INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 27 November, 2013; received in revised form, 29 January, 2014; accepted, 16 March, 2014; published 01 May, 2014

IN VITRO SUSCEPTIBILITY OF *PLASMODIUM FALCIPARUM* TO LUMEFANTRINE AND ANALYSIS OF POLYMOPHYSIMS IN PFMDR-1 GENE ISOLATES FROM ABIDJAN (CÔTE D'IVOIRE)

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Keywords:

Plasmodium falciparum, Pfmdr-1, Lumefantrine, Côte d'Ivoire

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ABSTRACT: Context: Malaria endemic disease remains the most common tropical parasitic disease in the world. According to the World Health Organization (WHO), malaria is the first endemic disease of which humanity is paying a heavy price. Malaria is a major public health problem in Côte d'Ivoire. **Objective:** To evaluate the *in vitro* chemosensitivity to Lumefantrine (LUM) and analyze *Pfmdr*-1gene polymorphism in isolates of *Plasmodium falciparum* from Abidjan. **Materials and Methods**: The *in vitro* activity of Lumefantrine (LUM) was performed using the optical variant of microtest of the World Health Organization (WHO). After extraction, plasmodial *falciparum* DNA fragments were amplified by PCR method. The study of *Pfmdr*-1 polymophism was performed after sequencing of amplicons. The analysis of the relationship between the observed mutations and chemosensitivity of isolates was performed using Cohen's kappa test. **Results:** Out of 64 *Plasmodium falciparum* isolates tested, 57 (89%) gave cleare results of *in vitro* culture. It was found that 67% of them were susceptible to LUM against 33% that were resistant. Molecular study performed on 28 isolates showed that 11% had N86Y mutation while 57% had Y184F mutation. The degree of concordance between phenotypic and molecular data was moderate (k = 0.5).**Conclusion:** The presence of *Plasmodium falciparum* isolates resistant to LUM could compromise the effectiveness of association of artemether-lumefantrine recommended in the treatment of uncomplicated malaria in Côte d'Ivoire. Furthermore, mutations affecting *Pfmdr*-1 gene may play a role in resistance to LUM.

INTRODUCTION: Despite a decrease in the transmission and a reduction of 25% in mortality rate compared to year 2000, malaria endemic disease remains the most common tropical parasitic disease in the world. According to the World Health Organization (WHO), malaria is the first endemic disease of which humanity is paying a heavy price. The estimates indicated that 216 million cases of malaria were recorded in 2010, of which 81% from Africa, with 174 million cases, more than 655,000 cases of malaria deaths in 2010, 91% in Africa. In children under 5 years, 86% of deaths were attributed to malaria [1]. Malaria remains the leading cause of infant mortality in the world. Most malaria deaths are caused by *Plasmodium falciparum* [2].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(4).1732-38

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(4).1732-38

For more than a decade, studies on therapeutic effectiveness revealed increasing levels of resistance of this parasite species to conventional antimalarial drugs [3]. In view of this situation, WHO recommends several strategies including early diagnosis and prompt treatment of malaria cases.

Therefore, because of the failure of monotherapy especially chloroquine and sulfadoxine-pyrimethamine, a change in malaria treatment strategy becomes necessary in malaria endemic countries, including Côte d'Ivoire. Therefore, the Ministry of Health has adopted a new protocol for malaria treatment. Today, it is recommended, in Côte d'Ivoire, the use of combination therapies based on artemisinin derivatives (CTA), namely artesunate-amodiaquine or artemether-lumefantrine for treating uncomplicated malaria[4].

