Emergence of Plasmodium vivax Resistance to Chloroquine in French Guiana

Lise Musset, Christophe Heugas, Richard Naldjinan, Denis Blanchet, Pascal Houzé, Philippe Abboud, Béatrice Volney, Gaëlle Walter, Yassamine Lazrek, Loïc Epelboin, et al.

To cite this version:
Lise Musset, Christophe Heugas, Richard Naldjinan, Denis Blanchet, Pascal Houzé, et al.. Emergence of Plasmodium vivax Resistance to Chloroquine in French Guiana. Antimicrobial Agents and Chemotherapy, 2019, 63 (11), 10.1128/AAC.02116-18. pasteur-02567258

HAL Id: pasteur-02567258
https://pasteur.hal.science/pasteur-02567258
Submitted on 7 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
Emergence of *Plasmodium vivax* Resistance to Chloroquine in French Guiana

Lise Musset,a Christophe Heugas,a,b Richard Naldjinan,a Denis Blanchet,d Pascal Houze,a Philippe Abboud,c Béatrice Volney,a Gaëlle Walter,c Yassamine Lazrek,a Loïc Epelboin,c Stephane Pelleau,a Pascal Ringwald,f Eric Legrand,g Magalie Demar,c,d Félix Djossouc

aLaboratoire de Parasitologie, Centre National de Référence du Paludisme, World Health Organization Collaborating Center for Surveillance of Anti-Malarial Drug Resistance, Institut Pasteur de la Guyane, Cayenne, French Guiana, France
bFaculty of Medicine, Université de Poitiers, Poitiers, France
cDepartment of Infectious and Tropical Diseases, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana, France
Laboratoire Hospitalo-Universitaire de Parasitologie et Mycologie, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana, France
dLaboratoire Hospitalo-Universitaire de Parasitologie et Mycologie, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana, France
eBiochemistry Laboratory, Hôpital Saint Louis, Paris, France
fGlobal Malaria Programme, World Health Organization, Geneva, Switzerland
gMalaria Genetic and Resistance Group, Biology of Host-Parasite Interactions Unit, Institut Pasteur, Paris, France

ABSTRACT In South America, *Plasmodium vivax* resistance to chloroquine was recently reported in Brazil and Bolivia. The objective of this study was to collect data on chloroquine resistance in French Guiana by associating a retrospective evaluation of therapeutic efficacy with an analysis of recurrent parasitemia from any patients. Patients with *P. vivax* infection, confirmed by microscopy and a body temperature of ≥37.5°C, were retrospectively identified at Cayenne Hospital between 2009 and 2015. Follow-up and treatment responses were performed according to the World Health Organization protocol. Parasite resistance was confirmed after dosage of a plasma concentration of chloroquine and microsatellite characterization. The *pvmdr1* and *pvcrt-o* genes were analyzed for sequence and gene copy number variation. Among the 172 patients followed for 28 days, 164 presented adequate clinical and parasitological responses. Eight cases of treatment failures were identified (4.7%; n = 8/172), all after 14 days. The therapeutic efficacy of chloroquine was estimated at 95.3% (95% confidence interval [CI], 92.5 to 98.1%; n = 164/172). Among the eight failures, five were characterized: two cases were true *P. vivax* chloroquine resistance (1.2%; 95% CI, 0 to 2.6%; n = 2/172), and three cases were found with subtherapeutic concentrations of chloroquine. No particular polymorphism in the *Plasmodium vivax* *pvmdr1* and *pvcrt-o* genes was identified in the resistant parasites. This identified level of resistance of *P. vivax* to chloroquine in French Guiana does not require a change in therapeutic recommendations. However, primaquine should be administered more frequently to limit the spread of resistance, and there is still a need for a reliable molecular marker to facilitate the monitoring of *P. vivax* resistance to chloroquine.

KEYWORDS *P. vivax*, resistance, Amazonia, French Guiana, Guiana Shield, chloroquine, *pvcrt-o*, *pvmdr1*

In 2017, malaria was still the most prevalent parasitic disease in the world, with 1.4 billion people remaining at risk (1). Representing 40% of malaria cases worldwide, *Plasmodium vivax* was the second species most responsible for human malaria after *Plasmodium falciparum* and is the most frequent species outside Africa. The same year in French Guiana, an overseas French territory located on the Guiana Shield in South America, 86% of malaria cases were due to *P. vivax*, and the remaining were due to *P. falciparum*.
*falciparum*, with scarce reports of *Plasmodium malariae* cases. In this region, the incidence of *P. vivax* exceeded that of *P. falciparum* in 2005 (2, 3). Meanwhile the overall number of notified malaria cases decreased from 4,000 in 2009 to 597 in 2017 (4).

Since 1995 in French Guiana, chloroquine (CQ) has no longer been recommended for treatment of *P. falciparum* and has been replaced by quinine-doxycline before artemether-lumefantrine (3). However, it is the standard treatment for uncomplicated *P. vivax* infection. Its posology follows the World Health Organization (WHO) recommendation: an oral dose of 25 mg/kg of body weight distributed over 3 days and 14 days of 30-mg/day primaquine (PQ) to cure dormant hypnozoites (3, 5). Unfortunately, PQ is not systematically administered, mainly because of administrative constraints and difficulties in assessing the glucose-6-phosphate dehydrogenase (G6PD) activity of *P. vivax* malaria patients in remote areas (3). Without appropriate treatment, these dormant liver forms can cause relapses and participate in transmission (6). As relapses may be caused by the homologous or heterologous genotype, the genetic characterization of parasites is not very useful to characterize failures (7), thus limiting the study of antimalarial drug effectiveness against *P. vivax*.

