that the parasite’s DNA might be circulating through immune complexes in the bloodstream of patients (8, 26).

Another explanation for these positive results could be submicroscopic persistence of parasite, in the sexual or the asexual form, since molecular methods have been described previously as more sensitive than microscopy for parasite detection (8, 27). The PCR results might have been positive due to the persistence of asexual parasites, but the patients were asymptomatic, did not return to areas of endemicity during the follow-up, and did not report vomiting or lack of compliance. Chronic asexual parasite carriage is more frequently reported in areas of endemicity in repeatedly infected immune patients. However, patients in this study were infected in West Africa and parasites in that area are sensitive to the treatment regimen used there (1). In addition, all samples were tested for molecular markers of antimalarial resistance and in vitro sensitivity to antimalarial drugs. No resistant parasites were detected. No patients returned later to report reappearance of symptoms, confirming the absence of late treatment failure.

Another hypothesis to account for PCR positivity is gametocyte persistence after treatment, which has been described previously (28). In addition, gametocytes can be sequestered in bone marrow (29), limiting their detection in blood films. As PCR detection is 10 times more sensitive than microscopy for detection of gametocytes (30), PCR positivity might be explained by the persistence of gametocytes. To assess this hypothesis, specific gametocyte detection could be performed by detection of specific transcripts. However, we could not perform this assay in our study, as preserved parasite RNA was not available.

The vast majority (91%) of the patients included in this study were born in endemicity settings; therefore, we could not compare the decreases in the rates between semi-immune patients and naive patients. However, it has recently been reported that PCR clearance is faster in patients of African origin, suggesting the effect of premunition (8), and patients from the same population have been shown to display higher titers of antibodies to *P. falciparum* (31). This hypothesis could explain the lower rate of positive PCR at D28 in our samples (10% and 12%) compared to data obtained by Vafa Homann et al. (26% of positive samples at day 40, all patients treated by artesunate and derivatives) (8). The decrease in DNA quantity over time that we observed was in agreement with previous published studies performed in nonendemicity settings (8, 9).

In the presence of positive results associated with an APCR, we tried to monitor the antimalarial efficacy by analysis of the rate of parasite DNA clearance. To evaluate the quantity of DNA remaining in follow-up samples, we performed quantitative PCR with HRM detection. We showed a decrease in parasite load during the follow-up, in accordance with other studies performed in the same context (8). For daily diagnosis,
PCR results must be examined according to the clinical presentation of the patient. In the absence of fever, qPCR-HRM positivity is not sufficient to call recovery into question if the thick blood smear is negative. These findings are important in the current context, with the availability of rapid bench DNA detection assays such as LAMP. The detection of parasite DNA alone after treatment without clinical or biological signs of malaria is not a documented sign of relapse.

In areas of malaria endemicity, after malaria treatment, parasite detection may be the consequence of either reinfection or treatment failure. In this context, and with the aim of malaria eradication, systematic treatment after a positive RDT result can be implemented. In settings where malaria is imported, no reinfection can occur; a new cure of malaria treatment has to be discussed according to the context.

Since this study was performed retrospectively and included only patients from whom follow-up samples were available, sampling bias may have occurred. However, the sample size considered in this study is larger than what has been reported previously in the literature (8, 10) (with all treatments mingled). Our sampling displayed characteristics similar to those of the previous year’s FNMRC data set with respect to patient populations, and we therefore assessed that this bias should be minor. We assumed that patients not returning for follow-ups might represent the result of scheduling conflicts due to daily occupations, appointment availability, or other independent reasons. No therapeutic failure occurred in the patients whose samples were included over the course of this study. Therefore, we could not evaluate PCR and RDT performance in this context.

According to our results, malaria treatment success assessment remains challenging. Microscopy techniques are the most reliable (and are in compliance with the WHO guidelines). The other studied markers remained positive despite ACPR using microscopy as a reference (HRP-2 RDT and PCR). qPCR-HRM showed a parasite load decrease during the follow-up, but the results that remained positive at D28 were proportional to those seen in the gel PCR. Further study should focus on the discrimination between viable circulating parasite and the remaining DNA. To this end, two approaches could be implemented. The first approach would include specific RNA preservation and detection by PCR of the corresponding cDNA. However, nonviable parasite would still harbor RNA. Another would involve the use of propidium monoazide (PMA)-PCR. This technique consists of using PMA, an intercalant that binds to DNA from nonviable parasites in a covalent manner after photoactivation. This dye is a PCR inhibitor. Consequently, with this pretreatment, only viable parasite are detected (32). This technique might be a better indicator of the presence of mRNA to assess parasite viability (dead parasites could still have remnants of RNA) but needs to be optimized for Plasmodium samples.

With the current data and methods available, in the absence of RNA detection for gametocyte, microscopy in the only method that can reliably distinguish true asexual parasitemia from prolonged clearance of antigen and nucleic acid in a convalescing patient.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank all patients and clinician who contributed to the story. We also thank Thomas J. Finn for his advice on the manuscript and for proofreading.

We declare that we have no competing interests.

The French Malaria National Reference Center funded the experiments.

C.K., S.H., and N.A. designed the experiment. C.K. and V.J. performed the experiment. C.K. and V.J. analyzed the data. C.K., N.A., and V.J. wrote the manuscript. S.H. supervised the study. All of us reviewed the manuscript.
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