Susceptibility of Plasmodium falciparum isolates to antimalarial drugs in a highly seasonal malaria endemic village in Mali

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Abstract

Background: In 2006, the National Malaria Control Program (NMCP) in Mali recommended artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated malaria. Since the introduction of ACT, few reports are available on the level of resistance of *Plasmodium falciparum* (*P. falciparum*) to antimalarial drugs in Mali. Dihydroartemisinin is the active metabolite of artemisinin derivatives. Here, we conducted an ex-vivo drug sensitivity testing in a rural area of southern Mali, namely the Kéniéroba village from 2016 to 2017.

Methods: Seventy-five (75) isolates of *P. falciparum* were successfully evaluated for ex-vivo sensitivity to key anti-malarial drugs, namely chloroquine (CQ), quinine (QN), amodiaquine (AQ), mefloquine (MQ), lumefantrine (LUM), dihydroartemisinin (DHA), and piperazine (PPQ). *P. falciparum* sensitivity to these drugs was assessed using the World Wide Antimalarial Resistance Network (WWARN) SYBR-GREEN method of inhibitory concentration of 50% (IC₅₀) determination. Reduced sensitivity to antimalarial drugs was defined as IC₅₀ less than the WWARN standard IC₅₀.

Results: The proportion of resistant *P. falciparum* isolates was 20.2% for CQ, 40.5% for QN, 6.8% for AQ, and 1.3% for MQ. All tested *P. falciparum* isolates were sensitive to LUM, DHA, and PPQ. A statistically significant correlation was found between QN and AQ IC₅₀ values ($r = 0.80$; $r^2 = 0.64$, $P < 0.0001$).

Conclusions: *P. falciparum* isolates were sensitive to all ACT derivatives tested in Kenieroba in Mali. In contrast, *P. falciparum* isolates were resistant to, CQ, QN, and AQ as evidenced by high IC₅₀ to these drugs.

Background

Early treatment of a malaria episode with an efficient antimalarial drug is required to prevent life-threatening disease outcome. Thus, the resistance of *P. falciparum* to common antimalarial drugs is a serious hurdle for malaria control in endemic countries [1]. The development of *P. falciparum* resistance to low-cost and well-tolerated antimalarial drugs such as CQ, AQ, antifolates, and MQ [2–6] has led the World Health Organisation (WHO) and the National Malaria Control Programs (NMCP) to recommend the artemisinin-based combination therapy (ACT) for malaria treatment [7]. The rationale for using ACT drugs is 2-folded. First, it relies on the high efficacy of artemisinin that early suppresses disease progression which could lead to life threatening manifestation. Second, there is a need to delay as long as possible the emergence and spread of artemisinin resistance worldwide. Nowadays, one of the major concerns is that we are witnessing the first signs of the emergence of parasites resistant to artemisinin derivatives in Southeast Asia [8, 9]. However, no molecular marker of such resistance has yet been identified [10]. Some of the mutations in the gene *kelch* (*K13*) have been associated with resistance to artemisinin *in vitro* in Asia [11–13]. Up-to-date, resistance to artemisinin has not been reported in Africa and ACTs have remained very effective [11, 14]. However, sub-Saharan Africa remains under threat because of the widespread use of ACTs which could lead to selective pressure on ACT, and also the increasing

intercontinental human migrations. In addition, the circulation of sub-standard or counterfeit drugs coupled to the non-adherence of patients to treatment may contribute to rapid selection of resistant malaria parasites. In 2006, the NMCP revised the treatment policy of uncomplicated malaria. Chloroquine has been replaced by two ACTs: artemether-lumefantrine (AL) and amodiaquine-artesunate (AQ-AS). For severe malaria, artesunate, artemether and QN should be used using intra-venous route. The necessity for regular monitoring of these antimalarial resistance phenomenon is therefore essential. *Ex-vivo* drug susceptibility testing is one of the most efficient indirect approaches to assess the efficacy of antimalarial drugs. Few data on *in-vitro* and *ex-vivo* ACT efficacy are available from isolates circulating in Mali. Here, we report the results of an *ex-vivo* susceptibility of *P. falciparum* isolates to seven (7) antimalarial drugs used in Kéniéroba, Mali.

