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Independent Emergence of the *Plasmodium falciparum* Kelch Propeller Domain Mutant Allele C580Y in Guyana

Stella M. Chenet,¹ Sheila Akinyi Okoth,^{1,2} Curtis S. Huber,¹ Javin Chandrabose,⁴ Naomi W. Lucchi,¹ Eldin Talundzic,^{1,2} Karanchand Krishnalali,⁴ Nicolas Ceron,⁵ Lise Musset,⁷ Alexandre Macedo de Oliveira,¹ Meera Venkatesan,³ Reynaud Rahman,⁶ John W. Barnwell,¹ and Venkatachalam Udhayakumar¹

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Suspected artemisinin resistance in *Plasmodium falciparum* can be explored by examining polymorphisms in the Kelch (*PfK13*) propeller domain. Sequencing of *PfK13* and other gene resistance markers was performed on 98 samples from Guyana. Five of these samples carried the C580Y allele in the *PfK13* propeller domain, with flanking microsatellite profiles different from those observed in Southeast Asia. These molecular data demonstrate independent emergence of the C580Y K13 mutant allele in Guyana, where resistance alleles to previously used drugs are fixed. Therefore, in Guyana and neighboring countries, continued molecular surveillance and periodic assessment of the therapeutic efficacy of artemisinin-based combination therapy are warranted.

Keywords. artemisinin resistance; K13; *Plasmodium falciparum*; malaria; South America; Guyana; ACT.

Plasmodium falciparum resistance to artemisinin, defined by the World Health Organization as delayed parasite clearance, was first observed in Southeast Asia [1]. Although artemisinin-based combination therapies (ACTs) are currently effective owing to the complementary action of partner drugs, the emergence and expansion of resistant parasites could eventually affect the overall efficacy of ACTs [2].

In South America, ACT was introduced as early as 2001. In 2013, Vreden et al reported delayed parasite clearance on day 3

in 31% of the patients participating in a 2011 therapeutic efficacy study in Suriname [3]. This report raised concerns about artemisinin-sensitivity changes in *P. falciparum* in the Guiana Shield, considering that resistance to previously used drugs, chloroquine and sulfadoxine-pyrimethamine, emerged independently and almost simultaneously in both South America and Southeast Asia [4, 5].

In Guyana, artemether-lumefantrine plus primaquine was introduced as the first-line treatment for *P. falciparum* in 2004 [2]. Given that recent studies have raised concerns about potential changes in the sensitivity of *P. falciparum* parasites in the Guiana Shield to ACT [3], we genotyped the *PfK13* gene in specimens from Guyana collected in 2010 and examined molecular markers associated with chloroquine (*pfcr*), mefloquine (*pfmdr1*), and sulfadoxine-pyrimethamine (*pfdhfr*, *pfdhps*) resistance to assess the *P. falciparum* background of previously used antimalarial drugs in this region.

