ORIGINAL RESEARCH ARTICLE

Ex-vivo Sensitivity of *Plasmodium falciparum* to Common Anti-malarial Drugs: The Case of Kéniéroba, a Malaria Endemic Village in Mali

Karim Traoré[1] · Seidina A. S. Diakité[1] · Sekou Bah[2] · Drissa S. Konaté[1] · Djeneba Dabitao[1] · Ibrahim Sanogo[1] · Modibo Sangaré[1] · Souleymane Dama[1] · Bourama Keita[1] · Mory Doumbouya[1] · Merepen A. Guindo[1] · Seydou Doumbia[1] · Mahamadou Diakité[1]

Abstract

Background In 2006, the National Malaria Control Program in Mali recommended artemisinin-based combination therapy as the first-line treatment for uncomplicated malaria. Since the introduction of artemisinin-based combination therapy, few reports are available on the level of resistance of *Plasmodium falciparum* to the most common anti-malarial drugs in Mali.
Methods From 2016 to 2017, we assessed the ex-vivo drug sensitivity of *P. falciparum* isolates in Kéniéroba, a village located in a rural area of southern Mali. We collected *P. falciparum* isolates from malaria-infected children living in Kéniéroba. The isolates were tested for ex-vivo sensitivity to commonly used anti-malarial drugs, namely chloroquine, quinine, amodiaquine, mefloquine, lumefantrine, dihydroartermisinin, and piperaquine. We used the 50% inhibitory concentration determination method, which is based on the incorporation of SYBR® Green into the parasite's genetic material.
Results *Plasmodium falciparum* isolates were found to have a reduced ex-vivo sensitivity to quinine (25.7%), chloroquine (12.2%), amodiaquine (2.7%), and mefloquine (1.3%). In contrast, the isolates were 100% sensitive to lumefantrine, dihydroartermisinin, and piperaquine. A statistically significant correlation was found between 50% inhibitory concentration values of quinine and amodiaquine ($r = 0.80$; $p < 0.0001$).
Conclusions *Plasmodium falciparum* isolates were highly sensitive to dihydroartermisinin, lumefantrine, and piperaquine and less sensitive to amodiaquine ($n = 2$), mefloquine ($n = 1$), and quinine ($n = 19$). Therefore, our data support the previously reported increasing trend in chloroquine sensitivity in Mali.

1 Background

The resistance of *Plasmodium falciparum* to common anti-malarial drugs represents a serious hurdle for malaria control in endemic countries [1]. *Plasmodium falciparum* has developed resistance to low-cost and well-tolerated anti-malarial drugs such as chloroquine (CQ), amodiaquine (AQ), antifolates, and mefloquine (MQ) [2–5]. This has led the World Health Organization and National Malaria Control Program (NMCP) to recommend artemisinin-based combination therapy (ACT) for malaria treatment [6]. There are two main reasons for using ACT in the clinical care of malaria. First, ACT relies on the high efficacy of artemisinin, which is a drug known to suppress disease progression in *P. falciparum*-infected individuals at an earlier stage of infection. Second, combining artemisinin with other anti-malarial drugs will likely delay the emergence and spread of parasites that are resistant to artemisinin. This is an important consideration, given that *P. falciparum* isolates that are resistant to artemisinin-derivative drugs have been detected in Southeast Asia [7, 8]. Specifically, mutations in the *kelch* (*K13*) gene have been associated with resistance to artemisinin in vitro in Asia [9–11]. To our knowledge, such resistance to artemisinin has not been reported in Sub-Saharan Africa yet, and ACT is still very effective in that part of the continent [9, 12]. However, Sub-Saharan Africa remains under threat because of the widespread use of ACT and the increasing intercontinental human migrations. In addition, the circulation of sub-standard or counterfeit drugs combined with

✉ Karim Traoré
ktraore@icermali.org

[1] Malaria Research and Training Center, Mali International Center for Excellence in Research (Mali-ICER), University of Sciences, Techniques and Technologies of Bamako (USTTB), BP 1805, Bamako, Mali

[2] Hospital of Point-G/University of Sciences, Techniques and Technologies of Bamako (USTTB), Bamako, Mali

poor adherence of patients to antimalarial treatment may contribute to a rapid selection of resistant malaria parasites in Sub-Saharan Africa.

