

Plasmodium falciparum Drug-Resistant Haplotypes and Population Structure in Postearthquake Haiti, 2010

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Abstract. Chloroquine (CQ) remains the first-line treatment of malaria in Haiti. Given the challenges of conducting in vivo drug efficacy trials in low-endemic settings like Haiti, molecular surveillance for drug resistance markers is a reasonable approach for detecting resistant parasites. In this study, 349 blood spots were collected from suspected malaria cases in areas in and around Port-au-Prince from March to July 2010. Among them, 121 samples that were *Plasmodium falciparum* positive by polymerase chain reaction were genotyped for drug-resistant *pfprt*, *pfdhfr*, *pfdhps*, and *pfmdr1* alleles. Among the 108 samples that were successfully sequenced for CQ resistant markers in *pfprt*, 107 were wild type (CVMNK), whereas one sample carried a CQ-resistant allele (CVIET). Neutral microsatellite genotyping revealed that the CQ-resistant isolate was distinct from all other samples in this study. Furthermore, the remaining parasite specimens appeared to be genetically distinct from other reported Central and South American populations.

INTRODUCTION

Plasmodium falciparum is endemic on the island of Hispaniola, which comprises the countries of the Dominican Republic and the Republic of Haiti. Almost all cases of malaria in Haiti are reported to be *P. falciparum*, whereas *Plasmodium vivax* and *Plasmodium malariae* are rarely reported in Haiti.^{1,2} *P. falciparum* remains sensitive to treatment with chloroquine (CQ), and therefore it is the first-line therapy in both countries. Once the mainstay of global *P. falciparum* treatment, resistance to CQ has developed throughout the world except in Hispaniola and Central America.

There is concern that CQ resistance will spread to or develop in Hispaniola as it has in other parts of the world. The World Health Organization (WHO) recommends monitoring for CQ resistance by in vivo efficacy studies every 2–3 years, in general, depending on the level of transmission. However, conducting such trials can be expensive and time consuming in countries where malaria incidence is low; therefore, molecular surveillance has become a valuable tool for assessing the prevalence of CQ- and sulfadoxine–pyrimethamine (SP)-resistant parasites in countries with low malaria transmission.^{3,4} Recently, WHO has included in its recommendations that in areas with low to very low transmission, studies with molecular markers should be conducted systematically every year provided that markers are known and validated.

In vivo trials in Haiti found no evidence of CQ resistance during the 1980s.⁵ More recently, studies have taken advantage of the well-characterized molecular basis for CQ resistance to assess the prevalence of resistance mutations. To assess CQ resistance, mutations in the *P. falciparum* CQ resistance transporter (*pfprt*) gene are most relevant, with the critical point mutations being present in codon 76 of the gene resulting in an amino acid change from lysine to threonine. In addition to the K76T codon mutation, mutations in codons 72, 73, and 74 also occur, and are relevant to the geographic context of where the original CQ-resistant popula-

tion originated and subsequently spread. Thus, SVMNT haplotype parasites are found in the *P. falciparum*-endemic regions of South America and CVIET haplotype parasites are found in Africa and southeast Asia.

There have been conflicting reports regarding the presence of K76T mutation in *P. falciparum* isolates from Haiti.^{6–9} Work conducted in the Haitian Department of Artibonite in 2009 reported only 6% of parasites carried the K76T mutation along with the CVIET haplotype ($N = 79$).⁶ Two cases of malaria that were imported to Canada, ostensibly from Haiti, carried the K76T mutation. However, 48 cases imported to France from Haiti carried only the wildtype CQ-sensitive allele.⁷ Between 2010 and 2011, 49 malaria patients from Leogane, 18 miles west of Port-au-Prince, showed no evidence of CQ treatment failures or resistant haplotypes.⁸ Similarly, there were no *pfprt*-CQ-resistance markers present in *P. falciparum* parasites from 79 malaria case samples collected in Port-au-Prince, Artibonite, North Cap Haitian, Leogane, Hinche, and Jacmel in 2013.⁹ Finally, the most recent in vivo CQ-efficacy study conducted from 2011 to 2012 in Leogane and Port-au-Prince found no evidence of CQ-resistance mutations in parasites collected from treatment failures.¹⁰

