Lack of artemisinin resistance in Plasmodium falciparum in northwest Benin after 10 years of use of artemisinin-based combination therapy

Aurore Ogouyemi-Hounto, Georgia Damien, Awa Deme, Nicaise Ndam, Constance Assohou, Didier Tchonlin, Atika Mama, Virgile Hounkpe, Jules Moutouama, Franck Remoue, et al.

To cite this version:
Aurore Ogouyemi-Hounto, Georgia Damien, Awa Deme, Nicaise Ndam, Constance Assohou, et al.. Lack of artemisinin resistance in Plasmodium falciparum in northwest Benin after 10 years of use of artemisinin-based combination therapy. Parasite, EDP Sciences, 2016, 23, pp.28. 10.1051/parasite/2016028. hal-02003180
Lack of artemisinin resistance in \textit{Plasmodium falciparum} in northwest Benin after 10 years of use of artemisinin-based combination therapy

Aurore Ogouyémi-Hounto\textsuperscript{1,2,*}, Georgia Damien\textsuperscript{3}, Awa Bineta Deme\textsuperscript{4}, Nicaise T. Ndam\textsuperscript{5}, Constance Assohou\textsuperscript{3}, Didier Tchonlin\textsuperscript{2}, Atika Mama\textsuperscript{2}, Virgile Olivier Hounkpe\textsuperscript{6}, Jules Doumitou Moutouama\textsuperscript{7}, Franck Remoué\textsuperscript{3}, Daouda Ndiaye\textsuperscript{4}, and Dorothée Kindé Gazard\textsuperscript{1,2}

\textsuperscript{1} Unité d’Enseignement et de Recherche en Parasitologie – Mycologie/Faculté des Sciences de la Santé; 01 BP 188 Cotonou, Bénin
\textsuperscript{2} Laboratoire du centre de lutte intégrée contre le paludisme; 01 BP 188 Cotonou, Bénin
\textsuperscript{3} Institut de Recherche pour le Développement. UMR 224-MIVEGEC; 08 BP 841 Cotonou, Bénin
\textsuperscript{4} Laboratory of Parasitology Mycology, Aristide le Dantec Hospital, BP 16477 Dakar, Senegal
\textsuperscript{5} UMR 216 MERIT-IRD Parasitology Department Noguchi Memorial Institute for Medical Research College of Health Sciences, University of Ghana, P.O. Box LG581 Legon, Accra, Ghana
\textsuperscript{6} Centre de santé de la Commune de Djougou, 02 BP 681 Porto Novo, Benin
\textsuperscript{7} Centre de santé de la Commune de Cobby, BP 40 Tanguéta, Bénin

Received 26 March 2016, Accepted 28 June 2016, Published online 21 July 2016

\textbf{Abstract – Aim:} In Benin, artemisinin-based combination therapy (ACT) has been recommended as the first-line treatment for uncomplicated \textit{Plasmodium falciparum} malaria since 2004. The emergence in Southeast Asia of parasites that are resistant to artemisinins poses a serious threat to global control of this disease. The presence of artemisinin resistance genotypes in parasite populations in Benin is currently unknown. The present study investigated the prevalence of relevant \textit{K13-propeller} gene polymorphisms in parasite isolates from the north-western region of Benin.

\textbf{Method:} \textit{Plasmodium falciparum} isolates were collected from children with a confirmed diagnosis of malaria aged 6 months to 5 years in two towns, Cobby and Djougou, in the north-western part of Benin. The study was conducted during the rainy season from July to November 2014 in local health facilities. The \textit{K13-propeller} gene was amplified in parasite isolates using nested PCR and subsequently sequenced.

\textbf{Results:} A total of 108 children were recruited into the study. The efficiency of amplification reactions was 72\% (78/108). The propeller domain of the \textit{K13} gene was successfully sequenced in 78 \textit{P. falciparum} isolates; all of them were wild type with no polymorphisms detectable.

\textbf{Conclusion:} The absence of mutations in the \textit{K13} gene indicates that \textit{P. falciparum} parasite populations in the study area are still fully susceptible to artemisinins.

\textbf{Key words:} \textit{Plasmodium falciparum}, Malaria, Resistance, Artemisinin, \textit{K13} propeller, Benin.


*Corresponding author: aurorefel@yahoo.fr

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Introduction

Artemisinin-based combination therapies are recommended by the World Health Organization (WHO) as first-line treatment for uncomplicated falciparum malaria in all areas in which malaria is endemic [32]. In 2004, as a result of high failure rates of treatment recorded with chloroquine and sulphadoxine-pyrimethamine (unpublished data from the National Malaria Control Programme), the Beninese National Malaria Control Programme implemented artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated malaria. The artemether-lumefantrine combination was thus deployed throughout the country in health facilities.