The Malian NMCP adopted ACT as the first-line treatment for uncomplicated malaria in 2006. Notably, artemether-lumefantrine (LUM) and AQ-artesunate are used for uncomplicated malaria treatment in Mali. In addition, three drugs namely, artesunate, artemether, and quinine (QN) were designated to be used in the clinical management of severe malaria via the intravenous route. It is therefore necessary to perform regular monitoring of antimalarial drug resistance in this setting. The ex-vivo drug susceptibility testing is one of the most efficient indirect approaches to assess the efficacy of anti-malarial drugs. Few data are available on in-vitro and ex-vivo efficacy of artemisinin alone or in association with other ACT drugs on isolates circulating in Mali. In 2017, Dama et al. reported a reduced ex-vivo susceptibility of *P. falciparum* after oral artemether-LUM treatment in Mali. This study did not include key anti-malarial drugs such as CQ, QN, AQ, MQ, and piperaquine (PPQ). Therefore, additional studies are needed for a comprehensive analysis of drug resistance during malaria treatment to help national and international policy makers in providing evidence-based guidance about clinical management. Here, we report ex-vivo sensitivity of *P. falciparum* isolates to seven (7) common antimalarial drugs in Kéniéroba, in southern Mali.

2 Materials and Methods

2.1 Collection of *Plasmodium falciparum* Isolates

From June 2016 to October 2017, *P. falciparum* isolates were collected from patients with malaria aged between 6 months and 18 years in Kéniéroba, a village located in the Sudano-Guinean area of Mali (12° 6′ 50″ N and 8° 19′ 58″ W). Study participants were enrolled at the health center of Kéniéroba. The inclusion criterion was a *P. falciparum* mono-infection with a parasitemia between 1000 and 100,000 parasites per microliter. We collected 2–3 mL of venous blood samples on citric acid and a dextrose collection tube (ACD tubes) from each patient before treatment initiation, according to the NMCP's recommendations.

2.2 Preparation of Anti-malarial Drugs

Plasmodium falciparum isolates were tested using seven common anti-malarial drugs: CQ, QN, AQ, MQ, LUM, dihydroartemisinin (DHA), and PPQ. All drugs were obtained from Sigma. A stock solution of CQ diphosphate, QN, MQ, and DHA were prepared in 70% ethanol. Amodiaquine and LUM were initially dissolved in methanol

while PPQ was dissolved in lactic acid 0.5% first and then in dimethyl sulfoxide. Two-fold serial dilutions were prepared using sterile distilled water and distributed in duplicate into 96-well flat-bottom plates. Final concentration ranges of 2.44–2500 nM for CQ, 4.88–5000 nM for QN, 1.22–1250 nM for MQ, 1.22–1250 nM for AQ, 0.34–350 nM for LUM, 0.10–100 nM for DHA, and 0.98–1000 nM for PPQ. Fifty microliters of each diluted anti-malarial drug were added in a 96-well plate in duplicates. The 96-well plate was first dried up in the ambient air, then maintained at 4 °C, and finally prepared for the culture within 2 weeks. The suitability of the prepared 96-well plate for in-vitro testing was continuously monitored using reference strain 3D7 [13, 14] during the test.

