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In Vitro Susceptibility to Atovaquone and Characterization of *Pfcytb* Gene of *Plasmodium falciparum* Isolates From Abidjan (Côte d'Ivoire)

Trébissou Jonhson Noel David[1-2], Yavo William[2-3], Tano Konan Dominique[2-3], Dablé Trésor Maruis[2], Amiah Ahou Mireille[2], N'cho Monsan[2], and Djaman Allico Joseph[1-4]

[1]Pharmacodynamics Biochemical Laboratory, UFR Biosciences, Felix Houphouet Boigny University, PO BOX, 582 Abidjan 22, Côte d'Ivoire

[2]Laboratory of Microbiology National of Public Health (INSP) of Cote d'Ivoire

[3]UFR of Pharmaceutical and Biological Sciences, Felix Houphouet Boigny University, PO BOX, 582 Abidjan 22, Côte d'Ivoire

[4]Laboratory of Basic and Clinical Biochemistry, Pasteur Institute of Cote d'Ivoire

ABSTRACT: To evaluate the *in vitro* chemosensitivity to Atovaquone (ATQ) and analyze polymorphism of *Pfcytb* gene of *Plasmodium falciparum* isolates from Abidjan. Fifty seven isolates of *P. falciparum* collected between 2010 and 2012 from the outskirts of the city of Abidjan. Were used for testing *in vitro* susceptibility according to the microtest optical version of the World Health Organization (WHO). Polymerase Chain Reaction (PCR) was used to determine mutation in the *Pfcytb* gene. The analysis of the relationship between the observed mutations and chemosensitivity of isolates was made using Cohen's kappa test. The geometric mean IC_{50} of atovaquone is 1,645 mM. All 57 isolates were sensitive to atovaquone. The 29 isolates of *P. falciparum* sequenced showed no mutation of *Pcytb* gene at codon Y268. No other mutation was observed. Our study showed no correlation between phenotypic and molecular data (K**< 0).** This study shows that the combination atovaquone / proguanil can be one of the drugs of choice for the prophylaxis of multidrug-resistant *falciparum* malaria recommended by the World Health Organization (WHO).

KEYWORDS: *Plasmodium falciparum*, *Pfcytb,* Atovaquone*, In vitro* susceptibility, Côte d'Ivoire.

1 INTRODUCTION

Despite drop in the transmission and reduction of 25% in mortality compared to year 2000, malaria endemic disease remains the most common tropical parasitic disease in the world [1]. According to the World Health Organization (WHO), malaria is the first endemic disease of which humanity is paying a heavy price. Records indicate that 216 million episodes of malaria in 2010, 81% from Africa, with 174 million cases. It was recorded more than 655,000 cases of malaria deaths in 2010, 91% in Africa. 86% of malaria deaths have involved children under 5 years [1]. Malaria remains as the leading cause of infant mortality in the world [2]**.** Most malaria deaths are caused by *Plasmodium falciparum* [3]. The emergence and growth of Chloroquine-resistant strains of *P. falciparum* is a major obstacle to the fight against malaria in most endemic areas and This calls into question the conventional chemo prophylaxis with Chloroquine [4].

Today, it is recommended by the WHO, the use of drug combinations. Thus it has been used in endemic areas such as Côte d'Ivoire, combination therapies based on derivatives of artemisinin (CTA), namely artesunate-amodiaquine or artemether-lumefantrine for treating uncomplicated malaria [5]**.** WHO recommends today for chemoprophylaxis of malaria

Corresponding Author: Dr. Trébissou Jonhson Noel David

atovaquone / proguanil combination in endemic areas of multidrug- resistance and particularly for non-immunized travelers [6].

The atovaqoune works by inhibiting electron transport in the mitochondrial respiratory chain, therefore the atovaqoune inhibits the synthesis of ATP [7].To the best of our knowledge, no study on the *in vitro* susceptibility of *P. falciparum* to atovaquone (ATQ) was conducted in Côte d'Ivoire. However, a recent study conducted at the border of Thailand has shown great effectiveness of atovaquone / proguanil for the preventive treatment of multidrug-resistant *falciparum* malaria [6]. There have been reports of failure of atovaquone proguanil treatment / (AP) mainly in Africa [2].The failure of the AP combination was linked to the point mutation in the *Plasmodium falciparum* cytochrome b *(Pfcytb*) gene of mitochondria at codon Y268. Thus, tyrosine (Y) may be replaced by serine (S) Tyr-268-Ser or Cysteine (Cyst) Tyr-268-Cyst. These mutations are considered key markers for monitoring resistance to atovaquone [8].

This study aims to determine the *in vitro* susceptibility of isolates of *P. falciparum* from the outskirts of Abidjan and carry out genotypic characterization of *Pfcytb* gene at codon 268.

