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Absence of *Plasmodium falciparum* K13 Propeller Domain Polymorphisms among Field Isolates Collected from the Brazilian Amazon Basin between 1984 and 2011

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Abstract. Artemisinin resistance, presently confined to Southeast Asia and associated with mutations in the *Plasmodium falciparum* K13 (PfK13) propeller domain, represents a serious threat to global malaria control. This study aimed to provide baseline information for future artemisinin resistance surveillance, by analyzing the PfK13 propeller domain in *P. falciparum* field isolates collected from the Brazilian Amazon Basin between 1984 and 2011. A total of 152 *P. falciparum* mono-infections were assessed, of which 118 (78%) were collected before and 34 (22%) after the introduction of artemisinin-based combination therapy (ACT) in 2006. An 849-base pair fragment encoding the PfK13 propeller was amplified by nested polymerase chain reaction and sequenced in both directions. The sequences were compared with the reference sequence of *P. falciparum* 3D7. All samples showed wild-type sequences, thus, no mutations were observed. The results are in agreement with other recent reports and do not provide evidence for presence of PfK13 propeller domain polymorphisms associated with artemisinin resistance among *P. falciparum* field isolates in the Brazilian Amazon Basin neither before nor after the implementation of ACT.

INTRODUCTION

Artemisinin-based combination therapy (ACT) has been instrumental for the recent improvements in global malaria control. These achievements are now under threat because of emergence and spread of artemisinin resistant *Plasmodium falciparum*, phenotypically characterized as delayed parasite clearance. Artemisinin resistance was first reported from western Cambodia in 2007.[1] Despite containment activities artemisinin resistant *P. falciparum* has spread to five neighboring countries in the Mekong region, i.e. Thailand, Myanmar, Vietnam, Laos, and southern China.[2–6]

History of antimalarial drug resistance provides evidence for independent evolution of chloroquine and sulfadoxine–pyrimethamine resistance in different "resistance epicentres," such as the Amazon Basin, South America, and Southeast Asia.[7–9] The reason for the Amazon Basin to be particularly resistant prone is not fully understood, but the intensive flux of migrant workers engaged in gold mining, a vulnerable group with limited access to appropriate malaria case management and therefore more likely to exercise self-medication with questionable drug quality, might represent contributing factors.[10]

Brazil is responsible for 46% of malaria burden in the region of the Americas. In 2017, Brazil reported 193,972 malaria cases.[11] The Brazilian Amazon Basin, located in the Northern region, accounts for 99.5% of all malaria cases in the country.[11] Quinine plus doxycycline and mefloquine were gradually replaced as first line therapies for *P. falciparum* malaria by fixed combinations of artemether–lumefantrine and artesunate–mefloquine in 2006.[12] The deployment of artesunate–mefloquine contributed to a drastic reduction of cases and morbidity in an area of high transmission in the country[13] and remains highly efficacious according to a clinical trial carried out between November 2010 and February 2013.[14]

A genome-wide association study identified mutations in the *P. falciparum* K13 (PfK13) propeller domain associated with artemisinin resistance in vitro and in vivo in parasites from Cambodia.[15] A subsequent study showed that the replacement of mutations in PfK13 propeller by wild-type allele in resistant clones significantly reduced the survival rates in a ring-stage survival assay, and the insertion of mutation in the sensitive *P. falciparum* Dd2 clone increased the survival rate.[16] More than 200 mutations have been described to date but only N458Y, Y493H, R539T, I543T, and C580Y are validated as molecular markers for resistance in *P. falciparum* parasites from Southeast Asia.[6] In South America, the presence of C580Y was reported in *P. falciparum* from Guyana collected in 2010 and microsatellite analysis suggested this allele has emerged independently from Cambodian isolates.[17] Nevertheless, a therapeutic efficacy study conducted in 2014 reported 100% efficacy by day 28 in uncomplicated *P. falciparum* patients treated with 7-day artesunate monotherapy.[18]

This study aimed to provide baseline information for future artemisinin resistance surveillance in this country by analyzing the PfK13 propeller domain in *P. falciparum* field isolates collected from the Brazilian Amazon Basin between 1984 and 2011.