2.3 Culture of the *P. falciparum* Isolates

Briefly, 2–3 mL of whole blood was obtained by venipuncture from each patient with *P. falciparum* mono-infection and transported to the laboratory in Bamako, which is located within 2 h of our study site in Kenieroba. The plasma was removed after centrifugation of the whole blood. The cell pellet was washed three times with incomplete RPMI 1640 medium (Gibco™; Invitrogen Corporation, Carlsbad, California, USA) buffered with 25 mM of HEPES (5.95 g/L) followed by a centrifugation at 2000 rpm for 5 min. Parasites were tested directly without culture adaptation. The suspension of parasites was distributed in 96-well plates preloaded with antimalarial drugs (as described above). Culture plates were incubated at 37 °C and 5% CO_2 for 72 h. After this incubation (which corresponds to the schizont stage), a blood smear was prepared to confirm healthy growth of controls (drug-free parasites). Samples were then stored at − 20 °C overnight.

2.4 Malaria SYBR® Green I-Based Fluorescence Assay

The Malaria SYBR® Green I-Based Fluorescence Assay was performed as described in the modified version of the Smilkstein and colleagues paper [15] by Johnson et al. [16]. The plate was thawed for 1–2 h at room temperature to lyse the cells. Then, 100 μL of 0.002% SYBR® Green lysis buffer (10 mL of lysis buffer plus 2 μL of SYBR® Green) was added in each well. The plate was then covered with aluminum foil and kept under agitation using a plate shaker at room temperature for 30 min. The parasite growth was determined by measuring fluorescence of the SYBR® Green incorporated into nucleic acids of the parasites. The plate was read using a fluorometer plate reader using a 485-nm excitation filter and a 538-nm emission filter. The IC_{50}, defined as a drug concentration at which the SYBR® Green

signal was 50% of that measured in drug-free control wells, was calculated by using the In-Vitro Analysis and Reporting Tool (IVART) software.

The IC_{50} threshold value measuring the parasite's sensitivity to a given drug was defined as reported in previous studies. These values were set at 100 nM for the CQ [17, 18], 30 nM for MQ [17–19], 80 nM for AQ [18, 20], 150 nM for LUM [18, 19, 21], 800 nM for QN [18–20], 12 nM for DHA [18], and 135 for PPQ [22, 23]. The SYBR® Green I-based method for determination of drug sensitivity concentrations provides results that are similar to other traditional methods, suggesting that this method can be routinely used to conduct surveillance for drug resistance with fresh or cultured parasites [24]. For example, Smilkstein and co-workers did not find a difference between the IC_{50} of standard anti-malarial drugs determined by the mean of a radio-isotopic approach from those generated using the SYBR® Green method [25].

2.5 Statistical Analysis

We analyzed results from samples that had good ex-vivo growth, free of contamination during culture, and with a good fit on the log dose–response curve. The concentration of a given anti-malarial drug that can inhibit the growth of 50% (IC_{50}) of the parasites in culture was estimated from a dose–response curve by non-linear regression analysis using an online program previously described elsewhere [26, 27]. The program generated IC_{50} estimates with associated 95% confidence intervals (CIs). Estimated values with insufficient precision based on the CI were discarded. Geometric mean IC_{50} was calculated for each drug. Cross-susceptibility was analyzed using the Pearson correlation (r) generated by the software GraphPad Prism version 8. A two-sided p value ≤ 0.05 was set as the significance threshold. The strength of correlation (with a significant linear relationship) was classified as moderate, if "$0.3 < r < 0.7$" or strong, if "$r ≥ 0.7$".

3 Results

A total of 100 parasite isolates were prepared for analysis. The IC_{50} was successfully determined for 74 (74%) isolates for CQ, 74 (74%) for QN, 73 (73%) for AQ, 75 (75%) for MQ, 73 (73%) for LUM, 69 (69%) for DHA, and 73 (73%) for PPQ (Table 1). The geometric mean of the IC_{50} was 46.07 nM with a 95% CI range between 4.0 and 724.3 for CQ. Similarly, it was 264.97 nM (39.90–1567.38) for QN, 25.5 nM (5.73–88) for AQ, 13.13 nM (1.82–36.16) for MQ, 10.14 nM (0.82–50.82) for LUM, 0.87 nM (0.34–3.03) for DHA, and 15.86 nM (3.72–35.72) for PPQ (Table 1). The proportion of resistant *P. falciparum*