Materials And Methods

P. falciparum isolates collection

From June to October 2017, *P. falciparum* isolates samples were collected from malaria patients in Kéniéroba, a village located 60 km Southwest of Bamako, the capital of Mali on the National Road 15 in the Sudano-Guinean area of Mali (12°6'50" N and 8°19'58" W). Only *P. falciparum* mono-specific malaria patients with fever were enrolled. Patients with hemolysis or antimalarial treatment or chemoprevention were not included. Around 2–5 mL of venous blood samples were collected in EDTA collection tubes after informed consent was obtained from patients or parents and/or legal guardians for children. All patients received treatment according to NMCP's recommendations.

Tested anti-malarial drugs

P. falciparum field isolates were tested against seven (7) usual malaria drugs: Chloroquine (CQ), Quinine (QN), Mefloquine (MQ), Amodiaquine (AQ), Piperaquine (PPQ), Lumefantrine (LUM) and Dihydroartemisinin (DHA). A stock solution of CQ diphosphate, QN, MQ and DHA were prepared in 70% ethanol. AQ and LUM were initially dissolved in methanol while PPQ was dissolved in lactic acid 0.5% first and then in DMSO. Two-fold serial dilutions were prepared using sterile distilled water and distributed in duplicate into 24-well flat-bottom plates. Final concentrations ranged from 2.44 to 2500 nM/l for CQ, 4.88 to 5000 nM/l for QN, 1.22 to 1250 nM/l for MQ, 1.22 to 1250 nM/l for l'AQ, 0.34 to 350 nM/l for LUM, 0.10 to 100 nM/l for DHA and 0.98 to 1000 nM/l for PPQ. Fifty (50) µl of each diluted antimalarial drug were added to 96-well plates in duplicates. The plates were dried in an open area and kept at 4°C for no longer than one month.

Ex-vivo drug sensitivity testing of antimalarial drugs

For *ex-vivo* drug sensitivity testing, spectrometry-based determination of parasite growth using SYBR-GREEN (SG) method was used as described previously [15]. Briefly 2–3 mL of whole blood was obtained

by venipuncture from each patient with *P. falciparum* mono-infection. The pellet from fresh blood was washed three times with incomplete RPMI 1640 medium (ICM) (Gibco™, Invitrogen Corporation, USA) buffered with 25 mM HEPES (5.95g) and centrifuged at 2000 rpm for 5 minutes. The parasites were tested directly without culture adaptation. The suspension of parasite was distributed in 24-well plates pre-loaded with antimalarial drugs. Culture plates were incubated at 37°C and 5% CO₂ for 72 hours. At the end of the incubation period (which corresponds to the schizonts' stage), the plate was taken out from the incubator and frozen at -20°C to read by adding SG. After, the plate was thawed for 2 hours to lyse the cells, Prepare SG lysis buffer (per plate - 10 mL lysis buffer, 2 µl SG (0.002% SG) and use immediately. Then, 100 µl SG lysis buffer was added to each well. The plate was later covered with aluminum foil, shake using a plate shaker, and incubated at room temperature in the dark for 30 minutes. The amount of SG incorporated into the nucleic acids of the parasite was determined by the Fluorometer plate Reader with excitation filter of 485 nm and emission filter 538 nm. The IC₅₀, defined as a drug concentration at which the SG signal was 50% of that measured from drug-free control wells, was calculated from *In-Vitro* Analysis and Reporting Tool (IVART) software to fit the concentration-inhibition data. The threshold values for the reduced in *ex-vivo* susceptibility were as followed: 61 nM, 77 nM, 12 nM, 115 nM, 30 nM, 135 nM and 611 nM for AQ, CQ, dihydroartemisin, LUM, MQ, piperaquine and QN respectively [16, 17].