In response to the Haitian earthquake of 2010, infectious disease surveillance was increased, especially where internally displaced persons were living at high risk for malaria infection. There was concern that large population movements and poor living conditions could increase vector exposure and therefore dramatically increase the risk of malaria transmission. The U.S. Centers for Disease Control and Prevention (CDC) assisted the Haiti Ministry of Public Health and Population in the implementation of enhanced malaria surveillance.¹¹ Herein, we report the prevalence of CQ- and SP-resistant alleles by testing mutation patterns in four genes; *pfprt*, *pfdhfr*, *pfdhps*, and *pfmdr1*. We also assessed the parasite population structure for evidence of parasite importation from other countries by the migration of foreign aid workers to support the postearthquake period relief effort.

METHODS

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Ethics approval. Approval for this study was provided by the CDC human subjects office as part of an emergency

response and assistance effort, and was approved as non-research activity. As per this approval, verbal consent was obtained. No local ethics review process was functioning immediately after the earthquake; however, agreement for the study was obtained with the Haiti Ministry of Public Health and Population.

Sample collection and processing. Dried blood spots from patients with suspected or confirmed malaria were collected from existing and temporary health facilities in metropolitan and suburban Port-au-Prince, principally at the international airport, in Carrefour, Leogane, and Jacmel after the earthquake ($N = 349$) from March to November 2010. Thirty blood spot specimens that were collected came from Ranquitte in the North Department, as part of a larger effort to measure the malaria trends described previously.¹¹ Almost all the participating facilities were supported by nongovernmental organizations that were assisting in the earthquake response effort. Dried blood spot specimens were collected from febrile patients with suspected malaria infection. Some were tested for the presence of malaria parasites using rapid diagnostic tests and/or microscopy. The dried blood spots were collected on Whatman 903 Protein Saver Cards (Sigma Aldrich, St. Louis, MO) for molecular testing. Collected specimens were stored in individual zip-lock bags containing silica gel desiccant and were kept at approximately 4°C when possible. Filter paper samples were collected periodically and transported to the CDC for processing. DNA was extracted from dried blood spots using the QIAamp DNA blood mini kit (QIAGEN, Valencia, CA) per manufacturer's instructions. A nested polymerase chain reaction (PCR) amplifying the 18s ribosomal RNA (rRNA) gene was used to confirm malaria diagnosis and differentiate malaria species.¹²

Targeted gene amplification and sequence analysis. PCR amplification was performed for *pfert*, *pfdhfr*, *pfdhps*, and *pfmdr1* using primers and conditions noted in Supplemental Table 1. Sanger sequencing of the amplified gene fragments was performed using an Applied Biosystems 3130 capillary sequencer (Applied Biosystems, Foster City, CA) to monitor mutations associated with drug resistance in the targeted genes. Sequences were deposited in the GenBank repository (accession nos.: KP704735–KP705069).

Microsatellite analysis. Seven neutral microsatellite loci spanning six chromosomes (TA1, chromosome 6; poly α , chromosome 4; PfpK2, chromosome 12; TA109, chromosome 6; and 2490, chromosome 10; C2M24, chromosome 2; and C3M69, chromosome 3) were used to assess the relatedness of *P. falciparum* isolates within Haiti and South America.^{13–15} Genomic DNA was amplified by nonnested or heminested PCR using previously described methods.^{12–15} Fluorescently labeled (HEX and FAM) PCR products were separated by capillary electrophoresis on an Applied Biosystems 3130xl analyzer. Alleles were sized and scored using GeneMapper v3.7 (Applied Biosystems), and binned to the nearest two or three base pairs.

Data analysis. Summary statistics for drug-resistant haplotypes were generated using SAS 9.3 (SAS Institute, Cary, NC).