Unfortunately, national guidelines for management of malaria treatment are often not followed thereby increasing the risk of selection and expansion of isolates of drug-resistant *P. falciparum* within the populations [5]. Indeed, despite their relatively recent introduction in Côte d'Ivoire, already reported cases of reduced susceptibility *in vitro* of *P. falciparum* to artemisinin derivatives have been reported [6-7]. To the best of our knowledge, no study on the *in vitro* susceptibility of *P. falciparum* to Lumefantrine has been done in our context. H

owever, studies of the *in vitro* susceptibility of *P. falciparum* conducted in Thailand in 2010 showed that of 96 isolates a quarter of them had reduced sensitivity to LUM [8]. A similar study conducted in Senegal in 2011 confirmed this trend[9]. Misuse and often subcurative doses of artemether-lumefantrine could be responsible for decrease in the sensitivity or resistance of *P.* falciparum to LUM in Côte d'Ivoire.

Furthermore, the identification of a genetic marker of resistance to this molecule will lead to rapid assessment of the reduced efficacy of the treatment of malaria, which could be useful in the context of treatment recommendations. In this context, studies have shown that *Pfmdr-1* gene is the genetic marker of lumefantrine [7-8]. Further investigations therefore deserve to be conducted to confirm these results.

Our study is the first to be conducted in Côte d'Ivoire, our aim is to determine the *in vitro* susceptibility to LUM and analyze polymorphism in the *Pfmdr*-1 gene of *P. falciparum* isolates in Abidjan.

MATERIALS AND METHODS:

Study sites: This study was conducted from October 2009 to December 2010 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 antimalarial drugs and genomic testing. The infected blood samples were obtained from patients at the health center of Anonkoua-Kouté located on the outskirts of Abidjan in Abobo.

***P. falciparum* isolates:** The infected blood samples were obtained from patients with uncomplicated *P. falciparum* malaria in which parasite was greater than or equal to 4000 parasitized erythrocytes / µl of blood and who had not received any antimalarial drugs for 7 days prior 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 obtained with strict aseptic conditions. Parasitized blood samples were transported in a cooler at 4°C, the same day of collection in the laboratory where the tests *in vitro* chemo sensitivity 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* spec es (*P.* falciparum). Samples with parasitaemia ranged from 0.1% to 0.25% were used directly to test the *in vitro* drug sensitivity. Those whose parasite density was greater than 0.25% were diluted with uninfected erythrocytes.

Anti-Malaria: The LUM used for testing *in vitro* susceptibility were obtained from France (University Paris-South, Block 445, IBAIC and CNRS-UMR 8080, Orsay). The LUM solution was prepared in ethanol. Two fold dilutions were made extemporaneously in a solution of RPMI 1640 and distributed in duplicate and triplicate in culture plates of 96 wells.

***In vitro* tests:** The evaluation of the *in vitro* activity of LUM was performed using the optical version of the WHO microtest [10]. The inoculum consisted of parasitized erythrocytes, RPMI 1640 double buffered (25 mM HEPES), 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 900 µl of parasitized red blood cells (PRB),of which the hematocrit was reduced to 50% (450 µl of PRB + 450 µl of RPMI 1640)to 18.3 ml of RPS (1.83 ml BSA + 16.47 ml of RPMI 1640).

The inoculum was distributed in the culture plate in volume of 200 µl per well final concentrations of LUM ranges from 2.5 nM to 310 nM. After brief shaking of the plate, it was placed in a candle jar (Modular incubator chamber, ICN Biomedicals, California, USA)wet and rich in CO_2 (5%) and incubated in a microbiological incubator (MemmertTM) at 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 GX100. We validated tests that their percentage of schizont maturation in control wells was at least 20%.

Molecular study: Twenty microliters of parasitized blood washed with RPMI 1640 was placed on Whatman paper No 3, dried for 24 hours and stored for molecular tests. Plasmodial DNA was extracted by the Chelex 100 method described by Paul et al [11]. We used PCR to amplify a fragment embedded 650 bp *Pfmdr*-1 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 10µM solutions of a pair of specific primers to be amplified, 5 µl of 2 mM dNTP, 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 MDR-679-5'-AGA-GAA-AAA-TAA-AGA-TGG-CCT-CAG-3 'and MDR-1105R 5'-ACC-ACA-AAC-ATA-AAT-TAA-CGG-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 and 72°C X 1min and 72°C X 10 min at the end.