*P. vivax* multiplication should not occur within 35 days after an adequate CQ treatment. During this time, the mean whole-blood concentrations of CQ and its metabolite desethylchloroquine (dCQ) are normally greater than 100 ng/ml and prevent parasite multiplication. CQ resistance (CQR) is suspected if parasitemia increases during this period (8). Therefore, *P. vivax* resistance could be identified using plasma concentrations (9). *In vitro* phenotyping methods are scarce and difficult to implement especially because of the very low synchronicity of parasites belonging to the Chesson South American strain (10). Putative molecular markers of CQR have been identified by homology with those from *P. falciparum*. Positions 976 and 1076 of the *pvmdr1* gene have been associated with resistance without clear evidence (11, 12). *pvmdr1* is considered only a minor determinant for resistance to chloroquine, eventually considered more informative for resistance to mefloquine (13, 14). *pvcrt-o*, the ortholog gene of *pfcrt*, the *P. falciparum* molecular marker for resistance to several antimalarial drugs, has also been described as a putative marker for resistance of *P. vivax* to chloroquine (15, 16). Gene duplication and the expression level of these genes have also been described as genetic determinants (17).

The first descriptions of well-documented *P. vivax* resistance in the Americas came from the Republic of Guyana, also part of the Guiana Shield (18). More recently in Oiapoque, Brazil, on the border with French Guiana, 1.1% (n = 1/95) of treatment failures (TFs [i.e., recurrent parasites after treatment]) were reported after supervised treatment with the combination CQ+PQ (19). Manaus, Amazonas, Brazil, is nowadays the hot spot of *P. vivax* resistance in South America, with 10.1% of TFs (n = 11/109) reported after supervised CQ treatment (20) or 5.2% (n = 7/135) after concomitant administration of CQ+PQ (21).

The present study’s main objective was to bring out new data on *P. vivax* CQR in French Guiana. This combined study associated the results from a follow-up therapeutic efficacy study of CQ implemented in clinical practices at the Cayenne Hospital with a retrospective analysis of recurrence in patients presenting fever and positive parasitemia within 35 days after the initial infection.

**RESULTS AND DISCUSSION**

In Cayenne Hospital, the patients infected by *P. vivax* are mostly young men. Between 2009 and 2015, 926 patients were screened and diagnosed positive for malaria (Fig. 1). During the clinical examination of the 583 patients with *P. vivax* infections, the median body temperature was 39.9°C (range, 37.5 to 41.5°C). The median parasitemia was 0.57% (range, 0.01 to 2.00%). During this period, young men were mostly infected by *P. vivax* (male/female [M/F] sex ratio, 2.05; median age, 31 years [range, 1 to 76 years]). Patients were diagnosed a median of 3 days after their first symptoms. No patient was underweight, and 22.5% (n = 131) of them declared vomiting.
Therapeutic efficacy evaluated in one-third of the patients based on the standard hospital protocol for malaria care. Of the 583 enrolled patients with *P. vivax* infections, 62 were excluded from the analysis on day 0 (D0) (Fig. 1). Of the 521 remaining patients, 172 patients were followed up to D28, and 292 were lost during follow-up. Fifty-seven patients were excluded during the study (Fig. 1). Among those 172 patients, men were more affected by malaria than women (M/F sex ratio, 2.37) (Table 1). The median age was 32 years (range, 5 to 76 years). Only 13.4% ($n = 110$) of these 172 patients took a chemoprophylaxis treatment: mainly (94.4%) members of the military using doxycycline. A history of malaria within the last 3 months was reported for 37.6% ($n = 56/149$) of patients. Within the same period, a history of travelling out of Cayenne was recorded for the majority of patients, of which 71.2% ($n = 104/146$)
declared they had traveled within French Guiana, while 28.8% (n = 42/146) reported not having traveled. Cayenne Hospital is located outside the malaria transmission areas in French Guiana. However, it concentrates people from all over French Guiana, including people contaminated in gold mines (at least n = 32 [data not shown]), such

![Table 1: Patient characteristics of uncomplicated P. vivax malaria treated by 3 days of chloroquine administration in French Guiana from 2009 to 2015]