The expected heterozygosity of each neutral microsatellite marker was calculated using the excel microsatellite toolkit for a locus j with i alleles:

$$h_j = \left(\frac{n}{n-1} \right) \left(1 - \sum p_i^2 \right)$$

where h is the heterozygosity at a locus j and p is the allele frequency for an allele i . To estimate the extent of genetic variability within the population, the overall unbiased expected heterozygosity (H_e) was calculated as:

$$H_e = \sum_{j=1}^L \frac{h_j}{L}$$

where L is the number of loci and h_j is the heterozygosity for each locus j .

A phylogenetic tree was constructed to visualize isolate clustering. To compare complete haplotypes without missing data, loci TA1 and 2490 were also removed to construct the final tree. A distance matrix from the microsatellite data was generated in Microsatellite Analyzer Software (University of Veterinary Medicine, Vienna, Austria) using Nei's chord distance.^{16,17}

$$D_A = 1 - \sum_u \sqrt{X_u Y_u}$$

Then a phylogenetic tree utilizing the unweighted pair group method with arithmetic mean (UPGMA) algorithm was constructed from the distance matrix using PHYLIP v3.695 (University of Washington, Seattle, WA) and edited in Geneious v6.1.6 (Biomatters, Auckland, New Zealand).¹⁸

To investigate the parasite population structure within Haiti, we used a Bayesian-based Markov Chain Monte Carlo (MCMC) algorithm in Structure 2.3.3.^{19,20} We ran simulations with the number of predicted clusters (K) from 1 to 20. The model parameters included admixture and correlated allele frequencies among populations with a running length of 10,000 burn-in steps, 100,000 MCMC iterations, and 20 replicate runs. The optimal number of K (clusters) was determined using Evanno's method and the ΔK value was calculated using Structure Harvester (Stanford, CA) (http://taylor0.biology.ucla.edu/struct_harvester/).²¹

The Haitian *P. falciparum* specimens were then compared with Central and South American parasite populations (Honduras, Nicaragua, Venezuela, and Brazil) using population pairwise F_{ST} calculations in Arlequin v3.1 (University of

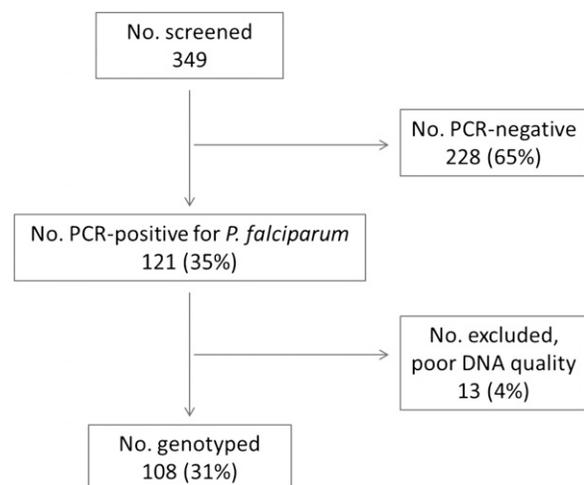


FIGURE 1. Flowchart of samples collected from March to November 2010 to assess molecular markers of antimalarial resistance among *Plasmodium falciparum* parasites in postearthquake Haiti.

TABLE 1
Prevalence of *Plasmodium falciparum* drug-resistant haplotypes among 108 parasites from postearthquake Haiti, 2010

No. of Samples	<i>Pfcrtr</i> haplotype					Freq.
	C72S	73	M74I	N75E	K76T	
107	C	V	M	N	K	0.99
1†	C	V	I*	E	T	0.01
108						1.00
No. of samples	<i>Pfdhfr</i> haplotype					Freq.
	C50R	N51I	C59R	S108N	I164L	
69	C	N	C	S	I	0.64
38	C	N	C	N	I	0.35
1†	C	I	R	N	I	0.01
108						1.00
No. of samples	<i>Pfdhps</i> haplotype					Freq.
	S436F	A437G	K540E	A581G	A613S/T	
107	S	A	K	A	A	0.99
1†	F	G	K	A	A	0.01
108						1.00
No. of samples	<i>Pfmdr1</i> haplotype					Freq.
	N86Y	Y184F				
108	N	F				1.00
108						1.00

* Amino acid changes are given in bold.