The secondary amplification was performed with a primer pair MDR-679-5'-TTT-GTA-TGT-GCT-GTA-TTA-TCA-3' and MDR-1072R 5'-GTA-ATA-CAT- AAA-GTC-AAA-CGT-3' with the same thermo cycler program as the first PCR.

Statistical Analysis: The determination of the 50% inhibitory concentrations of maturation (IC_{50}) was performed using nonlinear regression in Excel file. Threshold value adopted for the evaluation of *in vitro* chemosensitivity for LUM was 150 nM [12]. Sequence analysis of PCR was performed on ApE software (for Windows). Electropherograms reading was performed on the Chroma Lite software 2.01. The wild-type *pfmdr*-1 was defined by the alleles N86 and Y184.

The Cohen's kappa test was used to assess the degree of agreement between the different methods used to determine antimalarial drugs resistance [13]. 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.

RESULTS:

***In vitro* tests:** Of 64 isolates tested, 57 gave clear results a success rate of 89%. The geometric mean for IC_{50} of LUM of all isolates was 23 nM ($CI_{95\%}$: 17.5 to 26.2 nM).

The IC_{50} ranged from 3.66 nM to 184.25 nM. Ultimately, 38 of 57 isolates (67%) were susceptible to Lumefantrine against 19 (33%) that were resistant with geometric mean of 8.48 nM and 168.57 nM, respectively **(Table 1).**

TABLE 1: *IN VITRO* SUSCEPTIBILITY OF *P. FALCIPARUM* ISOLATES TO LUM

	ANTIMALARIA
	Luméfantrine (LUM)
Number of tests performed	64
Number of interpretable results	57/64 (89%)
IC_{50} Geometric Mean of *P. falciparum* isolates	23 nM
$IC_{95\%}$ of *P. falciparum* isolates	17,5 – 26,2
Isolates sensitivity (CI_{50} < 150nM)	
Number of 'isolates	38 (67%)
CI_{50} Geometric Mean	8,48 nM
CI_{50} Geometric Mean	6,57 – 10,28
Isolats résistants (CI_{50} ≥ 150 nM)	
Number of isolates	19 (33%)
CI_{50} Geometric Mean	168,93 nM
IC_{95} Geometric Mean	123,20 – 191,37

Molecular tests: We tested 37 isolates of which 28 gave clear interpretable results (79%). 9 (32%) had the wild-type genotype N86/Y184, three isolates (11%) were single N86Y mutants and 16 isolates (57%) single mutants Y184F **(Figure 1).**