| Parameter                        | Total | ACPR on D28 | Recurrent parasitemia on D28 | P   |
|----------------------------------|-------|-------------|------------------------------|-----|
| Patients, no. (%)                |       |             |                              |     |
| Total                            | 172   | 164         | 8                            | 0.554 |
| By yr                            |       |             |                              |     |
| 2009                             | 38 (22.1) | 37 (97.4) | 1 (2.6)                      |     |
| 2010                             | 37 (21.5) | 35 (94.6) | 2 (5.4)                      |     |
| 2011                             | 24 (14.0) | 24 (100.0) | 0 (0.0)                      |     |
| 2012                             | 44 (25.6) | 41 (93.2) | 3 (6.8)                      |     |
| 2013                             | 11 (6.4) | 10 (90.9) | 1 (9.1)                      |     |
| 2014                             | 8 (4.6) | 7 (87.5) | 1 (12.5)                     |     |
| 2015                             | 10 (5.8) | 10 (100.0) | 0 (0.0)                      |     |
| Gender, no. (%)                  |       |             |                              | 0.768 |
| M/F ratio                        | 2.37  | 2.35        | 3.00                         |     |
| Male                             | 121 (70.4) | 115 (95.0) | 6 (5.0)                      |     |
| Female                           | 51 (29.7) | 49 (96.1) | 2 (3.9)                      |     |
| Age, median yr (range)           | 33.00 (5–76) | 33.00 (5–76) | 30.50 (17–56) | 0.134 |
| Age group, no. (%)               |       |             |                              | 0.496 |
| Adults                           | 163 (94.8) | 155 (95.1) | 8 (4.9)                      |     |
| 5–15 yr old                      | 9 (5.2) | 9 (100.0) | 0 (0.0)                      |     |
| History of malaria, no. (%)      |       |             |                              | <0.001* |
| Yes                              | 56 (32.5) | 55 (98.2) | 1 (1.8)                      |     |
| No                               | 93 (54.1) | 91 (97.8) | 2 (2.2)                      |     |
| Unknown                          | 23 (13.4) | 18 (78.3) | 5 (21.7)                     |     |
| Prophylaxis, no. (%)             |       |             |                              | 0.121 |
| Yes                              | 18 (10.5) | 18 (100.0) | 0 (0.0)                      |     |
| No                               | 116 (67.4) | 112 (96.6) | 4 (3.4)                      |     |
| Unknown                          | 38 (22.1) | 34 (89.5) | 4 (10.5)                     |     |
| Days before consultation, median no. (range) | 3 (0–15) | 3 (0–15) | 1 (0–7)                      | 0.428 |
| Body wt, median kg (range)       | 72 (49–158) | 72 (49–158) | 68 (58–83)                   | 0.493 |
| Body temp, median °C (range)     | 39.0 (37.7–40.9) | 39.0 (37.7–40.9) | 38.9 (37.5–39.6) | 0.672 |
| Vomiting, no. (%)                |       |             |                              | 0.671 |
| Yes                              | 59 (34.3) | 57 (96.6) | 2 (3.4)                      |     |
| No                               | 106 (61.6) | 100 (94.3) | 6 (5.7)                      |     |
| Unknown                          | 7 (4.1) | 7 (100.0) | 0 (0.0)                      |     |
| Parasitemia, median % infected blood cells (range) | 0.15 (0.01–2.00) | 0.15 (0.01–2.00) | 0.13 (0.02–0.76) | 0.218 |
| Hospitalization, no. (%)         |       |             |                              | 0.946 |
| Yes                              | 23 (13.4) | 22 (95.7) | 1 (4.3)                      |     |
| No                               | 98 (57.0) | 93 (94.9) | 5 (5.1)                      |     |
| Unknown                          | 51 (29.6) | 49 (96.1) | 2 (3.9)                      |     |

*ACPR, adequate clinical and parasitological response; D28, day 28. *, P < 0.05 (significant difference).
as gold miners or members of the military dedicated to fighting illegal gold mining activities. Thus, the analyzed sample set of this study could be considered representative of the general parasite population circulating in the country.

**High but incomplete (95.3%) CQ therapeutic efficacy against *P. vivax* in French Guiana between 2009 and 2015.** In French Guiana, between 2009 and 2015, the D28 follow-up estimated a therapeutic efficacy of CQ at 95.3% (95% confidence interval [CI], 92.5 to 98.1; n = 164/172) to treat uncomplicated *P. vivax* monoinfection. This study included a large number of patients, compared to the WHO recommendation (n = 172 versus 73). Therefore, these results were associated with a confidence level of 95% and a margin error of 2.8%. A cross-analysis was done within samples received at the National Reference Center (around 50% of the total number declared each year in the country) to track potential recurrent parasitemia in patients enrolled in the protocol but followed outside the Cayenne Hospital. This allowed us to identify one additional recurrent parasitemia. This confirms the robustness of the results presented from this Cayenne Hospital follow-up. However, recurrent cases could be underestimated in miners as they rarely complete the follow-up regardless of the medical recommendations so they can rapidly travel back into the deep forest (22).

With an endpoint at D28, eight patients experienced recurrent parasitemia (4 with late clinical failure [LCF] and 4 with late parasitological failure [LPF])—all after D14. No difference between years of infection was observed (P = 0.5542 [Table 1]). Therapeutic efficacy was stable for a 7-year period, suggesting that drug pressure on the parasite population did not participate in a rapid spread of resistance through the parasite population. The only significant difference between the adequate clinical and parasitological response (ACPR) group and the group experiencing recurrent parasitemia was history of malaria (P = 0.0022). Therefore, in the absence of systematic PQ prescription, this observation could suggest a large part of recurrence was due to relapses.

**P. vivax** resistance occurs at the minimum prevalence of 1.2%. Two out of five analyzable failures on the D28 follow-up had a drug level that normally kills or at least suppresses parasite multiplication (M513, 146 ng/ml on D20; N518, 604 ng/ml on D29 [Table 2]). After comparison of plasma dosages of CQ with those of samples associated with ACPR around the same day of follow-up, these concentrations were compatible with an efficient antimalarial activity on sensitive parasites (Fig. 2). These results demonstrated that parasite resistance to CQ was present in at least 1.2% (95% CI, 0 to 2.6%; n = 2/172) of the patients in French Guiana. The six microsatellite markers showed homologous genetic background of parasites at D0 and the day of treatment failure in these cases (DF).

