Reduced *Ex Vivo* Sensitivity of Post Artemether-Lumefantrine Treatment Recurrent *P. falciparum* Isolates to Dihydroartemisinin and Piperaquine

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Abstract

Introduction: Since 2006, the Malian National Malaria Control Program (NMCP) adapted the Artemisinin based therapeutic combination (ATC) as the first line treatment molecule of uncomplicated malaria. Artemether + lumefantrine appears to be the most ACT used in Mali. However, recent studies reported increased recurrence of post AL treatment *P. falciparum* infections.

Methods: Using the SYBR® Green fluorometric method of IC_{50} determination, we compared the sensitivity *ex vivo* of post AL recurrent and non-recurrent *P. falciparum* isolates to common antimalarial molecules.

Results: Compared to non-recurrent, post AL treatment recurrent *P. falciparum* isolates harbored higher IC_{50}'s values (geometric mean, number [Min-Max]) to dihydroartemisinin (0.87 nM, 68, [0.34 - 4 nM] vs 1.14 nM, 7, [0.34 - 6 nM]; P = 0.003) and piperaquine (15.8 nM, 74 [13 - 22.6 nM] vs 17.54 nM, 6 [4.5 - 40.2 nM]; P = 0.001). Recurrent and non-recurrent P. falciparum isolates showed similar IC50's values with mefloquine (13.35 nM, 8 [1 - 28 nM] and 12.85 nM, 74 [4 - 36 nM]; P=0.29). The geometric mean IC50 to lumefantrine in recurrent isolates (14.52 nM, 8 [3 - 50.82 nM]) was higher than that of the non-recurrent isolates (9.9 nM, 71 [1 - 38.71 nM]) but the difference was not statistically significant (p = 0.53). The geometric mean IC50 to amodiaquine for recurrent isolates (24.75 nM, 73 [9 - 88 nM]) was higher than to non-recurrent isolates (22.92 nM, 6 [5 - 88 nM]), but the difference was not statistically significant (p = 0.09).

Conclusion: Our study revealed a lower sensitivity of Post AL treatment recurrent *P. falciparum* isolates to dihydroartemisinin and piperaquine and a trend to higher sensitivity of those parasite to amodiaquine.

Keywords: P. falciparum; Sensitivity; Recurrent; Antimalarial Drug; Ex vivo

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; AL: Artemether-Lumefantrine; ASAQ: Artesunate-Amodiaquine; DHA-PPQ: Dihydro-artemisine-Piperaquine; AQ: Amodiaquine; MQ: Mefloquine; LUM: Lumefantrine; DHA: Dihydroartermisinin; PPQ: Piperaquine; WWARN: World Wide Antimalarial Resistance Network; IC_{50}: Inhibitory concentration of 50%

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Background

The World Health Organization (WHO) has adapted the artemisinin based combination therapy (ACT) as a substitute to usual antimalarial molecules for *Plasmodium falciparum* (*P. falciparum*) malaria treatment since 2001 [1]. Hence, Malian National Malaria Control Program (NMCP) adapted the arthemeter - lumefantrine (AL) and artesunate-amodiaquine (ASAQ) combinations as the first line treatment molecules for uncomplicated *P. falciparum* malaria in 2006 [2]. However, no artemisinin- resistant *P. falciparum* strain has been reported yet in Africa [3,4], several studies revealed the emergence of the resistance to artemisinin derivative drugs in South-Est Asia [5,6]. The possibility of artemisinin-resistance emergence in Africa is haunting the medical and scientific community as well as the NMCPs in Africa. Hence, the close surveillance of the artemisinin efficacy through *in vivo* and *ex vivo* studies are necessary. Recurrent *P. falciparum* infections, defined as an occurrence of *P. falciparum* infection within 28 days after the precedent infection have been reported after AL treatment in Mali [7,8]. Those parasite harbored decrease sensitivity *ex vivo* to AL [9]. Furthermore, a reduced sensitivity of *P. falciparum* to artemisinin-associated molecules has been reported in certain area in Africa [10-12]. Treatment with ACT is known to improve cure rates, results in rapid parasite clearance [13] and reduces gametocyte carriage resulting in a decrease in parasite transmission [14-16]. The efficacy of the partner drug that may prevent the selection of artemisinin-resistant parasite. Thus, long-lasting activity appears to be a good characteristic for these partner molecules. This characteristic also may help prevent the recurrence of infection after the treatment. Recurrent parasites after ACT treatment are more prone to be less sensitive to the associated molecules. Assessing such isolate sensitivity to common antimalarial drug may help improving the police making procedure regarding the choice of the molecule to associate to artemisinin.

Materials and Methods

Collection of *Plasmodium falciparum* isolates

From June 2016 to October 2017, *P. falciparum* isolates were collected from *P. falciparum* malaria patients aged between 6 months and 18 years. Study participants were enrolled at the health center of Kéniéroba.in Kéniéroba, a village located in the Sudano-Guinean area of Mali (12° 6′ 50″ N and 8° 19′ 58″ W). From each patient, 2 to 3 mL of venous blood were collected in a citric acid and a dextrose collection tube (ACD tubes) before treatment initiation.