2 MATERIALS AND METHODS

2.1 STUDY SITES

This study was conducted from May 2010 at January 2012 at the Centre for Research and Fight against Malaria in the National Institute of Public Health of Côte d'Ivoire (INSP) for testing *in vitro* susceptibility of *Plasmodium falciparum* isolates to antimalaria drugs and genomic testing. The collection of parasitized blood sample was done at the health center of Anonkoua-Kouté located on the outskirts of Abidjan in Abobo.

2.2 ISOLATES OF *P. FALCIPARUM*

The infected blood samples were obtained from patients with uncomplicated malaria with a single-species infection with *P. falciparum*, the parasite was greater than or equal to 4000 parasitized erythrocytes / µl of blood and who had not received any antimalarial drugs during the past 7 days prior to consultation. The informed consent of patients or their parents or legal guardians for children should be obtained prior to inclusion in the study. The infected blood samples were carried out in strict aseptic conditions. Parasitized blood samples were transported in a cooler at 4°C, the same day of collection, in the laboratory where the *in vitro* chemosensitivity test were performed. Parasitized red blood cells were washed three times in RPMI 1640 and blood smears were stained with GIEMSA and examined microscopically to determine the parasite density and confirm the *Plasmodium* species *(P. falciparum*). Samples with parasitaemia ranged from 0.1% to 0.25% were used directly to test the *in vitro* drug sensitivity. Those with parasite density greater than 0.25% were diluted with uninfected erythrocytes.

2.3 ANTIMALARIA

Atovaquone used for testing *in vitro* susceptibility were obtained from France (University Paris-South, Bldg 445, IBAIC and CNRS-UMR 8080, Orsay). The stock solution of ATQ was prepared from methanol. Two Fold dilutions were made extemporaneously in a solution of RPMI 1640 and distributed in duplicate and triplicate in culture plates of 96 wells.

2.4 *IN VITRO* TESTS

The determination of the *in vitro* activity of Atovaquone was conducted using the optical version of the WHO microtest [9]. The inoculum consisted of parasitized erythrocytes, RPMI 1640 double buffered (HEPES 25 mM), sodium bicarbonate, 25 mM and solution of BSA (bovine serum albumin). For a 96-well plate, 19.2 ml of erythrocyte solution was prepared by adding 18.3 ml of RPS (1.83 ml BSA + 16.47 ml of RPMI 1640) to 900 µl of parasitized red blood cells (PRB),of which the hematocrit was reduced to 50% (450 µl de GRP + 450 µl de RPMI 1640). The inoculum was distributed in the culture plate into 200 µl per well final concentrations of ATQ ranges from 0.78 nM to 100 nM. After brief stirring of the plate, it was placed in a candle jar (Modular incubator chamber, ICN Biomedicals, California, USA) wet and rich in CO2 (5%) environment and incubated in a microbiological incubator (MemmertTM) 37°C for 42 hours. After this incubation time, three thick smears GIEMSA stained were made from pellets of blood from control wells. The percentage of schizont maturation was determined by the ratio of the number of schizonts (asexual forms more than 2 cores) to 200 asexual counted during the microscope reading at a magnification of GX100. We validated tests that the percentage of schizont maturation in control wells was at least 20%.

2.5 MOLECULAR STUDY

Twenty microliters of parasitized blood washed with RPMI 1640 was placed on Whatman No.3 paper, dried for 24 hours and stored for molecular tests. Plasmodial DNA was extracted by the Chelex 100 method described by Paul et al [10]. We used PCR to amplify a fragment embedded 500 bp *Pfcytb* gene. The final volume of the first PCR was 25 µl and the amount of plasmodial DNA was 6.25 µl. For the second PCR, we used as final volume 50 µl and the amount of plasmodial DNA was 5µl. The reaction mixture consisted of 1.5 µl from stock solution of 10µM of a pair of specific primers to be amplified, 5 µl of dNTP 2 mM, 0.25 µl of Taq polymerase, 2.5 µl of solution of 50 mM magnesium chloride, 5 µl of 10X buffer (100 mM Tris-HCl pH 9, 500 mM KCl, 0.2% Tween 20) and milliQ water QS. For the first PCR, the primer pair used were Pfcytb1- 5'-GTT-AAA-GCA-CAC-TTA-AAT-ATA-TAC-3' and *Pfcytb*2R 5'-GCT-TGG-GAG-GTG-TAA-TAA-TCA-TGT-3'. The thermal cycler was programmed to perform a denaturation at 94°C for 2 min, followed by 29 cycles at 94°C for 1 min, 50°C X 1min and 72°C X 1min and 72°C X 10 min at the end. The secondary amplification was performed with a pair of primers *Pfcyt*b-3 5-ATT-GAT-TAT-TAT-ATT-TGT-AAC-TGC-3' and Pfcytb-4R5'-AGT-TGT-TAA-ACT -TCT-TTG-TTC-TGC-3'with the same thermocycler program as the first PCR.