Until 1995, chloroquine was also recommended to treat *P. falciparum* (3). However, resistance of *P. vivax* has not evolved as quickly as for *P. falciparum*. French Guiana is the second country of the Guiana Shield reporting cases of CQR to *P. vivax* after the Republic of Guyana. In South America, resistance was reported in Manaus, Brazil (20), in more than 10% of cases after CQ treatment. Other studies also reported CQR in Amazonia but after CQ + PQ treatment: 5.2% in Manaus, Brazil (CQ + PQ) (21), 1.1% in Oiapoque, Brazil (CQ + PQ) (19), and 6.5% in Bolivia (CQ + PQ) (23). However, comparison with these results is impossible because of the potentialization of CQ action by PQ when the drugs are coadministered (24). In France, coadministration is rare because PQ is never given without a preliminary evaluation of the G6PD activity of the patient by quantitative laboratory methods.

Beside the eight treatment failures observed during the D28 follow-up, eight cases of treatment failures were also identified from the D35 extended follow-up or patients who were not followed but had returned to the hospital because of fever (Fig. 1). Biological analyses were conducted to identify resistance in seven of these samples because for one case, plasma and/or DNA was missing. In this context, one new case of *P. vivax* resistance was identified with a CQ + dCQ plasma concentration ([CQ]) of 269 ng/ml (Fig. 2). The other six were associated with very low or undetectable chloroquine concentration despite the fact that until day 35, the chloroquine concentration should
| ID   | Parasitemia (%) | Treatment response on D28 | CQ+dCQ (ng/ml) | Pvmdr1 copy no. | K10 Insertion of pvcrt-o | Sizes of microsatellite loci |
|------|-----------------|---------------------------|----------------|-----------------|-------------------------|-----------------------------|
| BT (°C) | Treatment | | | | | |
| N183 | D0 39.0 | 0.1000 | CQ | ND | A/WT | Yes | 92–196, 109–117, 206–213, 213–272, 182–186, 112–120, 199–207 |
| D22 | 36.1 | 0.1000 | No | LPF | ND | ND | ND |
| D30 | 39.0 | 0.1800 | CQ | ND | NR | NR | NR |
| D0 | 38.7 | 0.3000 | CQ | ND | NR | NR | NR |
| D14 | 38.1 | 0.0300 | CQ | ND | NR | NR | NR |
| D22 | 36.9 | 0.1000 | CQ | ND | NR | NR | NR |
| D30 | 39.0 | 1.2000 | CQ | ND | NR | NR | NR |

**Subtherapeutic chloroquine concentrations observed**

| ID   | Parasitemia (%) | Treatment response on D28 | CQ+dCQ (ng/ml) | Pvmdr1 copy no. | K10 Insertion of pvcrt-o | Sizes of microsatellite loci |
|------|-----------------|---------------------------|----------------|-----------------|-------------------------|-----------------------------|
| N183 | D0 38.9 | 0.7600 | CQ | ND | A/WT | Yes | 200, 129, 213, 186, 108, 207 |
| D0 | 39.6 | 0.1500 | CQ | ND | A | 0.97 ± 0.04 |
| D14 | 38.0 | 0.0000 | CQ | ND | A | 0.78 ± 0.05 |
| D28 | 39.0 | 0.1400 | CQ | ND | A | 0.78 ± 0.03 |

**Chloroquine therapeutic failure associated with parasite resistance**

| ID   | Parasitemia (%) | Treatment response on D28 | CQ+dCQ (ng/ml) | Pvmdr1 copy no. | K10 Insertion of pvcrt-o | Sizes of microsatellite loci |
|------|-----------------|---------------------------|----------------|-----------------|-------------------------|-----------------------------|
| M513 | D0 38.0 | 0.2500 | CQ | ND | A | 0.97 ± 0.04 |
| D2 | 36.0 | 0.0005 | CQ | ND | A | 0.78 ± 0.03 |

**Recurrence in patients followed D28 = 2 days**

| ID   | Parasitemia (%) | Treatment response on D28 | CQ+dCQ (ng/ml) | Pvmdr1 copy no. | K10 Insertion of pvcrt-o | Sizes of microsatellite loci |
|------|-----------------|---------------------------|----------------|-----------------|-------------------------|-----------------------------|
| N183 | D0 38.8 | 0.7600 | CQ | ND | A/WT | Yes | 92–196, 109–117, 206–213, 213–272, 182–186, 112–120, 199–207 |
| D0 | 39.5 | 0.2000 | CQ | ND | A | 0.97 ± 0.03 |

**Not determinable reasons**

| ID   | Parasitemia (%) | Treatment response on D28 | CQ+dCQ (ng/ml) | Pvmdr1 copy no. | K10 Insertion of pvcrt-o | Sizes of microsatellite loci |
|------|-----------------|---------------------------|----------------|-----------------|-------------------------|-----------------------------|
| N183 | D0 38.8 | 0.7600 | CQ | ND | A/WT | Yes | 92–196, 109–117, 206–213, 213–272, 182–186, 112–120, 199–207 |
| D0 | 39.5 | 0.2000 | CQ | ND | A | 0.97 ± 0.03 |