MATERIALS AND METHODS

Samples. *Plasmodium falciparum* samples were selected from the biorepository of Núcleo de Estudos em Malária of SUCEN/IMT-USP, São Paulo, Brazil. They were obtained from the following settings: 1) patients admitted to Núcleo de Estudos em Malária of SUCEN/Hospital das Clínicas of FMUSP, São Paulo, São Paulo from 1984 to 2011 ($N = 94$); 2) participants from a study that evaluated the in vitro response to several antimalarials in Peixoto de Azevedo, Mato Grosso during 1996 and 1997 ($N = 25$); 3) patients admitted to Laboratório de Malária/Hospital Municipal, Santarém, Pará from November 2010 to February 2011 ($N = 24$); 4) active

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surveillance carried out by Serviço de Endemias of Alenquer, Pará in November 2010 ($N = 9$). All individuals were diagnosed with *P. falciparum* mono-infection by thick-blood smear microscopy and the origin of infection was the Brazilian Amazon Basin. Blood samples were collected before initiation of treatment. Samples from settings 1 and 2 were collected by venous puncture in ethylenediamine tetraacetic acid tubes and frozen with glycerolyte in liquid nitrogen after washing with RPMI medium. Before initiation of the molecular analyses, these samples were thawed at room temperature and a volume of 50 μL was spotted on Whatman® 3-mm filter paper (Sigma-Aldrich, St. Louis, MO), to standardize the DNA extraction. The remaining samples, originating from settings 3 and 4, were collected directly on Whatman® 3-mm filter paper by capillary finger-prick blood sampling and stored at room temperature.

***Plasmodium falciparum* K13 propeller domain genotyping.** For DNA extraction, two 3.2-mm Ø punches were obtained from the filter paper using 1296-071 DBS Puncher (PerkinElmer, Waltham, MA). DNA was extracted with Chelex® 100 resin (Bio-Rad Laboratory, Hercules, CA) by the boiling method with minor modifications from the original protocol,[19] using 0.2% saponin/phosphate-buffered saline and 10% Chelex, and stored at –20°C until use. An 849-base pair (bp) fragment of the PfK13-propeller domain, covering amino acid position 407–689, where the described mutations associated with artemisinin resistance are located, was amplified by nested PCR using previously described primers.[15] All PCR reactions included DNA extracted from *P. falciparum* 3D7 and sterile water (Sigma-Aldrich) as positive and negative controls, respectively. After PCR, the products were separated by electrophoresis in 1.5% agarose gel and visualized with ultraviolet light in Gel Doc™ XR+ Gel Documentation System (Bio-Rad Laboratory, Hercules, CA). The 849-bp fragment was extracted and purified from the gel to remove non-incorporated deoxyribonucleotide triphosphates and primers using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI) or GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA) commercial kits. The purified PCR products from field samples and positive control were sent for Sanger sequencing to Macrogen, Netherlands, using both forward and reverse primers. The sequences were analyzed by one staff member in Sequencher version 5.1 software (Gene Codes Corporation, Ann Arbor, MI). Sequences generated by both primers were aligned for each sample and compared with the reference sequence from *P. falciparum* 3D7 (PlasmoDB gene ID: PF3D7_1343700). PCR and sequencing were repeated if some mismatch was observed and/or if the sequencing quality was poor. Samples presenting poor quality sequences after a second round of PCR and sequencing were removed from analysis.

GenBank accession numbers. The datasets generated during this study are available in the GenBank repository (accession numbers KY774704–KY774808).

Ethical approval. This study was approved by the Ethics Committee of Departamento de Moléstias Infecciosas e Parasitárias da Faculdade de Medicina da Universidade de São Paulo and by the Regional Ethics Committee, Stockholm, Dnr 2017/499-32.

RESULTS

In total 152 *P. falciparum* mono-infection samples were analyzed, of which 118 (77.6%) were collected before and 34 (22.4%) after deployment of ACTs as first line treatment in Brazil in 2006. The geographical distribution of the origin of the *P. falciparum* field samples by states in the Brazilian Amazon Basin and period of collection are described in Table 1. Three samples were obtained from individuals who presented with microscopy-confirmed recurrent parasitemia after treatment with artemisinin derivatives (Table 2). Re-infection is not considered in these cases since none of the patients resided in a malaria-endemic area during the treatment and follow-up periods.

PCR success rate was 121/152 (79.6%), of which 105 (86.8%) generated good quality sequences. All successfully sequenced samples, including the three samples collected from individuals with recurrent parasitemia, showed wild-type genotypes in the analyzed fragment of the PfK13 propeller domain, no mutations were thus observed.