Table 1 Geometric mean values of 50% inhibitory concentration (IC_{50}) of different anti-malarial drugs

Molecules	Number of experiments	IC_{50} geometric mean (95% CI)
CQ	74	46.07 (4.90–724.3)
QN	74	264.97 (39.90–1567.38)
AQ	73	25.5 (5.73–88)
MQ	75	13.13 (1.82–36.16)
LUM	73	10.14 (0.82–50.82)
DHA	69	0.87 (0.34–3.03)
PPQ	73	15.86 (3.72–35.72)

AQ amodiaquine, *CI* confidence interval, *CQ* chloroquine, *DHA* dihydroartemisinin, *LUM* lumefantrine, *MQ* mefloquine, *PPQ* piperaquine, *QN* quinine

Table 2 Ex-vivo response of isolates of *Plasmodium falciparum* from Kenieroba to antimalarial drugs

Drugs	Cut-off values (nM)	Resistant isolates		Sensitive isolates	
		N	%	N	%
CQ	100	9	12.2	65	87.8
AQ	80	2	2.8	71	97.2
MQ	30	1	1.3	74	98.7
QN	800	19	25.7	55	74.3
LUM	150	0	0	73	100
PPQ	135	0	0	73	100
DHA	12	0	0	69	100

AQ amodiaquine, *CQ* chloroquine, *DHA* dihydroartemisinin, *LUM* lumefantrine, *MQ* mefloquine, *PPQ* piperaquine, *QN* quinine

isolates, as defined in the methods section, were 12.2% (9/74) for CQ, 25.7% (19/74) for QN, 2.7% (2/73) for AQ, and 1.3% (1/75) for MQ. No resistant *P. falciparum* isolates to LUM, DHA, and PPQ were found in this study (Table 2).

A strong positive correlation was observed between the IC_{50} values for QN and AQ ($r = 0.80$; $r^2 = 0.64$, $p < 0.0001$), suggesting an ex-vivo cross-resistance among these drugs. Moderate positive correlations were found between the IC_{50} values of CQ vs AQ ($r = 0.57$, $p < 0.0001$), CQ vs QN ($r = 0.49$, $p < 0.0001$), DHA vs MFQ ($r = 0.35$, $p = 0.002$), LUM vs MQ ($r = 0.5$, $p < 0.0001$), DHA vs PPQ ($r = 0.43$, $p < 0.0001$), and MQ vs PPQ ($r = 0.45$, $p < 0.0001$). A weak positive correlation was observed between CQ vs MQ ($r = 0.29$; $r^2 = 0.08$, $p = 0.008$) and DHA vs LUM ($r = 0.30$, $p = 0.012$) (Table 3). In contrast, negative correlations were observed between IC_{50} values of DHA vs QN ($r = -0.35$, $p = 0.002$), LUM vs QN ($r = -0.36$,

Table 3 Correlation between anti-malarial drug 50% maximal inhibitory concentration values

Drug pairing	Number of experiments	Correlation coefficient (r)	p value
AQ vs CQ	**74**	**0.517**	**< 0.001**
AQ vs DHA	**68**	**− 0.28**	**0.01**
AQ vs LUM	**73**	**− 0.45**	**< 0.001**
AQ vs MQ	74	− 0.04	0.72
AQ vs PPQ	74	0.07	0.55
AQ vs QN	**73**	**0.80**	**< 0.001**
CQ vs DHA	70	0.096	0.41
CQ vs LUM	73	0.036	0.76
CQ vs MQ	**76**	**0.29**	**0.008**
CQ vs PPQ	75	0.21	0.06
CQ vs QN	**73**	**0.49**	**< 0.001**
DHA vs LUM	**67**	**0.30**	**0.01**
DHA vs MQ	70	0.35	0.002
DHA vs PPQ	**68**	**0.43**	**< 0.0001**
DHA vs QN	**67**	**− 0.35**	**0.002**
LUM vs MQ	**74**	**0.5**	**< 0.001**
LUM vs PPQ	73	0.11	0.34
LUM vs QN	**71**	**− 0.36**	**0.002**
MQ vs PPQ	**75**	**0.45**	**< 0.001**
MQ vs QN	73	− 0.02	0.87
PPQ vs QN	72	− 0.10	0.37