Statistical analysis

Data were expressed as the geometric mean IC₅₀ and 95% confidence intervals (95 CIs) were calculated after logarithmic transformation. Cross-susceptibility was analyzed using the Pearson correlation using Graphpad Prism version8. Statistical analysis of IC₅₀s was performed with Graphpad Prism. Two-tailed p-values were computed and any value less than 0.05 were considered significant.

Results

In total, 75 isolates of *P. falciparum* were successfully evaluated for ex-vivo sensitivity to CQ, QN, AQ, MQ, LUM, DHA, and PPQ. Figure 1 summarizes the distribution of the tests performed and the median IC₅₀. The QN has the highest median of IC₅₀ with 276.1nM (123.6 to 627.2nM) followed by CQ with an IC₅₀ of 42.35nM (34.51 to 49.45nM), AQ with an IC₅₀ of 27.87nM (21.52 to 34.64nM), PPQ with an IC₅₀ of 17.12nM (14.76 to 18.99nM), MQ with an IC₅₀ of 13.53nM (12.04 to 18.21nM) and finally LUM with an IC₅₀ of 11.22nM (8.84 to 13.75nM). In contrast the median of IC₅₀ of DHA was the lowest at the concentration of 0.94nM (0.81 to 1.01nM) (Figure 1)

The proportion of *P. falciparum* isolates with low sensitivity to antimalarial drugs were 40.5% (30/74) for QN, 20.2 % (15/74) for CQ, 6.8% (5/73) for AQ and 1.3% (1/75) for MQ. All *P. falciparum* isolates tested showed a good sensitivity to DHA (mean IC₅₀ = 0.87nM (95% IC: 0.34–3.03 nM), LUM (mean IC₅₀ of 10.14nM (95% IC: 0.82–50.82nM) and PPQ (mean IC₅₀ = 15.86 nM (95 % IC: 3.72–35.72nM) Table 1.

Regarding the correlation between IC₅₀s of studied drugs, a strong correlation with statistical significance was observed between QN and AQ IC₅₀ values ($r = 0.80$; $r^2 = 0.64$, $P < 0.0001$) (Table 2). A positive correlation was found between the *ex-vivo* IC₅₀ values of AQ vs CQ ($r = 0.57$; $r^2 = 0.32$, $P < 0.0001$), CQ vs MQ ($r = 0.29$; $r^2 = 0.08$, $P = 0.008$), CQ vs QN ($r = 0.49$; $r^2 = 0.24$, $P < 0.0001$), DHA vs MFQ ($r = 0.35$; $r^2 = 0.12$, $P = 0.002$), LUM vs MQ ($r = 0.5$; $r^2 = 0.25$, $P < 0.0001$), DHA vs PPQ ($r = 0.43$; $r^2 = 0.18$, $P < 0.0001$), DHA vs LUM ($r = 0.30$; $r^2 = 0.09$) and MQ vs PPQ ($r = 0.45$; $r^2 = 0.20$, $P < 0.0001$). In contrast negative correlation was observed between the IC₅₀ values of DHA vs QN ($r = -0.35$, $P = 0.002$), LUM vs QN ($r = -0.36$, $P = 0.002$), DHA vs AQ ($r = -0.28$, $P = 0.01$) and AQ vs LUM ($r = -0.45$, $P < 0.0001$).