† Same isolate (HH14).

Bern, Bern, Switzerland).^{22–25} The exact test of population differentiation was used with 1,000 permutations and a significance level of 0.05.

RESULTS

Thirty-five percent of febrile patients screened for malaria by nested 18s rRNA PCR, were positive for *P. falciparum* (121/349). No other species of malaria parasites were found by 18s rRNA PCR. The PCR-positive samples were sequenced to identify drug resistance-associated mutations in *pfcrtr*, *pfdhps*, *pfdhfr*, *pfmdr1*, and were genotyped using seven neutral microsatellites. We excluded 13 (4%) poor-quality samples that failed to amplify for all drug-resistance sequencing reactions (Figure 1) from additional analysis, leaving 108 samples.

Of the 108 samples, 107 (99%) had no mutations in the *pfcrtr* gene (wild type; CVMNK haplotype). One sample (HH14) had CQ-resistant mutations at codons M74I, N75E, and K76T (CVIET haplotype). Sixty-four percent of the isolates (69) had no mutations in *pfdhfr*, 35% (38) had a S108N mutation, and one isolate (HH14) carried N51I, C59R, and S108N mutations. For *pfdhps*, 107 (99%) of samples were wild type, and one sample (HH14) had mutations at codons S436F and A437G. Finally, for *pfmdr1*, samples that could be genotyped had a single Y184F mutation and were wild type at all other interrogated loci (Table 1).

For structure analysis and phylogenetic tree construction, we used neutral microsatellite loci (Table 2), and excluded

samples that had multiple alleles for at least one neutral microsatellite locus ($N = 4$) and samples that failed to amplify at three or more neutral microsatellite loci ($N = 2$). An UPGMA-based dendrogram revealed that the single CQ- and SP-resistant isolate was genetically distinct from all other parasites collected in this study (Figure 2).

For population pairwise F_{ST} calculations, we removed isolates with missing values for any microsatellite locus ($N = 3$). To compare the *P. falciparum* populations in Haiti to other *P. falciparum* populations in Central and South America, we then calculated pairwise F_{ST} values in comparison to Haiti, Venezuela, Brazil, Honduras, and Nicaragua populations (Table 3). The F_{ST} results show that the *P. falciparum* population in Haiti is genetically distinct from all the South American populations analyzed. All F_{ST} values were statistically significant (P value < 0.05).

Among the CQ-sensitive parasites collected in Haiti, analysis using neutral microsatellites in Structure v2.3.3 revealed population substructure with at least five distinct clusters ($K = 5$) (Figure 3).

DISCUSSION

In Hispaniola and Central America, north and west of the Panama Canal, *P. falciparum* remains CQ susceptible, and CQ is one of the drugs used for primary treatment of uncomplicated malaria. Because of almost total global spread of CQ resistance, there remains a concern for the de novo emergence of CQ-resistant alleles or importation and

TABLE 2
Heterozygosity for seven neutral microsatellites among parasites collected from symptomatic malaria case patients in Haiti, 2010

Chromosome	6	4	10	12	6	2	3
Marker name	TA1	Polyα	PfPK2	TA109	2490	C2M34	C3M69
No. of alleles	5	9	6	5	3	10	4
Expected heterozygosity (H_e)	0.68	0.76	0.70	0.65	0.48	0.82	0.46