2.6 STATISTICAL ANALYSIS

Determining the values of 50% inhibitory concentrations of maturation (IC_{50}) was performed using nonlinear regression in Excel file. The threshold value used for the evaluation of *in vitro* chemosensitivity was 6 nM to ATQ [10]. Sequence analysis of PCR was performed on ApE software (for Windows). Electropherograms reading was performed on the Chroma Lite software 2.01. *Pfcytb* wild type was defined by alleles Y268.

The Cohen's kappa test was used to assess the degree of agreement between the different methods used to determine drug resistance [11]. The degree of agreement between the two tests can be described as very good, Cohen's kappa coefficient ≥ 0.81 good, 0.61-0.80, moderate, 0.41 to 0.60; poor, 0.21 to 0.4, bad, from 0 to 0.20, very bad, < 0.

3 RESULTS

3.1 *IN VITRO* STUDY

Of 64 isolates tested, 57 gave interpretable results thus a success rate of 89%. The geometric mean IC_{50} of ATQ for all of these isolates was 1,645 nM with confidence interval (IC_{95}) between 1,42 and 1,87 nM. The IC_{50} ranged from 3,96 nM to 0,86 nM. All isolates (100%) were susceptible to ATQ (Table I).

3.2 MOLECULAR TESTS

We tested 41 isolates of which 29 gave clear interpretable results (71%). All isolates tested were the wild-type Y268.We did not observe any single mutation Y268C or Y268S (Figure1). Table II reports the *Pfcytb* gene polymorphism of the isolates according to the profile of sensitivity to ATQ. According to Cohen's kappa test, the degree of concordance between the nature of *pfcytb* gene and *in vitro* sensitivity to this molecule was 0 (Table II).

4 DISCUSSION

Concerning the study of *in vitro* susceptibility to Atovaquone, our results are consistent with similar studies done in Africa and Asia. Indeed, isolates of *P. falciparum* from Africa were tested *in vitro* in the presence of Atovaquone, the results showed that all isolates were sensitive to this drug [12]. A similar study was conducted in 2006 on *Plasmodium falciparum* isolates from tourists returning from a trip to Africa, the results reported that all isolates were sensitive [13]. It has been observed in a similar study in *P. falciparum* multi–resistant areas at the border of Thailand on 83 isolates between 1999 and 2005, the results showed that they were sensitive to Atovaquone with an IC_{50} mean of 3,4 mM [14]. Other similar studies conducted in Guyana on 103 isolates of *P. falciparum* have confirmed our results [6]. All these studies have been conducted in chloroquine resistance high-transmission area (Southern Saharan Africa and South-East Asia). In these areas, isolates of *P. falciparum* remain sensitive to Atovaquone. Whatever the types of *P. falciparum* multi-resistant tested Atovaquone remained sensitive to these parasites and this sensitivity since the use of combination atovaquone-proguanil for the treatment of malaria. Tourists who visit area of high-malaria endemic should be advised to take this molecule along. This lack of development of drug resistance in this molecule can be explained due to the fact that this molecule is either few or not available in Africa and Asia countries. It is sold in most European countries at high prices and its use is rational and reserved only to cases of malaria

prophylaxis. In contrast, in 2010 in Senegal made a study was conducted on the *in vitro* susceptibility of isolates of *Plasmodium falciparum* to Atovaquone, result revealed that isolates were resistant to this drug [12]. This results was confirmed in 2005 when a similar study on 105 isolates of *P. falciparum* in Central Africa, the results reported that 7% (4 isolates) were resistant to the anti-malaria drug with IC_{50} 6 mM [15]. These results are not consistent with ours. These results raise the question of threshold resistance valuea to Atovaquone that could be (IC_{50} 6 mM and 1900 mM) [13]. On the basis of these criteria, the isolates tested in Senegal and Central Africa was sensitive because IC_{50} found was high thus classified as susceptible isolates. In view of these results, we recommend the combination atovaquone-proguanil as an alternative antimalarial drug for the prophylaxis of multidrug-resistant *falciparum* malaria in areas of high chloroquine resistance.