Currently, the different ACTs in use remain highly effective for the treatment of malaria in Africa, as demonstrated both by rapid parasite clearance and low rates of recrudescence after therapy in clinical trials, as well as by the high rates of sensitivity of clinical isolates ex vivo [3, 16, 19, 27, 31, 35]. The first clinical cases of artemisinin resistance in western Cambodia were reported in 2008 [21], and Plasmodium falciparum with reduced in vivo susceptibility to artesunate was reported in 2009 [8, 9]. Emergence of resistance was subsequently reported in neighbouring regions [2, 11, 25]. These recent developments have grave implications for public health, since artemisinin derivatives are the mainstay of antimalarial treatment worldwide. Hence the spread of ACT resistance could be catastrophic for malaria control and elimination efforts around the globe.

Despite the absence, thus far, of mutations associated with artemisinin resistance in P. falciparum isolates from different areas of sub-Saharan Africa [7, 20], previous experience with the spread of chloroquine and sulphadoxine-pyrimethamine resistant parasites from Asia to Africa [18, 33] demonstrates that the spread of drug resistance is likely, and that vigilant surveillance for resistant parasites is warranted. Recently, mutations in the propeller domain of the K13 gene were identified as candidate molecular markers of artemisinin resistance associated with slow parasite clearance rates [1, 6, 17, 28]. These associations indicate that mutations in the K13 propeller (especially C580Y, R539T and Y439H) are important determinants of artemisinin resistance. These markers could therefore serve as a tool to monitor resistance to ACT. Although ACT remains highly efficacious for the treatment of falciparum malaria, and delayed parasite clearance after ACT has not been noted in Benin [15, 22], the molecular epidemiology of artemisinin resistance genotypes in Benin parasite populations is unknown. The aim of the study described here was to characterise the variability of the K13 gene for the first time in Benin.

Methods

Study site

The study was conducted in Benin during the rainy season between July and November 2014, in two towns named Djougou, situated 450 km from Cotonou (the economic capital), and Coby, 643 km from Cotonou. At the two sites, malaria transmission occurs from May to November during the rainy season. P. falciparum is the predominant parasite species transmitted by Anopheles gambiae (85%) and An. arabiensis (15%) [34]. The prevalence of P. falciparum infection in the general population was 19.1% in Djougou and 18% in Coby (unpublished data).

Patients, sample collection and laboratory procedures

Plasmodium falciparum isolates were obtained from children diagnosed with malaria who had lived in the area of the study sites for more than a period of 6 months and had not travelled during the previous month. Children visiting the health facilities in the study area, aged 6 months to 5 years, and who met the criteria below were enrolled in the study: (i) fever (axillary temperature \( \geq 37.5^\circ \text{C} \)) or a history of fever within the past 48 h, (ii) P. falciparum mono-infection with parasite density \( \geq 1,000 \) asexual forms per microlitre, identified by microscopy on blood smears; and (iii) written informed consent from parents. Venous blood from children fulfilling the above criteria was collected. Thick and thin blood smears were prepared, stained with 10% Giemsa and examined to determine P. falciparum density and to confirm mono-infection by P. falciparum. All thick blood smears were examined against 500 leucocytes. Parasite densities were recorded as the number of parasites/\( \mu \)L of blood, assuming an average leucocyte count of 8,000/\( \mu \)L of blood. All slides were read in the laboratories of the health centres, with external quality control performed on 10% of the negative slides and all positives in the reference Parasitology Laboratory of the Centre National Hospitalo-Universitaire in Cotonou. Evaluation of K13-propeller polymorphisms was performed using the same venous blood sample used for diagnostic analysis stored as spots on filter paper. All malaria-infected patients, based on microscopy results, were treated with standard doses of
Results

During the study period, a total of 225 potentially eligible patients were screened for participation in the study. Following application of inclusion criteria, a total of 108 participants were enrolled in the study. Children’s ages ranged from 6 months to 5 years (mean age: 31.6 ± 0.4 months). Parasite density ranged from 1,028 to 192,715 parasites/µL, a geometric mean density of 16,562 [9,909; 27,681]. Parasite populations. The fact that only 72% of included samples were genotyped could be explained by the sensitivity of the PCR method used, because we did not analyse samples with low parasitaemia.