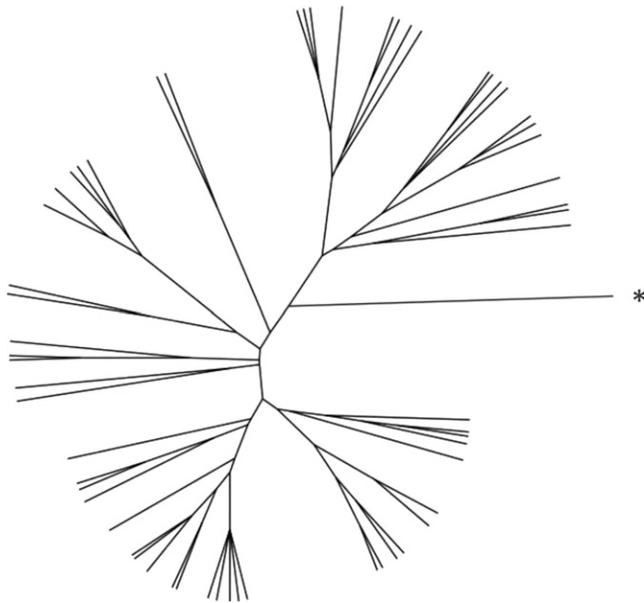


FIGURE 2. Hierarchical clustering of parasites collected in Haiti, 2010. Excluded neutral microsatellite loci with missing values. The blue line with asterisk denotes the isolate with highly resistant chloroquine and sulfadoxine-pyrimethamine mutations (HH14).

establishment of CQ-resistant parasite populations to these regions. Surprisingly, widespread CQ resistance in Central America and the Caribbean has not occurred in the 55 years since resistance to CQ emerged and spread throughout South America. During the months after the 2010 earthquake, it was not clear whether the influx of emergency-aid workers from various parts of the world would introduce new parasite populations including CQ-resistant parasites.

According to this study, and other reports,²⁶ the prevalence of CQ-resistant mutations has remained very low or is nonexistent in Haiti.^{8–10} Of the 108 samples that we tested, there was only a single *P. falciparum* isolate that carried numerous *pfert*, *pfdhps*, and *pfdhfr* mutations associated with drug resistance.

All our samples that were successfully sequenced for region 1 of *pfmdr1* had a single Y184F mutation in *pfmdr1*, which is consistent with previous reports of the *pfmdr1* Y184F mutation in Haiti.^{9,27} We conclude that this can be used as a signature of the Haitian *P. falciparum* population because parasite populations from other parts of the world typically report multiple mutations.

Our finding that 35% of the samples have only a single mutation (S108N) in *pfdhfr* is similar to reports ranging from

33% to 45% prevalence of the S108N mutation in specimens collected in 2007 and 2010 to 2012.^{27,28} The remainders of the parasite samples were wildtype for *pfdhfr* except for specimen HH14. The S108N mutation in *pfdhfr* is frequently selected when SP is first used and can confer low-level resistance to pyrimethamine.^{29,30} However, parasites carrying a single *pfdhfr* S108N mutation, although resistant to pyrimethamine in vitro, are not likely to carry an increased risk of treatment failure with SP.^{29–32} In addition, we did not find any sulfadoxine-resistant mutations in *pfdhps*, with the exception of those reported for HH14, consistent with reports of no *pfdhps* mutations in a study from Carter and others.²⁸ Therefore, this single isolate with a CVIET-CQ-resistant haplotype, triple *pfdhfr* mutations, and double *pfdhps* mutations differs greatly from all the other specimens in our study and from the majority of specimens in other recent reports from Haiti.^{7,8,27,28} This pattern of drug-resistant mutations is reminiscent of *P. falciparum* *pfert*-CVIET populations found in sub-Saharan Africa and southeast Asia.

