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SELECTION OF PFCRT 76T AND PFMDR1 86Y MUTANT *PLASMODIUM FALCIPARUM* AFTER TREATMENT OF UNCOMPLICATED MALARIA WITH ARTESUNATE-AMODIAQUINE IN REPUBLIC OF GUINEA

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KEY WORDS

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ABSTRACT

The use of Amodiaquine monotherapy is associated with the selection of molecular markers of *Plasmodium falciparum* resistance to chloroquine (pfcrt and pfmdr1). The decrease in sensitivity and the emergence of *P. falciparum* resistant to artemisinin-based combination therapy have been reported. Therefore, it is important to assess the impact of treatment of uncomplicated malaria with Artesunate-Amodiaquine (AS+AQ) on molecular markers of antimalarial resistance. We used standard World Health Organization (WHO) protocols to determine the in vivo efficacy of the combination (AS+AQ). In total, 170 subjects were included in the study. The molecular analysis focused on 168 dried blood spots. The aims were to determine the frequency of pfcrt 76T and pfmdr1 86Y mutations and the rates of reinfection using polymorphism markers *msp1*, *msp2*, and microsatellite markers (CA1, Ta87, TA99). Nested-PCR was used, followed in some cases by a restriction digestion. The level of *P. falciparum* clinical response was 92.9% (156/168) of Adequate Clinical and Parasitological Response (ACPR) before molecular correction and 97.0% (163/168) after molecular correction ($P = 0.089$). The frequency of mutation point pfcrt 76T was 76.2% (128/168) before treatment and 100% (7/7) after treatment ($P = 0.1423$). For the pfmdr1 mutation, the frequency was 28% (47/168) before treatment and 60% (6/10) after treatment ($P = 0.1124$). The rate of pfcrt 76T + pfmdr1 86Y was 22% (37/168) before and 50% (6/12) after treatment ($P = 0.1465$). Despite the presence of AS in the combination, AS+AQ selects for pfcrt 76T and pfmdr1 86Y mutant *P. falciparum* in Guinea.

Malaria is one of the major parasitic diseases in the World (World Health Organization [WHO], 2014). Two billion people representing approximately 40% of the world population are exposed (WHO, 2014). An estimated 219,000,000 clinical cases were reported with more than 93% of deaths occurring in Africa, mostly in children under 5 yr (61%) (WHO, 2019). In 2018, 405,000 deaths from malaria were reported globally compared with 416,000 estimated deaths in 2017 and 585,000 in 2010 (WHO, 2019). The uncomplicated malaria treatment consumed up to 25% of a public health facility's annual budget (Ezenduka et al., 2017).

In the Republic of Guinea, malaria remains a public health problem. It is the main cause of morbidity and mortality. Malaria accounts for 34% of all outpatient visits (Nsobya et al., 2007).

In 1945, the first synthetic antimalarial drug, chloroquine (CQ) was developed. Efficient, fast, and cheap, it became increasingly ineffective in various parts of the world with remarkable therapeutic failure rates. To overcome the ineffectiveness of CQ, sulfadoxine-pyrimethamine (SP) was introduced on a large scale during the 1970s in South-East Asia and the early 1990s in Africa. Initially, SP proved extremely useful given the single dose and its very few side effects. However, within a few years after the implementation of SP, SP-resistant parasites rapidly emerged in

South Asia and Africa (Saito-Nakano et al., 2011). Thus, WHO recommended the use of artemisinin-based combination therapy (ACT) against *Plasmodium falciparum*. The control of this global scourge is hampered by poor management of cases and also the emerging resistance of the vector to insecticides and that of *Plasmodium* to antimalarial drugs (Nsobya et al., 2007; Saito-Nakano et al., 2011).

In Africa, the National Malaria Control Program (NMCP) adapted WHO recommendations and changed their malaria treatment policy. A survey revealed a high level of chloroquine resistance (CQR) in the regions of N'zérékoré and Boke in 2001, with 28% and 21% (Nsobya et al., 2007), respectively. These results led to the NMCP of Guinea to change the first-line treatment of malaria to artesunate (AS) in combination with amodiaquine (AQ) in 2005 (NMCP-Guinea, 2012). However, there is a lack of data on the impact of the implementation of AS+AQ as first-line antimalarial drugs in Guinea on molecular markers of antimalarial resistance. Therefore, the aim of this study was to describe the prevalence of molecular markers associated with drug resistance following the treatment of uncomplicated malaria in Guinea.

MATERIALS AND METHODS

Study design and period

This was a clinical trial performed according to WHO protocols that was carried out from 11 March 2011 to 19 November 2012 in Maferinyah, located 75 km from the capital Conakry, with an estimated population of 14,851 inhabitants. Malaria is hyper-endemic and transmission is perennial (Ministry of Health-Guinea, 2014).