We looked into the area of *Pfcytb* gene sequence which contains the Y268 codon. The analysis of polymorphism of *Pfcytb* gene revealed that all the tested genes sequences were wild type at codon Y268. In this codon, there was no replacement of tyrosine by serine (Y268S) or replacement of tyrosine by cysteine (Y268C). These results are consistent with those found in 2006 study in which isolates from Africa have mutation that were all wild at the codon 268 [16]. A similar study conducted on 295 isolates from Africa reported in 2006 that there was no mutation in the codon 268 of the isolates. These results were confirmed at the border of Thailand on 70 isolates tested [17]. It was reported in 2008 in areas of multidrug resistance in Thailand, that the polymorphism in the 83 *Pfcytb* gene sequences revealed no mutation at codon Y268 [14]. Similar results have been reported in Angola and Ethiopia 249 and 97 isolates tested respectively [2]. In fact, all these studies showed that the mutation of the parasite *Pfcytb* gene at codon Y268 was responsible for the inhibition of Atovaquone. Atovaquone react in the complex III or bc1 complex of oxidative phosphorylation chain. It will bind to its oxidation site at the cytochrome b, which leads to inhibition of electron movement and a depolarization of the mitochondrial membrane potential [13]. Stopping the electron movement by Atovaquone, causes inhibition of breathing (over 70%) [18] and inhibition of the activity of DHOD by 90% [19]. Isolates non-resistance to Atovaquone in our study is due to the fact that this molecule atovaquone-proguanil is not sold in Africa as it is used in the prophylaxis on European tourists visiting malaria endemic areas. The use of this molecule would be rational.

The study of the correlation between *in vitro* chemosensitivity and mutation in codon 268 of *Pfcytb* gene responsible for the inhibition of Atovaquone, showed a poor correlation between the two tests. This test revealed that all isolates sensitive *in vitro* to Atovaquone have not undergone mutation Tyr268Ser or Tyr268Cyst on *Pfcytb* gene. These results are consistent with studies in 2008 in Thailand, where all isolates sensitive were of wild-type Tyr-268 [14]. It was reported also same results in Ethiopia [2].

Table I: In vitro susceptibility of isolates of P. falciparum to Atovaquone

	ATOVAQUONE (ATQ)
Number of tests taking	**64**
Number of interpretable test	**57 (89%)**
Sensitive isolates (IC_{50} **< 6 nM)**	
Number of isolates	**57**
IC_{50} Geometric Mean	**1,64 nM**
IC_{95} Geometric Mean	**1,42-1,87**
Resistant isolates (IC_{50} **≥ 6 nM**	
Number of isolates	**00**
IC_{50} Geometric Mean	**00**
IC_{95} Geometric Mean	**00**

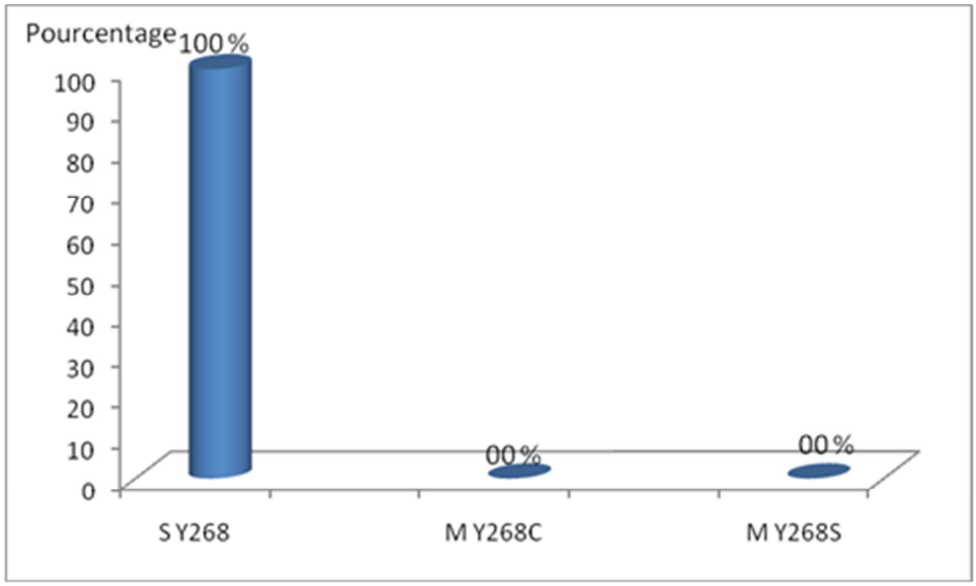

Figure 1: Distribution of isolates according to their genotypes

S = Wild
M = Mutant

Table II: Relationship between polymorphisms of the Pfcytb gene and in vitro susceptibility to Atovaquone

		***in vitro* test**		
		sensitive	resistant	
molecular test	wild	29	0	29
	Mutants	0	0	0
Grand total		**29**	**0**	**29**

K= kappa
K < 0

5 CONCLUSION

These results show that Atovaquone / proguanil combination should always be used in the prophylaxiques programs implemented by WHO.

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DECLARATION OF CONFLICT OF INTEREST

The authors wish to declare that there is no conflict of interest.

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