

Artemisinin Resistance-Associated Polymorphisms at the K13-Propeller Locus Are Absent in *Plasmodium falciparum* Isolates from Haiti

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Abstract. Antimalarial drugs are a key tool in malaria elimination programs. With the emergence of artemisinin resistance in southeast Asia, an effort to identify molecular markers for surveillance of resistant malaria parasites is underway. Non-synonymous mutations in the kelch propeller domain (K13-propeller) in *Plasmodium falciparum* have been associated with artemisinin resistance in samples from southeast Asia, but additional studies are needed to characterize this locus in other *P. falciparum* populations with different levels of artemisinin use. Here, we sequenced the K13-propeller locus in 82 samples from Haiti, where limited government oversight of non-governmental organizations may have resulted in low-level use of artemisinin-based combination therapies. We detected a single-nucleotide polymorphism (SNP) at nucleotide 1,359 in a single isolate. Our results contribute to our understanding of the global genomic diversity of the K13-propeller locus in *P. falciparum* populations.

Discussions of global malaria elimination programs have been renewed in light of the decline in malaria-associated morbidity and mortality resulting from scaled-up vector- and parasite-targeted interventions over the past decade.¹ A key strategy in malaria elimination programs is effective use of antimalarial drugs, particularly artemisinin-based combination therapies (ACTs), which are the first-line therapies in most malaria-endemic countries.¹ However, malaria elimination goals face a new threat with the emergence of artemisinin resistance in southeast Asia.^{2–5} A recent study used both *in vivo* and *in vitro* methods to identify non-synonymous single-nucleotide polymorphisms (SNPs) at the PF3D7_1343700 kelch propeller domain (K13-propeller) in Cambodian *Plasmodium falciparum* associated with artemisinin resistance.⁶ Subsequent investigations at the K13-propeller locus in other southeast Asian populations, including those of Vietnam, Myanmar, Laos, and Thailand, confirm the association of non-synonymous SNPs at the K13-propeller with artemisinin resistance.⁷ However, these polymorphisms were not detected in sub-Saharan Africa *P. falciparum* samples from Gambia, Mali, Ghana, Burkina Faso, Congo, Democratic Republic of Congo, Kenya, Tanzania, Malawi, and Uganda, where little to no artemisinin resistance has been reported.^{7–9} Furthermore, other non-synonymous SNPs were identified in the sub-Saharan African samples that were not observed in the southeast Asian *P. falciparum* samples. These findings suggest that polymorphisms in the K13-propeller could vary geographically, and therefore, genetic studies on the K13-propeller in other malaria-endemic countries are needed to assess the use of this locus as a tool to monitor the global emergence and spread of artemisinin resistance.

Haiti is one of two remaining malaria-endemic countries in the Caribbean. It has several favorable elimination characteristics.¹⁰ (1) One parasite species (*P. falciparum*) accounts for the majority, if not all, cases of malaria.¹¹ (2) Malaria transmission is low.^{12–14} (3) Being on the only malaria-endemic

island in the Caribbean, there is likely reduced importation of malaria parasite-carrying vectors from neighboring malaria-endemic regions compared with countries in Africa and South America that are surrounded by other malaria-endemic countries. Another important feature is the absence of chloroquine resistance in the present *P. falciparum* population in Haiti, which was evidenced by a recent *in vivo* efficacy study (Okech BA and others, unpublished data) and molecular investigations into the *pfcr* and *pfmdr* genes.^{15,16} Although pyrimethamine resistance was reported in an *in vitro* study from 1984,¹⁷ a recent molecular study reported only the *pfdhfr* S108N low-level pyrimethamine resistance mutation and no sulfadoxine resistance-associated mutations in the *pfdhps* in Haiti.¹⁸ Currently, chloroquine remains the first-line treatment for malaria in Haiti. However, ACTs are likely candidates to replace chloroquine in the future should chloroquine resistance emerge in Haiti.