Researchers have used other polymorphic markers, such as *pfmsp1* and *pfmsp2*, to characterize the population structure of parasites in Haiti.³³ We used neutral microsatellites to analyze the population structure of Haitian isolates collected during 2010 in Jacmel, Ranquitte, and areas peripheral to Port-au-Prince for evidence of autochthonous evolution of CQ and SP resistance. Our analysis of Haitian parasite isolates suggested that there was evidence of population substructure with up to five distinct clusters. Furthermore, calculated pairwise F_{ST} values indicate that the parasite populations in Haiti are distinct from others in South America, indicating that these are established or residual populations, as opposed to recent introductions to Haiti. These results provide no evidence of importation and establishment of new populations of *P. falciparum* parasites during the postearthquake period, with the exception of HH14. On the basis of the neutral microsatellite haplotype analysis and the pattern of drug-resistant mutations of this single isolate, we think that the CQ-resistant mutation did not arise in Haiti. The data instead suggests that this isolate was likely imported into Haiti, perhaps during the chaotic conditions and outside population movements occurring immediately postearthquake. Whether this or any of the other CQ-resistant haplotypes identified by Londono and others⁶ or Gharbi and others⁷ can be or are being transmitted in Haiti now or in the past cannot be answered at this time. However, given our data and that of others on *pfert* haplotypes and the ubiquitous use of CQ in Haiti, it is unlikely such transmission is efficiently taking place on Hispaniola.^{8–10}

One limitation of this study is that the samples used were all collected from a single, limited geographical region—areas within and surrounding Port-au-Prince. Therefore, they were not representative of all the departments in Haiti. Nevertheless, in previous studies.²⁶ The samples used were collected from different sites in multiple departments in Haiti, and the results obtained were concordant with those of this study.

Understanding the molecular profile of malaria parasites from Haiti is important for molecular surveillance and drug-treatment policies. These policies have typically relied on in vivo efficacy studies, which are extremely difficult to conduct in low-endemic settings. Molecular surveillance for drug resistance in Central American countries with low malaria

TABLE 3

Population pairwise F_{ST} * among isolates collected from Haiti, Brazil, Honduras, Nicaragua, and Venezuela

	Haiti	Brazil	Honduras	Nicaragua	Venezuela
Haiti	0.000				
Brazil†	0.371	0.000			
Honduras‡	0.391	0.557	0.000		
Nicaragua§	0.347	0.518	0.139	0.000	
Venezuela¶	0.298	0.203	0.482	0.414	0.000

* F_{ST} , pairwise genetic distances ranges from 0 (indicating no distance) to 1.00; all are significantly different (P value < 0.05).

†Brazil; includes Rondonia and Para.²¹

‡Honduras.¹⁹

§Nicaragua.¹⁹

¶Venezuela.²⁰

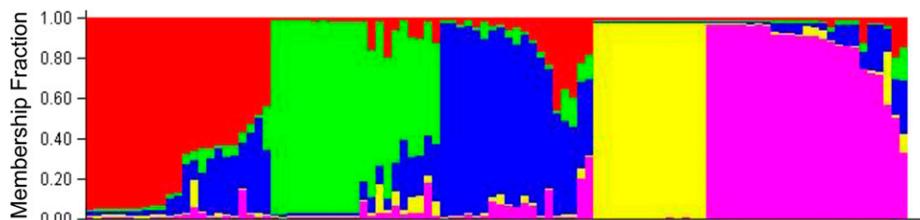


FIGURE 3. Bayesian clustering of 102 isolates in $K = 5$ clusters collected in Haiti, 2010.

transmission has become increasingly common and useful for characterizing local parasite populations.³⁴ Thus, our study has important implications for drug policy in the region. Fortunately, our findings suggest, as in Honduras and Nicaragua, a nonexistent or a very low prevalence of CQ-resistant alleles in Haiti.^{3,4} This supports the current recommendations to use CQ as first-line treatment, while emphasizing the need for continued molecular monitoring for the possible emergence and expansion or introduction and expansion of antimalarial-resistant parasite populations.

Received March 16, 2016. Accepted for publication June 1, 2016.

Published online July 18, 2016.

Note: Supplemental table appears at www.ajtmh.org.

Acknowledgments: We thank the study participants for their willingness to participate in this study. We thank all the study staff and Ministry of Health for supporting this study.

Financial support: Lindsay Carol Morton was supported by the CDC Emerging Infectious Disease Fellowship administered through the American Public Health Laboratories. Sheila Akinyi Okoth was respectfully supported by the American Society of Microbiology/CDC postdoctoral fellowship. We thank the Atlanta Research and Education Foundation for partial funding support.

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