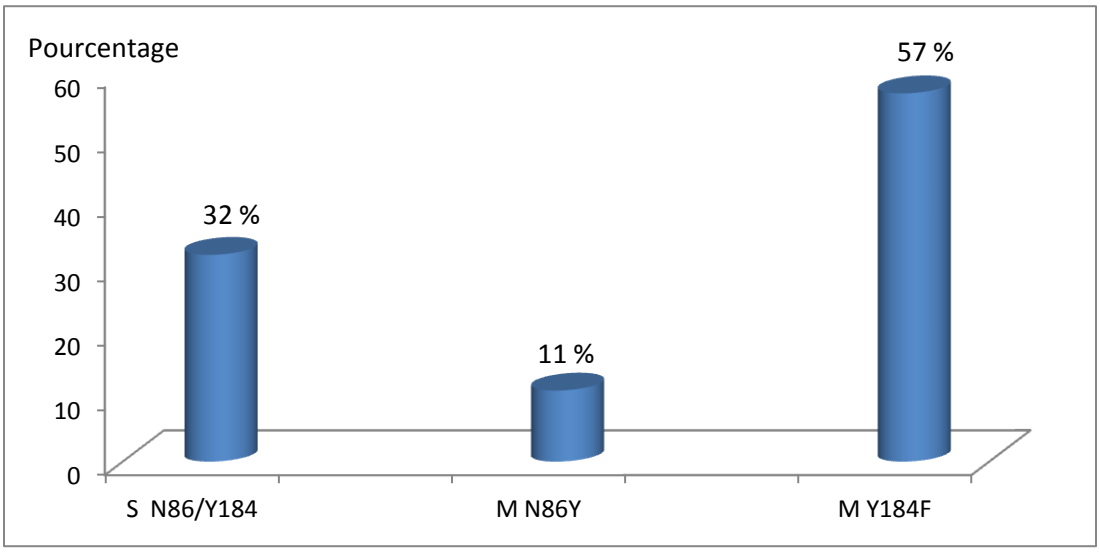

FIGURE 1: DISTRIBUTION OF ISOLATES ACCORDING TO THEIR GENOTYPE

Relationship *in vitro* chemosensitivity tests / molecular tests: Table 2 reports the polymorphism within *Pfmdr*-1A (region A) gene of the isolates according to the profile of sensitivity to Lumefantrine. According to Cohen's kappa test, the degree of concordance between the nature of *pfmdr*-1A gene and *in vitro* sensitivity to this molecule was 0.5.

TABLE 2: RELATIONSHIP BETWEEN POLYMORPHISMS ON *PFMDR*-1 GENE AND *IN VITRO* SENSITIVITY TO LUMEFANTRINE

Anti-malaria	n	CI_{50} (nM)	N°. of mutations strains				
			N86Y		Y184F		
			S	M	S	M	K
LUM	09	5,07- 14,19	09	00	09	00	0.5
	03	152,29-184,25	00	03	00	00	
	16	152,25-184,24	00	00	00	16	

K= kappa test

DISCUSSION: This study was carried out from October 2009 to December 2010, our aim was to evaluate the *in vitro* susceptibility of *Plasmodium falciparum* to Lumefantrine and analyze polymorphism in *Pfmdr*-1 gene at codons N86 and Y184.

The *in vitro* study has showed that 19 isolates (33%) were resistant to LUM with a geometric mean IC_{50} of 168.57 nM. *In vitro* resistance to LUM had already been reported in Africa. Indeed, in a study carried out in 2009 on 115 isolates in the area of Kilifi in Kenya, 80% of isolates tested were sensitive to LUM. But 20% of the population tested was resistant to the anti-malaria [14]. Rates of *in vitro* resistance to LUM were also reported by other authors in Asia especially in Thailand where isolates of *P. falciparum* were resistant to this antimalarial [15].

These results were confirmed in Tanzania [8]. Similarly, between 2006 and 2008, it was observed that 98% and 2% respectively for susceptible and resistant to this antimalaria drug [16]. In Senegal in 2011, various studies conducted on 165 and 93 isolates of *P. falciparum* gave isolates resistant rate of 3% and 1% respectively [6].

The results of all these studies carried out are in agreements with ours even if our results are higher (33% isolates resistant to LUM). These studies were conducted in malaria endemic areas where the prevalence of chloroquine resistance is very high which is part of Côte d'Ivoire (zone 3) [17]. We noted the appearance of resistance to Lumefantrine and this resistance increases with time, it was 2% in Uganda in 2008 and it rose to 3% in 2011 in Senegal. This increased resistance to Lumefantrine could be explained due to the high use of CTA including artemether-lumefantrine sold cheaply on the black market whose antimalarial activities are not certain and taking in wrong doses could explained the appearance of chemoresistance.

Our results were in contrast to what was obtained in 1999 and 2013 in Cameroon and Niger respectively. The results of these studies showed the absence of isolates resistance to Lumefantrine [18]. In any event, the presence of isolates resistant to LUM in our study may explain the occurrence of treatment failure in the artemether-lumefantrine combination reported by several authors [4].

To this must be added the case of low *in vitro* susceptibility to dihydroartemisinin, the active metabolite of artemisinin derivatives, previously reported in Côte d'Ivoire [3]. This drop in sensitivity or resistance to components of CTA could jeopardize their long-term use in the treatment of uncomplicated malaria in Côte d'Ivoire. It is therefore urgent that these drugs are prescribed rationally only to confirmed cases of malaria [1].