(Continued on next page)
| ID   | BT (°C) | Parasitemia (%) | Treatment | Treatment response on D28 | CQ+dCQ (ng/ml) | Pvmdr1 copy no. | Pvmdr1 sequence | Pvcrt-o copy no. | Insertion of Pvcrt-o K10 | Sizes of microsatellite loci 13.239, 3.27, 5.504, 11.162, MS9, and MS8 |
|------|---------|-----------------|-----------|--------------------------|----------------|----------------|----------------|----------------|--------------------------|--------------------------------------------------------------------------------|
| D0   | 39.2    | 1.0000          | CQ        | ACPR                     | NR             | NR             | NR             | NR             | NR                      |                                                                                   |
| D35  | 40.0    | 0.6000          | CQ        | Lost                     | <10 + <10      | 1 A/WT         | 1.48 ± 0.04    | No             | 192, 109–129-149, 213, 186, 116–120-124, 207 |
| P092 | D0      | 39.0             | 0.4000    | Lost                     | <10 + <10      | 1 A            | 0.83 ± 0.09    | No             | 188, 109, 213, 186, 116–120-124, 199 |
| P213 | D0      | 39.0             | 0.6000    | Lost                     | <10 + <10      | 2 A            | 1.33 ± 0.21    | Yes            | 192, 109, 213, 186, 116–120-124, 199 |
| P267 | D0      | 38.3             | 0.1000    | Lost                     | <10 + <10      | 1 A            | 0.75 ± 0.03    | Yes            | 196, 121, 262, 186, 116–120-124, 215-219 |
| Q332 | D0      | 37.6             | 0.3500    | Lost                     | <10 + <10      | 1 A            | 1.11 ± 0.03    | No             | 192-196, 97–145, 206–213, 182–186, 112–116–120, 207 |
| M226 | D0      | 38.6             | 0.1800    | Lost                     | <10 + <10      | 1 A            | 1.02 ± 0.02    | No             | 192, 129, 59-206, 182, 124–128–132, 199 |
| D7   | 38.6    | 0.2000          | CQ        | Lost                     | <10 + <10      | 1 A            | 0.74 ± 0.13    | No             | Identical to D0          |
| D26  | 38.6    | 0.0500          | CQ        | Lost                     | <10 + <10      | 2 A            | 0.64 ± 0.06    | Yes            | Identical to D0          |

*., Guy-A; ACPR, adequate clinical and parasitological response; BT, body temperature; D0 to D35, days 0 to 35; dCQ, desethylchloroquine; ID, identification; LCF, late clinical response; Lost, lost to follow-up; LPF, late parasitological response; ND, not determinable; No, no modification of the treatment; NR, not received; Pvmdr1, Plasmodium vivax multidrug resistance 1 gene; WT, wild type. Pvcrt-o gene copy numbers have been determined based on two technical replicates.

**Reasons for recurrence not determinable because no drug concentration was available.

*Recurrence linked to subtherapeutic concentration of drug: the CQ+dCQ concentration was <100 ng/ml.

*Reasons for recurrence with extended follow-up (D31 to D33) or patients without follow-up (D1 to D35).

*Values separated with a hyphen mean that this microsatellite has a multiclonal structure represented by the different observed sizes at the studied locus.
normally be above 100 ng/ml (8). Therefore, these recurrent cases of parasitemia were probably due to relapses. In fact, the Chesson strain circulating in South America generates relapses around D28 (25). With a limited and variable [CQ] in blood after D28, these results underlined the importance of ending the follow-up at D28 during a chloroquine efficacy study in case of infection by a Chesson strain, before the occurrence of natural relapses.

A relevant molecular marker is required to easily monitor *P. vivax* resistance to chloroquine. The genotype and copy number of the *pvmdr1* and *pvcrt-o* genes were analyzed in the 13 available pairs of samples (D0-DF) associated with a treatment failure whatever the reason. The *pvmdr1* mutation T958M previously described as prevalent in French Guiana was identified in 86.5% of the samples (26). No difference within D0-DF pairs of samples was observed. The mutations Y976F and F1076L were absent even in the chloroquine-resistant parasites associated with the three chloroquine treatment failures (M226, M513, and N518). The *pvmdr1* copy number was 2 in seven samples (29.2%), without any associated with the *in vivo* phenotype. The general percentage of multicopy samples was significantly higher than what was observed in the same period in the general parasite population of French Guiana (12.8%; n = 43/335; P < 0.05) (data not shown). The *pvcrt-o* part of the gene including an extra amino acid at position 10 (K10) has been sequenced in D0-DF pairs as well as in a sample set of 28 samples in order to compare to the genetic profile specific of French Guiana (27). No difference within D0-DF pairs of samples was observed for the *pvcrt-o* K10 insertion (Table 2). This polymorphism was observed in 57.1% of samples (95% CI, 38.8 to 75.5%; n = 16/28 [Fig. 3A]). The *pvcrt-o* gene was monocopy in the sample set, whether associated or not with therapeutic failure (Fig. 3B).

The *pvmdr1* and *pvcrt-o* genotypes (sequence and copy number) were not associated with CQR in French Guiana. As previously described, these markers are probably not markers of chloroquine resistance or even minor determinants for resistance (13). However, their expression levels have been described as being associated with *P. vivax* resistance in the Amazon region (17). These have not been analyzed in this study because of the absence of RNA collection.

*P. vivax* resistance to CQ exists in French Guiana and needs to be controlled using primaquine. *P. vivax* CQR exists in French Guiana but at a low prevalence.
Therefore, these results do not justify a change in treatment regimen and regional recommendations. However, a better implementation of the coadministration of CQ and PQ is crucial to avoid the spread of these resistant parasites. To do so, the use of rapid quantitative screening methods for G6PD deficiency and the systematic recording of patient’s G6PD status should be considered.