Significant correlation values are highlighted in bold

AQ amodiaquine, *CQ* chloroquine, *DHA* dihydroartemisinin, *LUM* lumefantrine, *MQ* mefloquine, *PPQ* piperaquine, *QN* quinine

$p = 0.002$), DHA vs AQ ($r = − 0.28$, $p = 0.01$), and AQ vs LUM ($r = − 0.45$, $p < 0.0001$).

4 Discussion

Ex-vivo assessment of the susceptibility of malaria parasites to antimalarial drugs remains an important component in the surveillance of the efficacy of anti-malarial drugs. As this method is largely independent on clinical factors, it provides information that complements clinical assessment of drug efficacy. The SYBR® Green method for assessing the outcome of the ex-vivo drug sensitivity test was revalidated and used to assess the responses of *P. falciparum* clinical isolates to seven anti-malarial drugs in Mali. To the best of our knowledge, this is the first study reporting the use of SYBR® Green method in Mali. We confirmed that this method is easy to use, reliable, and cheaper, as evidenced by the quick and successful evaluation of ex-vivo sensitivity *P. falciparum* isolates from Kéniéroba to a comprehensive panel of anti-malarial drugs, namely CQ, QN, AQ, MQ, LUM, DHA, and PPQ.

The prevalence of *P. falciparum* parasites with reduced susceptibility to QN was 25.7% in Kéniéroba. This prevalence was higher than those reported in Ghana, in 2011 (19.4% [6/31]) [28] and Senegal in 2017 (9.7% [3/31]) [29]. Globally, similar sensitivity of *P. falciparum* isolates to QN were found (mean $IC_{50} = 264.97$ nM; 95% CI 39.90–1567.38) as in a study conducted in Cote d'Ivoire in 2008 (mean $IC_{50} = 272.12$ nM) by Touré et al. [30]. In contrast, a higher IC_{50} mean value for QN (355.37 nM) was reported by Kwansa-Bentum et al. in 2011 in Ghana [28]. To treat complicated malaria, QN by the intravenous route still remains the third-line treatment in Mali. Oral QN is also a recommended drug to treat uncomplicated malaria during the first trimester of pregnancy in Mali. As a reduced susceptibility in vitro is an early warning of impeding resistance, a very close follow-up of the resistance to QN is necessary in Mali.

In the present study, 12.2% of the *P. falciparum* isolates exhibited a reduced susceptibility to CQ ($IC_{50} \geq 100$ nM). In 2004–6, a higher proportion of CQ-resistant *P. falciparum* isolates (60–69%) was found in Mali [31]; however, we do not know if current circulating *P. falciparum* isolates are now sensitive to CQ because of the reduced selective pressure on that drug after the introduction of ACT in Sub-Saharan Africa. Reports from Malawi and Kenya suggest that likelihood [32–34]. This trend was also found in studies conducted by French scientists, using samples from patients with malaria from Senegal, Mali, Ivory Coast, and Cameroon [35, 36]. The data indicate that it may be possible to re-introduce CQ as the first-line treatment of malaria in Mali because CQ is easily manufactured locally at a very low cost. Hence, we recommend routine monitoring of CQ resistance despite the widespread use of ACT.

Only 2.7% of the parasite isolates exhibited a reduced ex-vivo susceptibility to AQ ($IC_{50} \geq 80$ nM). This was interesting because AQ is one of the most commonly associated molecules with artemisinin in the ACT used in Mali. Higher prevalence of AQ-resistant parasites have been reported in 2017 in Senegal (28.1%) [29] and in India (8%) [37]. A strict adhesion to the NMCP's malaria treatment guideline in conjunction with the World Health Organization guidelines [38] may considerably reduce the spread of the AQ resistance in Africa.