Sequence analysis of the *Pfmdr*-1 gene allowed us to explore the coding region of the gene that contains the codons N86 and Y184. And polymorphism analysis revealed 76% of single mutations in these codons; Y184F (57%) and N86Y (11%).The most frequent was codon 184 with the replacement of tyrosine by phenylalanine. At codon 86, there was the replacement of asparagine by tyrosine. In our study, we have not recorded any cases of double mutations 86Y/184F.

The prevalence of the 184F mutation compared to the 86Y mutation has already been reported in 2007 in Abidjan, for 83 isolates tested, 20.5% were of wild-type genotype and 79.5% were mutants with the following haplotypes: 86Y single mutants (n = 11), 184F (n = 30). Three years later, in a population of 61 isolates, it was reported that the 184F mutation was present in 75% of isolates and 86Y mutation in 47%of cases [4]. This predominance has also been reported in Nigeria, with respective rates of 64% and 29% [19]. Swaziland in 2010, it was observed 18% of isolates had 86Y mutants against 91% of isolates had the 184F mutation[20]. These results were confirmed in Tanzania with a rate of 61% for the 184F mutation [9].

However, some studies have shown the prevalence of 86Y mutation. Thus the analysis of 40 sequences of *pfmdr*-1 gene of Indian origin showed 80% and 20% for 86Y and 184F mutations respectively [21]. This trend had already been reported in 2009 in Côte d'Ivoire, with respective rates of 52.9% and 17.6% [22].

In view of all these studies, we observed that there is a variability profile of this gene based on regions in the same continent. These mutations in codons 86Y and 184F could play an important role in the function of *Pfmdr*-1 protein encoded by the *Pfmdr*-1 gene.

Polymorphism in the *Pfmdr*-1 gene observed in our study could be explained by the significant genetic mixing of parasite populations in different stages of growth of isolates of *P. falciparum* especially during sexual reproduction in the female Anopheles mosquito. This situation is characterized by chromosomal crossing and the creation of new alleles by intragenic recombination. The extent of this mixing may be related to the high malaria transmission in the Abobo area (study area) [4].

The study of the correlation between *in vitro* chemosensitivity and single mutations in codons 86 and 184 *Pfmdr*-1 gene showed a good correlation. Indeed all resistant (33%) isolates to Lumefantrine were all carriers of mutant alleles Tyr-86 or Phe-184.

In contrast, studies in Côte d'Ivoire in 2003 and 2010 showed that chloroquine-resistant isolates had the wild-type allele Arn-86 [4]. These results were confirmed by similar studies in Africa [23-24].

Our results showed that CTA sold on the black market have antimalarial activities not measured and uncontrolled and would cause the emergence of drug resistance to artemether-lumefantrine used as second-line in the fight against uncomplicated malaria in endemic areas including Côte d'Ivoire. The National Medical Council should ensure the rational use of CTA and remove alternative medicines from the market.

CONCLUSION: The presence of *Plasmodium falciparum* isolates resistant to LUM could compromise the effectiveness of association of artemether-lumefantrine recommended in the treatment of uncomplicated malaria in Côte d'Ivoire.

ACKNOWLEDGEMENTS: The authors wish to thank Basco L.K (South-Paris University, Bldg 445, IBAIC and CNRS-UMR 8080, Orsay, France).

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

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How to cite this article:
Jonhson NDT, Yavo W, Tano DK, N'cho M, Yapi FH and Djaman JA: *In vitro* susceptibility of *plasmodium falciparum* to lumefantrine and analysis of polymophysims in pfmdr-1 gene isolates from abidjan (côte d'ivoire). *Int J Pharm Sci Res* 2014; 5(5): 1732--1738.doi: 10.13040/IJPSR.0975-8232.5 (5).1732-1738.