**MATERIALS AND METHODS**

**Study site, patients, and treatment monitoring.** All cases of malaria confirmed by microscopy and an axillary temperature of ≥37.5°C or subjects with a history of fever during the past 24 h between March 2009 and October 2015 were retrospectively included in this study. Cases were excluded from the analysis under the following circumstances: (i) cases of severe *P. vivax* malaria according to the WHO definitions regarding *P. falciparum*, (ii) the presence of concomitant infectious disease or comorbid conditions, (iii) pregnant women, and (iv) patients not treated by CQ (Nivaquine) at a total dose of 25-mg base/kg body wt (10-mg base/kg body wt on D0, 10 mg/kg body wt on D1, and 5 mg/kg body wt on D2). Treatment administration was not supervised.

Patients treated by antimalarial drugs followed the common clinical practices of Cayenne Hospital. Patients were invited to come back for clinical and biological examinations on D3, D7, D14, D21, and D28. Hospitalized patients were also followed on D1 and D2. Patients who did not experience TF during the standard 28-day follow-up had an extended follow-up until D35. Additionally, patients were informed to come back to the hospital in case of symptom resurgence without waiting for the next scheduled visit.

Any patients who did not attend the visit on D28 were classified as lost to follow-up. However, patients who attended the D28 visit but were not followed for more than 2 weeks during this period were withdrawn from the analysis. Those who missed one appointment but had no relapse detected during the preceding and following appointments were considered to have negative parasitemia for the missed appointment and were not withdrawn from the study. Finally, patients (i) treated with PQ, (ii) misdiagnosed on D0, or (iii) diagnosed with another malaria species during the follow-up were also excluded.

General baseline data were also recorded in the patients’ files: (i) sex, (ii) age, (iii) history of malaria in the last 3 months, (iv) onset of symptoms, (v) weight, (vi) history of travel during the 4 weeks preceding the consultation, and (vii) antimalarial chemoprophylaxis.

**Classification of treatment responses.** Treatment responses were classified according to the WHO guidelines (5), as early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), or adequate clinical and parasitological response (ACPR). Treatment failures (TFs) included ETF, LCF, and LPF.

In order to properly characterize parasite resistance, biological analyses were conducted on samples from all patients who had experienced recurrent parasitemia, including (i) patients presenting TF during the standard or extended follow-up and (ii) patients not followed but diagnosed with TF outside the protocol.

**Measurement of antimalarial drug concentration.** CQ+dcQ plasma concentrations ([CQ]) were measured at the day of treatment failure (DF) by liquid chromatography combined with tandem mass spectrometry (TSQ Quantum Ultra; Thermo Fisher, France) as previously reported by Hodel et al., with minor modifications (28). Using OASE 96-well microplates (Waters, France), 100 μl of plasma was mixed with acetoniitrile. Proteins and phospholipids were eliminated by positive pressure using the 96-Positive Pressure system (Waters, France). Eluents were evaporated at room temperature. Dry residues were dissolved in 100 μl of mobile phase, and 10 μl was injected into the system. For both molecules, the method was linear between 10 and 1,000 ng/ml. For homemade and external controls from the
WorldWide Antimalarial Resistance Network, coefficients of variation were below 10% and bias values were ±10%.

A plasma concentration greater than 100 ng/ml was considered adequate up to D35 (8). When the measurement was conducted before D28, results were compared to drug concentrations observed in patients with adequate clinical and parasitological responses on the same day of follow-up. Then TFs were classified as (i) TF due to subtherapeutic concentration if [CQ] is <100 ng/ml, (ii) TF due to resistance if [CQ] is >100ng/ml, and (iii) “unclassified” if no drug concentration was available.

**DNA extraction.** Parasite DNA was extracted from 200 μl of blood using QIAamp genomic DNA kits according to the manufacturer’s instructions (Qiagen, Courtaboeuf, France).

**Microsatellite characterization.** The genetic background of parasites was compared between D0 and DF using a set of six microsatellite loci (3.27, 8.504, 11.162, 13.239, MS8, and MS9) (29–31). This panel was selected because of its high polymorphism in the general parasite population of French Guiana (0.62 < expected heterozygosity [H] < 0.68 [data not shown]). Microsatellites were analyzed by nested PCR following previously described procedures and with the primers listed in Table S1 in the supplemental material. The homologous genetic profile (based on the allelic sizes) between D0 and DF suggested a recrudescence of resistant forms, while heterogeneous profiles suggested a new infection. However, whatever the genetic profile, relapses could not be excluded.

**Analysis of pvmdr1 and pvcrt-o as putative molecular markers of CQR.** The *pvmdr1* and *pvcrt-o* gene sequences and gene copy numbers were analyzed on all isolates associated with a recurrence collected on D0 and DF according to the previously described methods (26, 27). PCR products were visualized using 2% agarose gel electrophoresis before a double-strand Sanger sequencing. The generally accepted range of a sequencing error limit is 10% for the minor genotype (32). Sequences were analyzed with Geneious 8.1.7 software (Biomatters, Ltd., Auckland, New Zealand). Positive and negative controls were systematically included in each series of genotyping. In the absence of known published genotype for the *pvcrt-o* gene, a sample set of 28 samples associated with adequate treatment response has also been analyzed in order to compare the results.

**Ethical and consent approval.** Data and samples were all obtained as standard medical care for any patient presenting fever on hospital admission in French Guiana. According to the French legislation (article L.1211-2 of the French Public Health Code), biobanking and secondary use for scientific purpose of data and human clinical samples are possible as long as the corresponding patients are informed and have not given any objection. In our study, information was given to every patient through the Cayenne Hospital brochure, and no immediate or delayed patient opposition was reported. In cases involving infants, parents or guardians had to report their opposition to the hospital. Samples received from the National Reference Center (NRC) biobank were approved and registered by the French Ministry for Research and the French Ethics Committee (declaration no. DC-2010-1223, collection Nu2). According to the French legislation, no institutional review board approval was required.