Sample collection

We hypothesized that the treatment of uncomplicated malaria with AS+AQ selects for pfcrt 76T and pfmdr1 86Y mutant parasites in Maferinyah. Patients that met the following criteria were included: axillary temperature $\geq$37.5 C; a mono-infection with *P. falciparum* parasitemia between 2,000 and 200,000 trophozoites/mm^3 of blood; a hemoglobin $\geq$5 g/dl; weight $\geq$5 kg; signed informed consent or acquiescence for minors; able to take oral medication; not pregnant; with no concomitant chronic diseases; available for 28 days of follow-up; resident of Maferinyah for at least 6 mo; and aged between 3 mo and 45 yr, inclusive. Patients who had clinical signs of cerebral malaria or severe malaria (macroscopic hemoglobinuria, jaundice, hemorrhagic shock [systolic BP <70 mm Hg in adults, <50 in children], spontaneous bleeding, hypoglycemia [<40 mg/dl], renal failure [creatinine >3 mg/dl], severe anemia with hemoglobin <5 g/dl, severe concomitant pathology), or a known allergy to one of the test drugs were not included in the study.

The patients were followed for 28 days according to the WHO standard protocols for the evaluation of therapeutic efficacy of antimalarial drugs. They underwent clinical and laboratory follow-up on days 1, 2, 3, 7, 14, 21, and 28, and on days of unscheduled visits when necessary.

Microscopy was used to determine malaria infection. Briefly, a blood sample was taken from a finger prick and thick and thin smears were made by skilled microscopists according to WHO standards (WHO, 2010). The smears were stained with Giemsa for the presence of *Plasmodium* parasites, and species identification and parasite density were assessed as well as the presence of asexual (trophozoites and schizonts) and sexual (gametocytes) stages and pigment in white blood cells (WBC). The AS+AQ treatment was administered under the supervision of the study personnel. Patients were observed for 1 hr after the administration of treatment.

Full doses of the drug were re-administered if the patient vomited within 30 min. Vomiting of re-administered drugs resulted in the removal of the patient from the study and treatment with rescue medication.

Coarsucam (AS [4 mg/kg] + AQ [10 mg/kg]) (Sanofi; Aventis, Paris, France) was the ACT used in this study with 1 dose per day for 3 successive days. The primary endpoint was the therapeutic response, classified as the following: Early treatment failure (ETF); late treatment failure (LTF), consisting of late clinical failure (LCF); and late parasitological failure (LPF); and adequate clinical and parasitological response (ACPR) at day 28. Safety and tolerance were considered as secondary endpoints.

DNA extraction

Genomic DNA was extracted from dried blood spot (DBS) filter papers using the Chelex method (Plowe et al., 1995). Briefly, erythrocytes were lysed in a 0.5% saponin solution and the DNA was separated from other cellular components using 20% of CHELEX resin. The DNA extract was then used for PCR immediately or stored/frozen at –20 C.

PCR amplification

We amplified genes associated with resistance, namely *Pfcrt* and pfmdr1, to determine the prevalence of drug resistance markers. The *msp1* and *msp2* polymorphic markers, and microsatellites such as CA1, Ta87, and TA99 were used to determine the rate of recrudescence and reinfection. Nested-PCR followed by polymorphism restriction fragment length (RFLP) were used (Suppl. Table 1).

Genotyping of pfcrt K76T and pfmdr1 N86Y

The amplification technique used in this study was previously described by Djimdé et al., 2001a, 2001b). Briefly, a volume of 20 µl of the reaction mixture was added to 5 µl of DNA. The first amplification was done for 45 cycles. We used 1 µl of the product of the first PCR as a template with specific primers for the second amplification using 25 cycles. PCR products were separated by electrophoresis on a 2% agarose gel. These products were visualized under UV light with ethidium bromide and then photographed. For the RLFP, we used 15 µl of a mixture containing the restriction enzyme and 5 µl of the second amplification product. After brief centrifugation, the samples were incubated at 60 C and 37 C for 6 hr for pfcrt and pfmdr1 respectively.

Genotyping MSP1, MSP2, and microsatellites CA1, Ta87, and Ta99

A first amplification was performed using the msp1 primers (O1 = sense and O2 = antisense) and msp2 (S2 = sense and S3 = antisense) and microsatellites Ca1 (Ca1-1L = sense and Ca1-1R = antisense), Ta87 (Ta87-1L= sense and Ta87-1R = antisense), and

Table I. Distribution of study subjects by gender and age in Maferinyah from 11 March 2011 to 19 November 2012.

Characteristics of study subjects	Number of subjects	Percentage
Gender		
Male	80	47
Female	90	53
Age		
3 mo–5 yr	57	34
6 yr–10 yr	60	35
11 yr–15 yr	31	18
16 yr–45 yr	22	13
Geometric mean of asexual parasite density ± SD*	24,281.14 ± 3.12	

*SD = Standard deviation.