Discussion

Routine assessment of antimalarial drug efficacy is one of great interest for the NMCP. Although, the molecular markers for antimalarial drug resistance are very common, cheap and affordable tool to monitor parasite resistance to antimalarial drugs. *In-vivo* and *ex-vivo* tests are necessary to confirm the resistance of parasites to a given molecule. *P. falciparum* isolates from Kenieroba were successfully evaluated for *ex-vivo* sensitivity to CQ, QN, AQ, MQ, LUM, DHA, and PPQ assessing their IC₅₀. We observed higher geometric means of the IC₅₀ for QN at 264.97 nM (39.90–1567.38 nM). Similar IC₅₀ for QN was reported by Touré, A. O. *and al.* in Cote d'Ivoire in 2008 (272,12 nM) [18]. In contrast a higher QN IC₅₀ value was reported by Kwansa-Bentum, B. *and al.* in 2011 in Ghana with a concentration of 355,37 nM [19]. This difference with our results could be explained by the heterogeneity of the distribution of *P. falciparum* strains and the fluctuation of the phenomenon over time. Also, the proportion of QN-resistant *P. falciparum* isolates defined as having an IC₅₀ < 611 nM reached up to 40.5% in our study. This proportion was much higher than 19.4% (6/31) [19] in Ghana in 2011 and 9.7% (3/31) [20] in Senegal in 2017. Sharma, S. *et al.* reported reduced sensibility in 4% of *P. falciparum* isolates *in vitro* to QN in 2017 in India [21]. Our result has confirmed a reduced *ex-vivo* sensitivity of *P. falciparum* to QN, which may guide malaria treatment policy that is recommended by the NMCP of Mali. QN is used in third-line treatment option in severe malaria and uncomplicated malaria treatment in pregnant women during the first trimester. We found a strong correlation with statistical significance between IC₅₀ values of QN and AQ ($r = 0.80$; $r^2 = 0.64$). Cross-resistance might suggest a similar mechanism between the two antimalarial drugs.

Results of our *ex-vivo* susceptibility study of *P. falciparum* isolates to AQ obtained showed IC₅₀ that varied between 5.73 and 88 nM with geometric means of 25.5 nM. This result was superior to 11.2 nM reported by Phong, NC. *and al.* in 2019 in Vietnam [22]. In terms of sensitivity, 6.8 % (5/73) of isolates had reduced sensitivity to AQ (IC₅₀ > 61 nM). Diawara, S. *et al.* observed an increased *in vitro* resistance to AQ with 28.1% (9/32) in 2017 in Dakar, Sénégal [20]. Sharma, S. *et al.* in 2017 in India found 8% reduced sensitivity to AQ [21]. Our study confirmed the reduced sensitivity of *P. falciparum* to AQ in Kenieroba, which may quickly impact the clinical efficacy of the artemisinin combinations.

One isolate had reduced sensitivity to MQ (1.3%). The geometric mean of IC₅₀ to MQ was 13.13 nM. The geometric mean of IC₅₀ to CQ was 46.07 nM. Our result was similar to 51nM by Phong, NC. *et al.* in 2019 in Vietnam [22] and much lower than 143.94 nM by Kaddouri, H. *et al.*, in Bancoumana, 156.55 nM in Faladjé and 163.76 nM in Kollé in 2008 both conducted in Mali [23]. Our result was lower than that reported by Touré, A. O. *et al.* in 2008, in Ivory Coast (Abidjan) with a geometric means of 93.72 nM [18] and Nathalie W. *et al.* in 2014 in Dakar, Sénégal with 97.7 nM [24]. Among the isolates tested with CQ, 20.2% (15/74) showed IC₅₀ values > 77 nM (cut off resistant to CQ). On the other hand Kaddouri, H. *et al.*, between 2004 and 2006 in Mali, found a high level of resistance *in vitro* from *P. falciparum* to CQ (60–69%) [23]. Touré, A. O. *et al.* in 2008 in Ivory Coast found chloroquino-resistant in 26 % (6/23) [18]; Sharma, S. *et al.* in 2017 in India found a level of chloroquino-resistant of 18% [21]. This increased sensitivity of the isolates could be related to the decreased or absent pressure on CQ. The geometric means of IC₅₀ was 0.87 nM for DHA, 10.14 nM for LUM and 15.86 nM for PPQ. All isolates tested showed a very good sensitivity to DHA, LUM, and PPQ. This supports that DHA is the ideal component of ACTs with either LUM or PPQ. This result was similar to that reported by Diawara, S. *et al.*, in Sénégal [20] and Dama *et al.*, in Mali, both in 2017 [25]. Interestingly, Fall, B. *et al.*, in 2013 in Senegal observed a reduced *in vitro* sensitivity of *P. falciparum* to LUM with 2.9% [26]. In order to predict the different combinations possible in case of resistance to AL. We do believe that it is necessary to study the possible correlations of the different antimalarial molecules in term of cross-resistance. The positive correlation between IC₅₀ values ex-vivo of AQ- CQ, AQ-QN, CQ-MQ, CQ-QN, DHA-MFQ, LUM-MQ and MQ-PPQ indicates a cross-resistance between these molecules.