There is evidence that ACTs have been used by international health non-government organizations in Haiti¹⁶ (Ministry of Public Health and the Population [MSPP], personal communication) and antimalarial treatment studies.¹⁹ Because of limited oversight by the MSPP in Haiti, it is not known how widespread ACT use is in Haiti. It is, therefore, important to generate baseline data on genetic markers associated with artemisinin resistance before ACTs become widely used in Haiti. We originally targeted the *pfatpase6* gene, because it carried an SNP (S769N) that was associated with lowered sensitivity to artemether in an earlier investigation in French Guiana²⁰; however, we did not detect the S769N mutation in our preliminary sample (data not shown). With more recent studies unable to confirm the association of the S769N mutation with artemisinin resistance in other populations^{21,22} and the recent identification of the K13-propeller as a strong candidate for artemisinin resistance, we decided to refocus our investigation on K13-propeller polymorphisms. To contribute to the growing knowledge about the global genetic diversity of the K13-propeller and establish a baseline for future surveillance of potential artemisinin resistance in Haiti, we sequenced an 810-base pair region of the K13-propeller from *P. falciparum* samples collected in Haiti between 2010 and 2013.

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FIGURE 1. A map of the study sites in Haiti. Most samples were collected from sites in the west (Ouest) and southeast (Sud-Est) departments represented by the blue diamonds. Smaller samples sets are represented by red stars.

Blood spot samples were collected from seven study sites in Haiti: Terre Noire, Leogane, Jacmel, Chabin, Hinche, North Cap Haitian, and Nippes (Figure 1). Details about the collection of these samples have been discussed previously.^{15,18} Terre Noire is considered an urban region, whereas Leogane, Jacmel, Chabin, Hinche, North Cap Haitian, and Nippes are more rural. Although malaria transmission in Haiti is low overall, there have been reports of transmission hotspots in the Southeast Department (which includes Jacmel and Chabin), particularly near coastal areas.^{23,24} DNA was extracted using the QIAamp DNA Investigator Kit and eluted with 60 μ L elution buffer (Qiagen Inc., Valencia, CA). Nested polymerase chain reaction (PCR) amplification of an 810-base pair region of K13-propeller (nucleotides 1,298–2,108) was carried out as described in the work by Ariey and others⁶ with the following modified reagent concentrations for both the primary and nested reactions: 1 \times GoTaq Flexi Buffer, 2.5 mM MgCl₂, 0.2 mM each nucleotide, 0.25 μ M each primer in the primary PCR, and 0.625 U Go Taq Hot Start Polymerase (Promega, Madison, WI). Amplicons were sequenced with the nested primers using Big Dye Master Mix and run on an ABI Genetic Analyzer (Applied Biosystems, Foster City, CA). DNA sequences were aligned to the 3D7 reference sequences (National Center for Biotechnology Information Reference Sequence XM_001350122.1) using Sequencher 4.10.1 (Gene Codes Corp, Ann Arbor, MI).

Of 94 samples initially targeted for sequencing of the K13-propeller, 82 samples were successfully sequenced: 38 samples from Terre Noire, 18 samples from Leogane, 15 samples from Jacmel, 1 sample from Chabin, 4 samples from Hinche, 4 samples from North Cap Haitian, and 2 samples from Nippes. One SNP, T to A at nucleotide 1,359 (glycine at amino acid 453), was detected in a single mixed infection sample (i.e., both mutant and wild-type alleles were observed) and confirmed

two times. The 1,359 SNP results in a synonymous substitution, and therefore, it likely plays no role in artemisinin resistance. No other K13-propeller polymorphisms were detected in any of the other samples. This sample was collected from Leogane in January of 2012 during the high-transmission season in Haiti. Although Haiti holds a chloroquine treatment policy in Haiti, it is not clear whether unregulated ACT administration is occurring in Leogane.

The absence of known artemisinin resistance polymorphisms in the K13-propeller in our *P. falciparum* samples in Haiti is not surprising, because the level of artemisinin use is likely too low to exert selective pressure on the *P. falciparum* population. Low genetic variation observed at the K13-propeller mirrors the results of investigations into other antimalarial resistance-associated loci in Haiti, including *dhfr*, *dhps*, *pfcr*, and *pfmdr*,^{15,18} supporting the idea that Haiti's *P. falciparum* population has low genetic variation at antimalarial resistance-associated genes.

In summary, these data serve as baseline information for monitoring artemisinin resistance should artemisinin-based therapies become widespread in Haiti. Given the diversity of the K13-propeller locus observed in *P. falciparum* samples from sub-Saharan Africa, it is possible that other pathways and polymorphisms could lead to artemisinin resistance. Continued investigations of the K13-propeller locus and additional genome-wide association studies to identify novel resistance loci in other malaria-endemic countries of varying transmission intensities and levels of ACT use are needed to elucidate the mechanisms behind artemisinin resistance. This information is crucial to ensure that appropriate genetic markers are included in molecular surveillance of artemisinin resistance globally.

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