In our study, *P. falciparum* isolates displayed a very high sensitivity to MQ, LUM, DHA, and PPQ similar to previous findings in Mali [39] and in Senegal [40]. To determine the most suitable antimalarial therapeutic combinations and to prevent the emergence of resistant parasites in the study site, correlations between IC_{50} values of the *P. falciparum* isolate were assessed. A positive correlation was found between the IC_{50} values for: CQ vs AQ, CQ vs QN, and CQ vs MQ. The variation in the ex-vivo response to AQ (32%), QN (24%), and MQ (8%) could be explained by the variation in

response to CQ. However, the values of correlation coefficient remain low with no possible threat of a cross-resistance between CQ and AQ, CQ and QN, or CQ and MQ.

The artemisinin derivatives are recommended to be associated with LUM, AQ, PPQ, and MQ. In this study, we demonstrated a positive correlation between DHA vs LUM, DHA vs PPQ, and DHA vs MQ. Twelve percent, 9%, and 18% of the variation in the ex-vivo response to MQ, LUM, and PPQ could be explained by variations in response to DHA. The values of the coefficient of determination between DHA vs MQ, DHA vs LUM, and DHA vs PPQ were too low to explain the cross-resistance. A cross-resistance between LUM and artemisinin derivatives has been observed previously [41]. There was a significantly positive correlation between the ex-vivo activity of MQ and PPQ ($r = 0.45$; $r^2 = 0.20$, $p < 0.0001$). A significant association between MQ and PPQ was already estimated with parasite isolates from Africa ($r = 0.45$) [42]. The correlation between MQ and PPQ might be partly explained by their similar mode of action. However, the values of the coefficient of determination remain low to fear cross-resistance between MQ and PPQ. A strong significant positive correlation was observed between QN and AQ ($r = 0.80$; $r^2 = 0.64$, $p < 0.0001$). Sixty-four percent (64%) of the variation in the ex-vivo response to QN could be explained by variations in response to AQ. The high value of the correlation coefficient raises a concern about a potential threat of cross-resistance between QN and AQ. From these data, we suggest that common mechanisms of action of these two drugs may explain the positive correlation between them, suggesting that parasites that have reduced susceptibility to QN would also be less susceptible to AQ.

Cross-resistances between CQ-AQ, CQ-QN, and QN-AQ have already been reported in previous studies [43–45]. Cross-resistance is thought to occur when a drug confers resistance to other drugs that have a similar mode of action or belong to the same chemical group. Cross-resistance affects essentially drugs belonging to the same chemical family or drugs with similar modes of action or modes of transportation [46–49]. Theoretically, parasites are more prone to be sensitive to a molecule whose IC_{50} is inversely proportional to that the one of a molecule the parasites display resistance. Data from our study revealed light to moderate, but significant negative correlations between sensitivities of *P. falciparum* isolates to AQ and DHA ($r = -0.28$, $p = 0.01$), DHA and QN ($r = -0.35$, $p = 0.01$), and AQ and LUM ($r = -0.45$, $p < 0.001$). The negative correlation observed between DHA and AQ implies that the use of artemisinin in combination with AQ is very relevant in Mali. Our findings are reassuring considering the emergence of resistance to artemisinin derivatives [50–53].

5 Conclusions

Plasmodium falciparum isolates were highly sensitive to DHA, LUM, and PPQ. In addition, few isolates showed a reduced sensitivity to AQ and MQ (only one isolate). A much lower sensitivity to QN was also detected. Our data confirm an increasing trend in the sensitivity of the *P. falciparum* field isolate to CQ in Mali. This study provides valuable data that may considerably contribute to a better clinical management of patients with malaria and guide the decision-making process for an effective national strategy for malaria treatment.

Compliance with Ethical Standards

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