TA99 (Ta99-1L =sense and Ta99-1R = antisense). To a volume of 24 µl of the reaction mixture was added 1 µl of the extracted DNA template. DNA was amplified for 30 cycles for *msp1* and *msp2* and 40 cycles for the microsatellites. A second amplification was performed using 1 µl of the product of the first PCR as template with primers specific for amplifying *msp1* (N1 = sense and N2 = antisense) and *msp2* (S1 = sense and S4 = antisense) and microsatellite Ca1 (Ca1-L = sense and Ca1-R = antisense), Ta87 (Ta87-L = sense and Ta87-R = antisense), and TA99 (Ta99- L = sense and Ta99-R = antisense). To a volume of 24 µl of the reaction mixture was added 1 µl DNA and amplification was done for 30 cycles for *msp1* and *msp2* and 20 cycles for Ca1, Ta87 and Ta99. PCR products were separated on agarose gel electrophoresis (1.5% and 1.8% for *msp1* and *msp2*, respectively).

Sample size calculation

The size of the required sample was determined based on 70.3% of the allele prevalence of pfcrt 76T reported in Mali (Djimdé et al., 2001a, 2001b). After setting the risk of error of 5% (95% accuracy), the minimum size required was 170 subjects. The molecular analysis included 168 subjects: 168 filter paper samples before treatment and 12 cases of post-treatment parasitemia.

Statistical analysis

Data entry was performed on Excel version 2007 (Microsoft, Redmond, Washington) and analyzed on Stata version 11.0 (Stata Corp., College Station, Texas). Categorical variables were presented as proportions. For comparison of mutation proportions before and after treatment, we used Fisher's exact test and chi-square (χ^2) with a threshold for statistical significance of 5%.

RESULTS

Overall, a total of 170 participants were included in the study. There were more women (53%) in the age group of 6 to 10 yr (35%), and participants 3 mo to 5 yr were the most frequent (Table I). The proportion of participants with fever and negative slide from 0 to 3 days is shown in Figure 1. From day 0 to day 3 of follow-up, the proportion of participants with fever decreased drastically, ranging from 100% on day 0 to 0.00% on day 3. As for the proportion of negative parasite clearance, the same trend was observed after administration of AS+AQ.

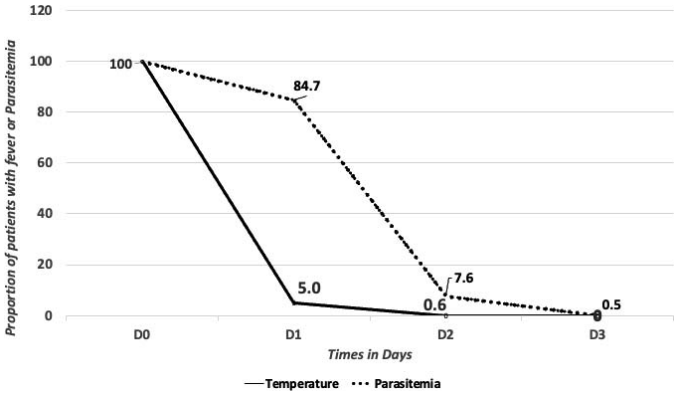

Figure 1. The proportion of participants with fever and negative blood smear from day 0 to day 3.

Therapeutic efficacy at 28-day follow-up

The therapeutic efficacy of the fixed combination of AS+AQ is shown in Table II. No case of ETF and LTF was recorded. Of the sample, the ACPR rate was 93% (158/170). However, 7% (12/170) were cases of LPF. After molecular correction, the therapeutic efficacy ACPR of AS+AQ was raised to 97% ($P = 0.089$).

Molecular marker assessments

DNA was isolated from 168 out of the 170 included participants. Overall, 12 cases of failure were observed. Before administration of AS+AQ, the mutant allele pfcrt 76T was the predominant at 76.20% (128/168) compared to 28% (47/168) of the pfmdr1 N86Y allele. After the treatment, no mutant allele of pfcrt 76T was observed in 7/7 (100%) compared to the pfmdr1 N86Y allele 6/10 (60%). Moreover, the wild allele was 23.8% (40/168) before administration of AS+AQ. For the pfcrt gene on the 12 cases of failure, all 7 samples that were successfully genotyped harbored the pfcrt 76T mutant allele. For the pfmdr 1 gene on the 12 cases of failure, 10 strains gave an interpretable result by PCR.

The association of mutant genotypes *Pfcrt76T* and *Pfmdr186Y*

A total of 37/168 strains or 22.02% before treatment, and 6/12 strains, 50% after treatment gave products simultaneously. We

Table II. Uncorrected and corrected results of the therapeutic efficacy of the fixed combination artesunate + amodiaquine (AS+AQ) in the treatment of uncomplicated malaria in Maferinyah, Guinea, from 11 March 2011 to 19 November 2012.