**Statistical analysis.** Data were collected with Microsoft Excel 2016 (Microsoft, Redmond, WA, USA). Statistical computing was analyzed with R software (R Foundation, Vienna, Austria). Percentages were calculated according to the total number of patients followed up to D28 with a 95% confidence interval. Medians were associated with range. The Wilcoxon test and Fisher’s test were performed to compare data between ACPR and TF after 28 ± 2 days of follow-up. A *P* value of <0.05 was considered significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02116-18.

**SUPPLEMENTAL FILE 1**, PDF file, 0.7 MB.

**ACKNOWLEDGMENTS**

The National Reference Center for Malaria in French Guiana acknowledges its partners involved in diagnosis and care of malaria in French Guiana, who collaborate for several years in malaria surveillance and allow generation of precious data for public health and malaria control. The authors are grateful to Hervé Bogreau and Antoine Adde for support and advice during the statistical analyses and Marie-Hélène Rodier, Christine Imbert, Blandine Rammaert, and Lucie Sedille for comments on the manuscript.

This work was supported by Santé Publique France (French Ministry of Health) and the French Ministry for National Education, Higher Education and Research. The Regional Health Agency of French Guiana financed C.H. We acknowledge an Investissement d’Avenir grant from the Agence Nationale de la Recherche (CEBA: ANR-10-LABX-25-01).

P.R. is a staff member of the World Health Organization. P.R. alone is responsible for the views expressed in this publication, and they do not necessarily represent the decisions, policy, or views of the World Health Organization.