A cross-resistance between CQ-AQ and CQ-QN and between QN-AQ has already been reported in previous studies [27–29]. A negative correlation between the IC₅₀ values of DHA-QN, LUM-QN reflects higher activity of DHA and LUM against the isolates of *P. falciparum* QN-resistant. A negative correlation was also observed between DHA and AQ implying that they have different mechanisms of action and that isolates resistant to AQ may be sensitive to DHA. This is reassuring considering the emergence of resistance to artemisinin derivatives [30–33].

In conclusion, we found that P. falciparum isolates from Kéniéroba had a reduced sensitivity to QN, AQ, and MQ. All clinical field isolates tested showed a very good sensitivity to DHA, LUM, and PPQ. Resistance to QN and AQ showed a positive correlation between IC₅₀ values, indicative of cross-resistance between these two important anti-malarial drugs.

Declarations

Ethics approval and consent to participate

The study was approved by the ethics committee of the faculty of medicine and Pharmacy of the University of Sciences, Technics and Technologies of Bamako (USTTB), Mali. All study participants signed a written consent or assent (for children) forms in order to participate to this study.

Consent for publications

All authors read and approved the final manuscript.

List of Abbreviations

MRTC: Malaria Research and Training Center

ICER: International Center for Excellence in Research

USTTB: University of Sciences, Technics and Technologies of Bamako

ACT: Artemisinin-based Combination Therapy

WHO: World Health Organization

NMCP: National Malaria Control Program

CQ: Chloroquine

QN: Quinine

AQ: Amodiaquine

MQ: Mefloquine

LUM: Lumefantrine

DHA: Dihydroartemisinin

PPQ: Piperaquine

WWARN: World Wide Antimalarial Resistance Network

IC50: Inhibitory concentration of 50%

Competing interests

The authors do not report a conflict of interest

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Author's Contributions

Study setup; Traoré, K; Diakité, M

Sample collection, data collection: Traoré, K ; Diakité, SAS; Sanogo, I; Konaté, D;; Keita, B; Doumbouya, M

Data analysis: Traoré, K; Diakité, SAS

Manuscript writing: Traoré, K

Manuscript review: Traoré, K; Diakité, SAS; Dabita, D; Dama, S; Sangaré M; Bah, S; Guindo, MA; Diakité, M

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Tables

Table 1 *Ex vivo* response of isolates of *P. falciparum* from Kenieroba to antimalarial drugs

Drugs	Cut-off values	Resistant Isolates		Sensitive Isolates		Total	
		N	%	N	%	N	%
CQ	77 nM	15	20.2	59	79.2	74	100
AQ	61 nM	5	6.8	68	93.2	73	100
MQ	30 nM	1	1.3	74	98.7	75	100
QN	611 nM	30	40.5	44	59.5	74	100
LUM	115 nM	0	0	73	100	73	100
PPQ	135 nM	0	0	73	100	73	100
DHA	12 nM	0	0	69	100	69	100

Table 2 Correlation between *ex-vivo* responses of isolates of *P. falciparum* from Kenieroba to anti-malarial drugs

Drug pairing	Interpretation	Correlation Coefficient (r)	Significance value
AQ vs CQ	74	0.517	<0.0001
AQ vs DHA	68	-0.28	0.01
AQ vs LUM	73	-0.45	<0.0001
AQ vs MQ	74	-0.04	0.72
AQ vs PPQ	74	0.07	0.55
AQ vs QN	73	0.80	<0.0001
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	<0.0001
DHA vs LUM	67	0.30	0.012
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	<0.0001
LUM vs PPQ	73	0.11	0.34
LUM vs QN	71	-0.36	0.002
MQ vs PPQ	75	0.45	<0.0001
MQ vs QN	73	-0.02	0.87
PPQ vs QN	72	-0.10	0.37

Figures

Figure 1

Median values of IC50 of different anti-malarial drugs