Variables	n (%)
Uncorrected corrected results, n = 170	
Early treatment method	0 (0)
Late treatment failure	0 (0)
Late parasitological failure	12 (7)
Adequate clinical and parasitological response	158 (93)
Corrected results, n = 168	
ACPR* before molecular correction	156 (92.85)
Cases of re-infections	7 (4.16)
ACPR after molecular correction	163 (97.01)

*ACPR = Adequate clinical and parasitological response.

observed 60% (6/10) of pfmdr1-86Y mutant allele and 40% (4/10) of wild-type allele pfmdr1-N86.

After treatment, 7/7 strains of *P. falciparum* carried the mutant allele pfrct 76T (100%). For the gene pfmdr1, 6/10 *P. falciparum* strains carried the mutant allele (60%) and 4/10 strains carried the wild allele (40%). For both genes, there were 6 strains with both mutant alleles pfcrt 76T + pfmdr 16Y simultaneously. Of the 12 cases, only 7 cases of failure could give an interpretable result; 83% of the gene was a mutant of allele pfcrt 76T before treatment vs. 100% after treatment. For the pfmdr1 gene, 25% were mutant for allele 86Y before treatment vs. 60% after treatment.

DISCUSSION

We conducted a prospective cohort study evaluating the efficacy of the AS+AQ combination in the treatment of uncomplicated *P. falciparum* malaria in Guinea. The frequency of mutation of the pfcrt 76T gene was 76.19% (128/168) and 92% (11/12) before and after administration of the AS+AQ combination, respectively. Treatment with AS+AQ, therefore, increases the frequency of pfcrt 76T. Our results are comparable to those obtained in Kenya (Thwing et al., 2009) and in Uganda (Nsobya et al., 2007).

These results show a high prevalence of mutant strains, and a basic elevation of pfcrt 76T and pfmdr1 86Y mutations to the AS+AQ combination are reported for the first time at Maferinyah. A multicenter study of AS+AQ efficacy revealed that the pfcrt point mutation on codon 76 (pfcrt 76T) was strongly associated with the in vivo resistance of chloroquine (Thwing et al., 2009; Hailemeskel et al., 2019). Another study demonstrated that AS+AQ selects pfcrt and pfmdr1 genes, point mutations associated with CQ and AQ (Djimde et al., 2001a, 2001b).

For the pfmdr1 gene, its frequency varied by 27.97% (47/168) before the 50% (6/12) treatment. After treatment with AS+AQ, it increased 22%. The same trend was reported by Zwang et al. (2009) in sub-Saharan Africa and in Gambia by Duraisingh et al. (2000).

However, the simultaneous association of pfcrt and pfmdr1 genotypes at the isolate level was 22.02% before treatment and 50% after treatment. This simultaneous mutation growth could have a modulatory role of in vivo resistance to AQ. Our results are similar to those in Senegal (Diawara et al., 2017).

All cases of parasitological therapeutic failure that we were able to analyze carried the allele of the pfcrt 76T mutant gene (7/12). The same phenomenon was observed with the pfmdr 18Y gene (9/12). The parasitological therapeutic failure in these subjects can be explained by the presence of markers of resistance to AQ. The recrudescence and reinfection rates were 22% (2/9) and 78% (7/9), respectively.

After molecular correction, the adequate clinical and parasitological response rate was 93.7%. The same trend was observed in Kenya (Holmgren et al., 2006), in Equatorial Guinea at 97.3% (Bonnet et al., 2007), and in Nigeria at 100% (Sowunmi et al., 2019).

CONCLUSION

Our results show a high base level of gene pfcrt 76T and pfmdr1 86Y before antimalarial treatment and a significant selection of molecular markers to AQ by the use of AS+AQ.

But also, there was a simultaneous presence of the mutation gene pfcrt 76T and pfmdr1 86Y, which is a molecular marker of potential resistance to AS+AQ.

The level of clinical response and adequate parasitological affect (97.01%) after correction shows that the combination AS+AQ is still effective on the site of Maferinyah. However, despite this efficacy of the combination, it should be subject to special attention from policymakers, as the selection of molecular markers linked to the resistance of the Artemisinin-based combination therapy (ACT) partner's drugs raise a concern about life combinations in Africa, especially in Guinea.

The study protocol was approved by the National Ethics Committee on Health Research of the Republic of Guinea. A community consent (including the administrative authorities, customary, old subjects, the parents or guardians of children) was first applied to inform them of all the aspects of the study. Then, each participant signed or affixed his fingerprint on a voluntary informed consent form that was presented to him by the research team. For minor children, parents or guardians signed the forms. For participants who cannot read in French, a translation of the content of the informed consent form was made in the local national language in the presence of an independent witness.

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