In this study, we did not find any mutation in the propeller part of the gene, as was the case in the Chatterie study in India [5]. Clinical artemisinin resistance is defined as a reduced parasite clearance rate, expressed as an increased parasite clearance half-life or a persistence of microscopically detectable parasites on the third day of ACT. The half-life parameter correlates strongly with results from the in vitro ring-stage survival assay. The absence of mutations in the K13 gene of parasite strains isolated in Benin confirms the results of the therapeutic efficacy tests, conducted at the same study sites, where adequate clinical and parasitological response was 100% after PCR correction [22]. Thus, after 10 years of ACT use, no polymorphisms have appeared in the K13 gene, suggesting that *P. falciparum* populations in the north-western part of Benin are still effectively susceptible to artemisinin. However, a larger sample size with extension into other parts of the country, including the south where drug pressure is higher [23], would allow us to draw better conclusions in this context. Artemisinin resistance, with delayed clearance of parasites after treatment with artemisinin monotherapy or artemisinin combination therapies (ACTs), is of great concern but has not yet been documented in sub-Saharan Africa, where speed of clearance of parasites after treatment with ACTs has generally been within the expected range [3, 10, 24, 31]. Small numbers of parasites with K13-propeller gene polymorphisms have been described in some countries in Africa, but importantly these were not the mutations previously associated with drug resistance in Southeast Asia [7, 20, 30]. The absence thus far of K13 resistance-associated mutants from Southeast Asia in Africa is promising, but continuous surveillance for the emergence of resistance should be implemented to enable early detection. The use of molecular markers such as K13 mutations is nowadays a cornerstone of malaria surveillance programmes, but potential differences between African and Asian K13-mutant parasites should be taken into account. The polymorphisms associated with artemisinin resistance in *P. falciparum* in Southeast Asia are not present in sub-Saharan Africa, but numerous K13-propeller coding polymorphisms have been documented in Africa [12–14, 24, 29]. Although their distributions do not support a widespread selective sweep for an artemisinin-resistant phenotype, the impact of these mutations on artemisinin susceptibility is unknown and will require further characterisation. Longitudinal studies conducted in Kenya [4, 20] showed that parasites from only one of 32 patients carried a mutation (at codon 578 (A578S)) in the propeller region of the pfK13 gene, despite evidence of longer than normal parasite persistence in over 30% of children. To identify K13 polymorphisms that affect artemisinin sensitivity in Africa, clinical trials should be supplemented with *in vitro* and molecular studies, providing additional data to strengthen confidence that observed mutations are associated with slowed parasite clearance.

Discussion

In Benin, ACT was introduced as the first-line treatment for uncomplicated *P. falciparum* malaria in 2004. Although this treatment remains highly efficacious, it is important to monitor the potential presence of artemisinin-resistant *P. falciparum* parasite populations. The fact that only 72% of included samples were genotyped could be explained by the sensitivity of the PCR method used, because we did not analyse samples with low parasitaemia.

**Analysis of Plasmodium falciparum isolates**

Parasite DNA was extracted from filter paper using the Chelex method [26]. The propeller domain of the K13 gene was amplified by nested PCR using the following primers: for the primary PCR (K13_PCR_F 5′-G GAAATCTGGTGGA-TAACAGC-3′ and K13_PCR_R 5′-GGAGTGA-CCAAATCTGGGA-3′) and for the nested PCR (K13_N1_F 5′-G GCAAACTGGTAAAGAAGCAGA-3′ and K13_N1_R 5′-G GCAAGCTGCCATTCTTGG-3′). The reaction volume and amplification programme used were reported previously [1]. Amplified products were bi-directionally sequenced by Sanger sequencing using BigDye® v3.1 from ThermoFisher Scientific by Beckman Coulter Genomics. The sequence reactions were then run on an ABI3730xl following the manufacturer’s protocols. The propeller domain of the K13 sequence data for single nucleotide polymorphisms (SNPs) was analysed using Geneious software (www.geneious.com). A cut-off of quality score HQ > 30% (the percentage of untrimmed bases that are high quality) was applied to all sequences. Sequences were assembled using the *de novo* assembly method and aligned to the reference K13 annotated *Plasmodium falciparum* 3D7 (PF3D7-1343700). The polymorphism search was limited to inside CDS sequences. A search was performed for the mutations described in Asia and in the previous study [13, 14, 29].

**Ethical approval:** The study obtained the ethical approval of the National Ethics Committee for Health Research of Benin.

**Polymorphism of the K13 propeller**

The propeller domain of the K13 gene was successfully sequenced in 78 *P. falciparum* isolates. After alignment with PF3D7-1343700, all the strains were found to be wild type having no polymorphism previously found in the K13 gene.
Conclusion

In this study, the absence of mutations in the K13-propeller gene suggests that artemisinin resistance is not a problem in Benin. However, similar studies from different parts of the country with a larger number of samples will be helpful to ascertain the emergence of artemisinin resistance, if any. In addition, routine monitoring and surveillance, as recommended by the WHO global plan for artemisinin resistance containment, should be continuously strengthened. Moreover, this study contributes to the ongoing surveillance of suspected artemisinin resistance parasites in Africa by providing baseline data on K13-propeller mutations in Benin.

Conflict of interest

The authors declare no conflict of interest in relation with this paper.

Acknowledgements.
We are grateful to the children who participated in the study, as well as to their mothers. We are pleased to thank caregivers from Djougou and Coby health services. This work was integrated into the PALEV ALUT project, funded by the 5% – Expertise France Initiative. This funding contributed to data collection, laboratory testing and payment of investigators.

References