L.M., E.L., P.R., and F.D. conceived and coordinated the study. F.D., R.N., P.A., G.W.,
L.E., and M.D. collected clinical data. M.D. and D.B. collected biological data. Y.L., B.V., L.M., and S.P. confirmed the diagnostic data and updated the biobank collection. P.H., C.H., and B.V. carried out the molecular genetic studies. C.H., L.M., P.R., and S.P. analyzed the data. C.H. carried out statistical analysis. C.H. and L.M. wrote the manuscript. P.H., S.P., L.E., P.R., and F.D. reviewed the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. WHO. 2018. World malaria report. World Health Organization, Geneva, Switzerland.
2. Carme B. 2005. Substantial increase of malaria in inland areas of eastern French Guiana. Trop Med Int Health 10:154–159. https://doi.org/10.1111/j.1365-3156.2004.01365.x
3. Musset L, Pelleau S, Girod R, Ardillon V, Carvalho L, Dusfour I, Gomes MS, Djossou F, Legrand E. 2014. Malaria on the Guiana Shield: a review of the situation in French Guiana. Mem Inst Oswaldo Cruz 109:525–533. https://doi.org/10.1590/0074-0276140031.
4. Cellule-interrégionale d’Épidémiologie de Guyane. 2019. Situation du paludisme en Guyane: point du 16 Avril 2019. http://invs.santepubliquefrance.fr/Publications-et-outils/Points-epidemiologiques/Tous-les numeros/Guyane/2019/Situation-epidemiologique-du-paludisme-en-Guyane-Point-au-16-avril-2019.
5. WHO. 2015. Guidelines for the treatment of malaria. World Health Organization, Geneva, Switzerland.
6. WHO. 2015. Control and elimination of Plasmodium vivax malaria—a technical brief. World Health Organization, Geneva, Switzerland.
7. Imwong M, Snounou G, Pukrittayakamee S, Tanomsing N, Kim JR, Nandy A, Guthmann JP, Nosten F, Carlton J, Looareesuwan S, Nair S, Sudimack D, Day NP, Anderson TJ, White NJ. 2007. Relapses of Plasmodium vivax infection usually result from activation of heterologous hypnozoites. J Infect Dis 195:927–933. https://doi.org/10.1086/512241.
8. Baird JK, Lekshana B, Masbar S, Fryauff Dj, Sutanto I, Djossou F, Legrand E. 2014. Malaria on the Guiana Shield: a review of the transmission dynamics of Plasmodium vivax in two different biogeographical regions of French Guiana. Mem Inst Oswaldo Cruz 109:525–533. https://doi.org/10.1590/0074-0276140031.
9. WHO. 2009. Methods for surveillance of antimarial drug efficacy. World Health Organization, Geneva, Switzerland.
10. Ehman FC, Ellis JM, Young MD. 1945. Plasmodium Vivax Chesson strain. Science 101:377. https://doi.org/10.1126/science.101.2624.377.
11. Breja S, Medlin VR, Grisoni L, Udomsang-Petch R, Sutanto I, Peeling RW, Picot S. 2005. Identification of the Plasmodium vivax mdr1-like gene (pvmdr1) and analysis of single-nucleotide polymorphisms among isolates from different areas of endemicity. J Infect Dis 191:272–277. https://doi.org/10.1086/426830.
12. Schousboe ML, Ranjitkar S, Rajakaruna RS, Morales Phillips EJ, Keystone JS, Kain KC. 1996. Evidence for different mechanisms of chloroquine resistance in 2 Plasmodium species that cause human malaria. J Infect Dis 183:1653–1661. https://doi.org/10.1086/320707.
13. Melo GC, Monteiro WM, Siqueira AM, Silva SR, Magalhães BM, Alencar AC, Kuehn A, Portillo HA, Fernandez-Recorcia C, Lacerda MV. 2014. Expression levels of pvcr-o and pvmr-1 are associated with chloroquine resistance and severe Plasmodium vivax malaria in patients of the Brazilian Amazon. PLoS One 9:e105922. https://doi.org/10.1371/journal.pone.0105922.
14. Phillips EJ, Keystone JS, Kain KC. 1996. Failure of combined chloroquine and high-dose primaquine therapy for Plasmodium vivax malaria acquired in Guyana, South America. Clin Infect Dis 23:1171–1173. https://doi.org/10.1093/clinids/23.5.1171.
15. Gomes MS, Vieira JL, Machado RL, Nacher M, Stafani A, Musset L, Legrand E, Menezes RA, Júnior AA, Sousa AP, Couto VS, Couto AA. 2015. Efficacy in the treatment of malaria by Plasmodium vivax in Oiapoque, Brazil, on the border with French Guiana: the importance of control over external factors. Malar J 14:402. https://doi.org/10.1186/s12936-015-0929-7.
16. de Santana Filho FS, Arcanjo AR, Chehuan YM, Costa MR, Martinez-Espinoza FE, Vieira JL, Barbosa MG, Alecriden WD, Alecriden MG. 2007. Chloroquine-resistant Plasmodium vivax, Brazilian Amazon. Emerg Infect Dis 13:1125–1126. https://doi.org/10.3201/eid1307.061386.
17. Marques MM, Costa MR, Santana Filho FS, Vieira JL, Nascimento MT, Brasil LW, Nogueira F, Silveira H, Reyes-Lecca RC, Monteiro WM, Lacerda MV, Alecriden MG. 2014. Plasmodium vivax chloroquine resistance and anemia in the western Brazilian Amazon. Antimicrob Agents Chemother 58:342–347. https://doi.org/10.1128/AAC.02279-12.
18. Douine M, Lazrek Y, Blanchet D, Pelleau S, Channil R, Corlin F, Hureau L, Volney B, Hiwat W, Vreden S, Djossou F, Demar M, Nacher M, Musset L. 2018. Predictors of antimarial self-medication in illegal gold miners in French Guiana: a pathway towards artesminins resistance. J Antimicrob Chemother 73:231–239. https://doi.org/10.1093/jac/dko343.
19. Ane A, Moscou M, Lagan A, Garcia C, Melgar V, Cuba M, Gutierrez S, Ascasci C. 2015. Resistance of infection by Plasmodium vivax to chloroquine in Bolivia. Malar J 14:2461. https://doi.org/10.1186/s12936-015-0774-4.
20. Naing C, Aung K, Win DK, Wah MJ. 2010. Efficacy and safety of chloroquine for treatment in patients with uncomplicated Plasmodium vivax infections in endemic countries. Trans R Soc Trop Med Hyg 104:695–705. https://doi.org/10.1016/j.trstmh.2010.08.009.
21. Hanf M, Stephani A, Basurko C, Nacher M, Carme B. 2009. Determination of the Plasmodium vivax relapse pattern in Camopi, French Guiana. Malar J 8:278. https://doi.org/10.1186/1475-2875-8-278.
22. Fawzy E, Musset L, Pelleau S, Volney B, Casters J, Caro V, Menard D, Briolant S, Legrand E. 2016. Plasmodium vivax multidrug resistance-1 gene polymorphism in French Guiana. Malar J 15:540. https://doi.org/10.1186/s12936-016-1595-9.
23. Silva SR, Almeida AC, da Silva GAV, Ramosawamy R, Lopes SCP, Siqueira AM, Costa GL, Sousa TN, Vieira JL, Lacerda MVG, Monteiro WM, de Melo GC. 2018. Chloroquine resistance is associated to multi-copy pvcr-o gene in Plasmodium vivax malaria in the Brazilian Amazon. Malar J 17:267. https://doi.org/10.1186/s12936-018-2411-3.
24. Hodel EM, Zanolari B, Mercier T, Biollaz J, Keiser J, Olliaro P, Genton B, Decosterd LA. 2009. A single LC-tandem mass spectrometry method for the simultaneous determination of 14 antimarial drugs and their metabolites in human plasma. J Chromatogr B Analyst Technol Biomed Life Sci 877:867–886. https://doi.org/10.1016/j.jchromb.2009.02.006.
25. Ferreira MU, Karunaweera ND, da Silva-Nunes M, da Silva NS, Wirth DF, Hartl DL. 2007. Population structure and transmission dynamics of Plasmodium vivax in rural Amazonia. J Infect Dis 195:1218–1226. https://doi.org/10.1086/516285.
26. Imwong M, Nair S, Pukrittayakamee S, Sudimack D, Williams JT, Mayxay...
M, Newton PN, Kim JR, Nandy A, Osorio L, Carlton JM, White NJ, Day NP, Anderson TJ. 2007. Contrasting genetic structure in *Plasmodium vivax* populations from Asia and South America. Int J Parasitol 37:1013–1022. https://doi.org/10.1016/j.ijpara.2007.02.010.

31. Rezende AM, Tarazona-Santos E, Fontes CJ, Souza JM, Couto AD, Carvalho LH, Brito CF. 2010. Microsatellite loci: determining the genetic variability of *Plasmodium vivax*. Trop Med Int Health 15:718–726. https://doi.org/10.1111/j.1365-3156.2010.02535.x.

32. Rohlin A, Wernernson J, Engwall Y, Wiklund L, Björk J, Nordling M. 2009. Parallel sequencing used in detection of mosaic mutations: comparison with four diagnostic DNA screening techniques. Hum Mutat 30:1012–1020. https://doi.org/10.1002/hum.20980.