Preparation of anti-malarial drugs

Plasmodium falciparum isolates were tested against amodiaquine (AQ), mefloquine (MQ), lumefantrine (LUM), dihydroartemisinin (DHA), and piperaquine (PPQ). All drugs were obtained from Sigma-Aldrich. A stock solution of CQ, MQ, and DHA were prepared in 70% ethanol, the AQ 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 ranged from 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.

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 2h of our study site in Kéniéroba. 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

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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 72h. 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.

Malaria SYBR® green I-based fluorescence assay

The Malaria SYBR® Green I-Based Fluorescence Assay was performed as described in the Smilkstein and colleagues paper [17] modified by Johnson., *et al* [18]. Briefly, the plate was thawed for 1 - 2h at room temperature to lyse the cells. Then, 100 µL of 0.002% S YBR® 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 minutes. 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.

Statistical analysis

Only non-contaminated culture samples presenting a good fit on the log dose-response curve were analyzed. 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 [19, 20]. 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 of IC_{50} was calculated for each drug. A two-sided p value ≤ 0.05 was set as the significance threshold.

Results

A total of 177 malaria patients were enrolled and followed up 28 days and 34 of them presented a recurrent infection (new infection in less than 28 days after the precedent infection). The overall IC_{50} value, (mean, number [95%IC] was: 24.6 nM, 79 [5 - 88 nM]) for AQ; 10.29 nM, 79 [1 – 50.82 nM] for LUM; 12.90 nM, 82 [1 - 36 nM] for MQ; 15. 92 nM, 80 [4 - 40 nM] for PPQ and 0.89 nM, 75 [0.34 - 6 nM] for DHA. Compared to non-recurrent *P. falciparum* isolates, recurrent *P. falciparum* isolates harbored higher IC_{50} value to DHA; 1.14 nM, 7 [0.34- 6 nM] vs 0.87 nM, 68 [0.34-4 nM], p = 0.003 and PPQ 17.54 nM, 6 [13 – 22.6 nM] vs 15.8 nM, 74 [4.5- 40.2 nM], p = 0.001. Both recurrent and non-recurrent parasites showed similar IC50 values to MQ, 13.35 nM, 8 [4 – 36 nM] vs 12.85 nM, 74 [1 – 28 nM] , p = 0.29. The geometric mean IC_{50} to lumefantrine in recurrent isolates (14.52 nM) was higher than that of the non-recurrent isolates (9.9 nM) but the difference was not statistically significant (p = 0.53). The geometric mean IC_{50} to amodiaquine for recurrent isolates (24.75 nM, 73 [9 - 88 nM]) was higher than to non-recurrent isolates (22.92 nM, 6 [5 - 88 nM]), but the difference was not statistically significant (p = 0.09).
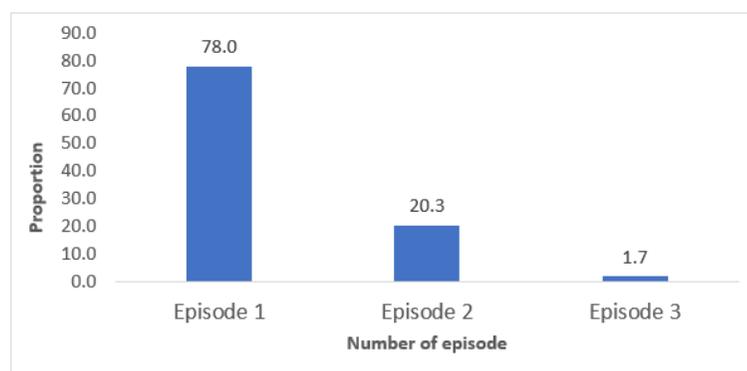

Figure1: *Evolution of main variables during the study period.*

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Socio-demographic characteristics and clinical data		Effectif	%
Patient follow-up until D28	Non recurrent	143	80.8
	Recurrent	34	19.2
	Total	177	100
Number of episodes by patient	Episode 1	138	77.97
	Episode 2	36	20.34
	Episode 3	3	1.69
Sex	Male	90	50.85
	Female	87	49.15
	Sex ratio	1.03	

***Table 1**: Socio-demographic characteristics and clinical data.*

Drugs	Number of experiments	IC_{50} geometric mean (nM) (95% CI)
AQ	79	24.6 (5 - 88)
LUM	79	10.29 (1 - 50.82)
MQ	82	12.90 (1 - 36)
PPQ	80	15.92 (4.5 - 40.2)
DHA	75	0.89 (0.34 - 6)

***Table 2**: Geometric mean of the IC_{50} values of the different antimalarial drugs.*

Drugs	Number of experiments	Non Recurrent IC_{50} geometric mean (95% CI)	Number of experiments	Recurrent IC_{50} geometric mean (95% CI)	P value
AQ	73	24.75 (5 - 88)	6	22.92 (9 - 88)	0.09
LUM	71	9.9 (1 - 38.71)	8	14.52 (3 - 50.82)	0.53
MQ	74	12.85 (1 - 28)	8	13.35 (4 - 36)	0.29
PPQ	74	15.8 (4.5 - 40.2)	6	17.54 (13 - 22.6)	0.001
DHA	68	0.87 (0.34 - 4)	7	1.14 (0.34 - 6)	0.003