DISCUSSION

This study aimed at providing baseline information for future artemisinin resistance surveillance by analyzing the PfK13 propeller domain in *P. falciparum* field isolates collected from the Brazilian Amazon Basin between 1984 and 2011. The results do not provide evidence for presence of PfK13 propeller domain polymorphisms associated with artemisinin resistance among *P. falciparum* field isolates in the Brazilian Amazon Basin neither before nor after the deployment of ACTs as first line treatment in Brazil in 2006.[12]

The absence of PfK13 propeller domain polymorphisms among samples collected before ACT implementation in Brazil is in agreement with a worldwide mapping study that included 31 *P. falciparum* samples from Acre State collected before 2006.[20] The assumed relatively low exposure of the Brazilian *P. falciparum* population to artemisinin before 2006 is likely to explain the absence of polymorphisms in the PfK13 propeller domain because sulfadoxine–pyrimethamine was the treatment of choice from 1960s to late 1980s, followed by quinine + doxycycline or mefloquine between 1990 and 2006.[21,22] Even after the introduction of ACTs as first-line treatment in Brazil no polymorphisms were observed. However, the number of *P. falciparum* samples collected after 2006 is small, which is an apparent limitation of the study, and why data should be interpreted with caution. Still this finding is in agreement with a recent study conducted in the Amazonas State 2014 that reported one *P. falciparum* sample of 237 (0.4%) analyzed harboring the A481V mutation. The remaining

TABLE 1

Geographical distribution of the origin of the *Plasmodium falciparum* field samples by states in the Brazilian Amazon Basin and period of collection

State	Before ACT	After ACT
Acre	2	1
Amazonas	2	0
Amapá	2	0
Mato Grosso	50	0
Pará	20	33
Rondônia	36	0
Roraima	3	0
Unknown origin	3	0
Total	118	34

ACT = artemisinin-based combination therapy.

TABLE 2

Initial treatment, day of microscopy confirmed recurrent parasitemia after treatment initiation, and day of sample collection for *Plasmodium falciparum* K13 propeller domain genotyping among individuals with artemisinin-derivative treatment failure

Sample	Treatment	Day of recurrent parasitemia	Day of sample collection
R26	Artesunate + quinine	23	0
R32	Artesunate + mefloquine	29	29
R59	Artemether	31	31

236 were all wild type.[23] Another report,[14] from a clinical trial conducted in Acre State between 2010 and 2014 with 162 participants did not find any PfK13 propeller domain polymorphisms but described a K189T mutation among 61 (43.6%) samples. Importantly, K189T is located outside the propeller domain and has not been associated with artemisinin resistance.[6] Of note is that all 154 individuals from this study that were treated with artesunate–mefloquine and followed up for 42 days had adequate clinical and parasitological response.[14]

Currently artemisinin-derivatives are the most powerful antimalarial drugs available. If resistance to these drugs occurs independently, or is spread outside the Mekong Region, it would have devastating effects on global malaria control. To reduce these risks both aggressive containment activities in Southeast Asia are needed, as well as robust early warning systems of artemisinin resistance in endemic countries presently not affected by artemisinin resistant *P. falciparum.* Molecular surveillance of PfK13 propeller domain polymorphism may play an important role in this regard, provided baseline data are available for comparison. However, *P. falciparum* clearance times after ACT treatment are also important to monitor.

In South America, a historical epicenter of antimalarial drug resistance, an increase in day 3 microscopy determined parasite positivity rate was observed in Suriname after artemether–lumefantrine treatment in 2011,[24] and in Guyana the C580Y mutation, located in the PfK13 propeller domain, has recently been reported.[17] In the border areas of Brazil, Suriname, Guyana, and French Guiana a massive flux of gold mining and logging workers takes place.[25] These migrant workers are prone to take antimalarial drugs, often of unknown quality or even illegal, whenever a febrile episode occurs, because of factors including limited access to formal health care.[21,24] Thereby, they represent a risk group of selection of artemisinin resistant *P. falciparum*, which needs to be targeted. Although the distribution of antimalarial drugs in Brazil is controlled by the government, and consequently not available in the private sector, resistant parasites selected in these settings may be introduced in the country through this migrant population. The Brazilian Ministry of Health in partnership with the World Health Organization launched in November 2015 the Plan for Elimination of Malaria in Brazil, focusing on *P. falciparum*. Monitoring of ACTs efficacy and surveillance of molecular markers associated with *P. falciparum* resistance are key components to achieve this ambitious goal.

In conclusion, the results from this study are in agreement with other recent reports, and do not provide evidence for presence of PfK13 propeller domain polymorphisms associated with artemisinin resistance among *P. falciparum* field isolates in the Brazilian Amazon Basin neither before nor after the implementation of ACT.

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