***Table 3:** Geometric mean IC50s according to recurrent and non-recurrent P. falciparum isolates after AL treatment.*

Discussion

Ex-vivo assessment of the susceptibility of malaria parasites to antimalarial drugs remains an important tool of antimalarial drug's efficacy surveillance. As this method is largely independent on clinical factors, it provides information that complements clinical assessment of drug efficacy. Increasingly, there is a high number of recurrent parasites after treatment with the artemether-lumefantrine combination [8,21]. In this study, *ex vivo* drug sensitivity, of recurrent and non-recurrent *P. falciparum* isolates to the most common antimalarial drug such DHA, AQ, MQ, LUM and PPQ were assessed using the SYBR® Green method, a validated method.

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A total of 177 malaria patients were enrolled, treated with AL and followed up 28 days. The recurrence rate of infection in this study was 19.2%. This prevalence was higher than those reported in Uganda, in 2010 (0.8%) [22]. In contrast, a higher infection rate to AL (29%) was reported by Woodring *et al.* in 2010 in Kenya [21]. The Recurrent rates infection to AL are generally low in most African countries and ACTs remain highly effective as first-line treatment for uncomplicated malaria, the few recurrent infections reported are usually new infections [23,24]. In the current study, 22% of the patient did at least two episodes of malaria. This high rate of new infection seems to be linked to the high endemicity of malaria in the study site.

The geometric mean CI_{50} for amodiaquine was 24.6 nM (5 - 88 nM). This result was higher than that of Phong *et al.* in 2019 in Vietnam [25]. The geometric mean CI_{50} for lumefantrine was higher than those reported in previous studies conducted in Mali [9,26], but lower than that reported in Burkina Faso in 2014 [27]. The geometric mean IC_{50} values of the piperaquine measured in the present study are higher to published data from studies conducted in by Tito *et al.* in Burkina Faso [27]. The geometric mean IC_{50} of DHA was extremely low, indicating a high sensitivity of *P. falciparum* isolates from Kéniéroba to this molecule. This result is consistent with other studies conducted in sub-Saharan Africa [27-30]. The geometric mean IC_{50} of mefloquine was 12.90 nM (1 - 36 nM).

Ex vivo sensitivity of post artemether-lumefantrine treatment recurrent *P. falciparum* isolates to amodiaquine, lumefantrine, mefloquine, piperaquine and dihydroartemisinin was measured. The geometric mean IC_{50} of the recurrent isolates to DHA (1.14 nM) was significantly higher than that of the non-recurrent isolates (0.87 nM) to this molecule (p = 0.003). This data suggests a decrease sensitivity of recurrent isolates to dihydroartemisinin. The geometric mean IC_{50} to lumefantrine in recurrent isolates (14.52 nM) was higher than that of the non-recurrent isolates (9.9 nM) but the difference was not statistically significant (p = 0.53). Studies carried out in Mali have shown a significant number of recurrent parasites after treatment with artemether-lumefantrine (AL). These studies have hypothesized that these recurrent parasites are less sensitive to AL [7,8]. Dama S *et al.* in 2017 in Mali reported that geometric means of IC_{50} of recurrent isolates were statistically higher to non-recurrent isolates at AL [9].

The geometric means IC_{50} to PPQ (15.8 nM) for non-recurrent isolates was significantly lower than for recurrent isolates (17.5 nM) (p = 0.001). This data suggests that recurrent Kéniéroba isolates are less susceptible to PPQ. The reduced sensitivity of recurrent isolates to dihydroartemisinin and piperaquine may challenge the use of the DHA-PPQ combination in the treatment of recurrent malaria infections in Kéniéroba. The geometric mean IC_{50} for mefloquine was 12.85 nM for non-recurrent isolates versus 13.35 nM for recurrent isolates, but the difference was not statistically significant (p = 0.29). The geometric mean IC_{50} of the amodiaquine for recurrent isolates (24.75 nM) was higher than for non-recurrent isolates (22.92 nM), but the difference was not statistically significant (p = 0.09). Our data suggest a trend to higher sensitivity of those parasite to amodiaquine.

Conclusion

Our study revealed a lower sensitivity of Post AL treatment recurrent *P. falciparum* isolates to dihydroartemisinin and piperaquine and a trend to higher sensitivity of those parasite to amodiaquine

Acknowledgements

Competing Interests

The authors do not report a conflict of interest.

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Author's Contributions

Study setup; Traoré, K; Diakité, SAS; Diakité, M.
Sample collection, data collection: Traoré, K; Diakité, SAS; Konaté, D.

Data analysis: Traoré, K; Diakité, SAS.

Manuscript writing: Traoré, K.

Manuscript review: Traoré, K; Diakité, SAS; Bah, S; Diakité, M.

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.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

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