METHODS

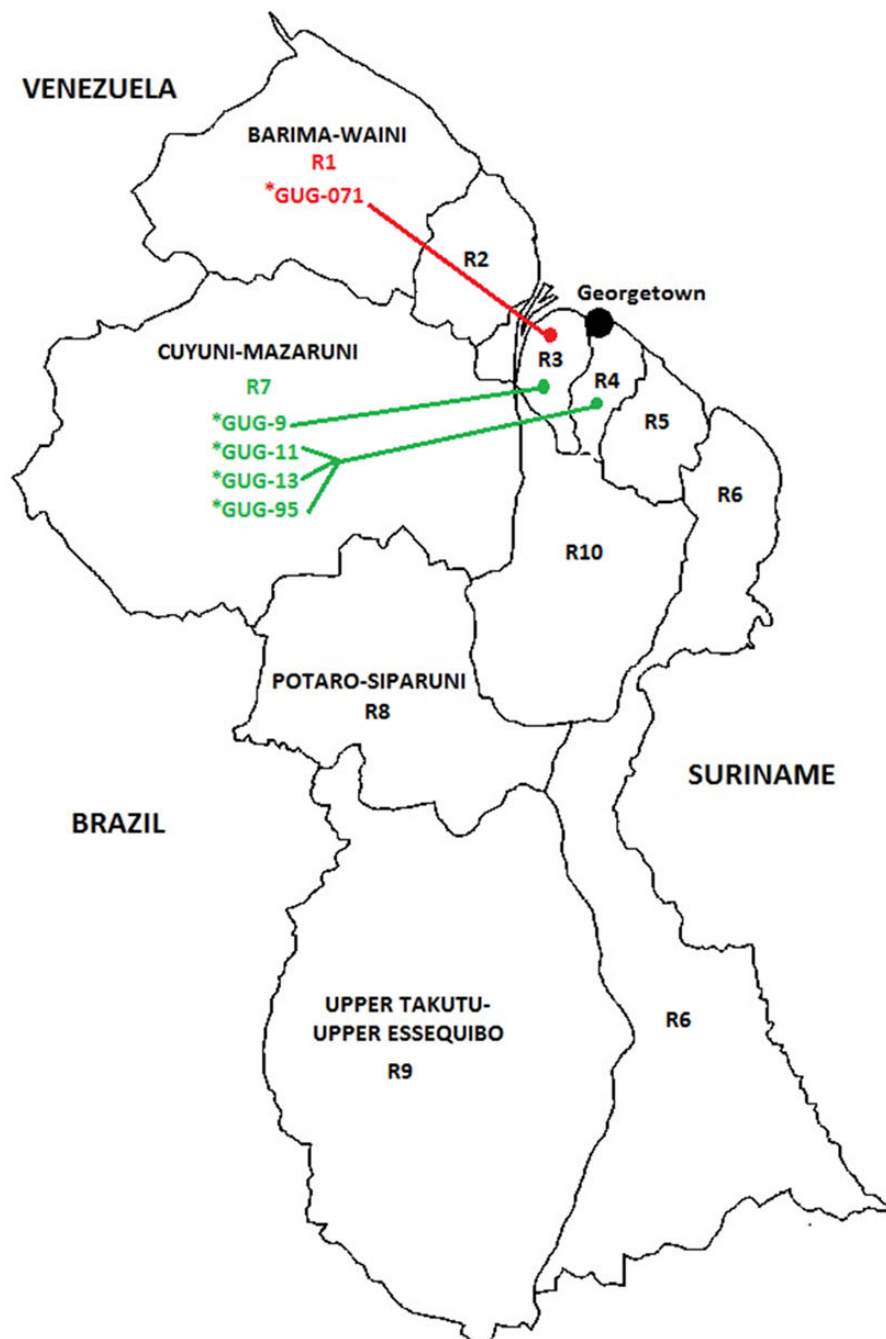
We genotyped 98 *P. falciparum* blood samples (Supplementary Table 1) collected in Guyana from March 2010 to June 2010 [6]. Guyana is divided into 10 regions, of which malaria remains endemic in interior regions 1 (Barima-Waini), 7 (Cuyuni-Mazaruni), 8 (Potara-Siparuni), and 9 (Upper Takutu-Upper Essequibo) (Figure 1). Samples were collected from a single malaria clinic located in Georgetown where patients with malaria from endemic regions seek treatment [6]. No clinical outcome or resistance phenotype information is available for these specimens. We used a modified nested polymerase chain reaction (PCR) protocol for amplification of the propeller domain of the *PfK13* gene and Sanger sequencing using primers reported elsewhere [7]. PCR amplifications were carried out in a 20 μ L volume reaction using 20 ng of total genomic DNA, $\times 1$ PCR buffer with magnesium chloride, 0.2 mmol/L dNTP, 0.75 μ mol/L forward and reverse primers K13Pf_F1 (5'-GCAAATAGTATCTCGAAT-3') and K13Pf_R1 (5'-CTGGGAAGTAATAAGAT-3'), and 0.6 U/ μ L high-fidelity Taq polymerase (Expand High Fidelity PCR System; Roche) for the primary reaction. The secondary reaction was carried out using the forward and reverse primers K13Pf_F2 (5'-GATAACAAGGAAGAATATTCT-3') and K13Pf_R2 (5'-CGGAATCTAATATGTTATGTTCA-3') and the same reagent concentrations as in the primary reaction, with 1 μ L of the primary PCR product.

The cycling conditions for the first round were as follows: an initial denaturation step at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 1 minute, and extension at 68°C for 2 minutes; followed by a final extension at 68°C for 10 minutes. The cycling conditions for

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***Drug resistance profile**

<i>Pfk13</i>	<i>Pfcr</i>					<i>Pfdhps</i>					<i>Pfdhfr</i>					<i>Pfmdr1</i>				
580	72	73	74	75	76	436	437	540	581	613	50	51	59	108	164	86	184	1034	1042	1246
Y	S	V	M	N	T	S	G	E	G	A	R	I	C	N	I	N	F	S	D	Y

Figure 1. Guyana map showing the regions of residence (R3 and R4) and the travels reported (to R1 and R7) by the patients harboring the *Pfk13* C580Y mutant parasites. The drug resistance profile for these samples is shown below the map. The relevant codon numbers and single letter code for amino acids are indicated below the gene list (mutations are indicated by bold type).

the second round were as follows: an initial denaturation step at 94°C for 5 minutes; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 68°C

for 1 minute; followed by a final extension at 68°C for 10 minutes. PCR products were confirmed after ExoSAP cleanup using agarose (1.8%) gel electrophoresis and GelRed (Biotium). The

samples were also genotyped by Sanger sequencing to examine single-nucleotide polymorphisms in *Pfcr*, *Pfdhfr*, *Pfdhps*, and *Pfmdr1*, using methods from previous studies [8, 9]. *Pfmdr1* copy number was determined with TaqMan real-time PCR (Stratagene MX3005P; Agilent Technologies) [9].

All Guyanan samples and 4 additional Cambodian isolates—MRA1236 (Pailin), MRA1238 (Pursat), MRA1240 (Battambang) and MRA1241 (Mondolkiri)—were tested for 9 flanking microsatellite loci at positions 3.4, 3.7, 8.6, 15.1, 72.3, −0.15, −3.74, −6.36, and −31.9 kb (negative values denote loci located upstream of *PfK13*; positive values, loci located downstream) [10, 11]. We also analyzed 7 neutral microsatellite loci (TA1, Polyα, PfpK2, TA109, C2M34, C3M69, 2490) [5, 12]. Fluorescently labeled PCR products were separated on an Applied Biosystems 3130 capillary sequencer and scored using Gene Marker v1.95 software (SoftGenetics). The amplification of >1 allele at any locus in a single specimen was interpreted as coinfection with ≥2 genetically distinct clones. Missing data (no amplifications) were reported by locus but not considered for defining haplotypes. Haplotypes were defined using single infections only. We used Structure 2.1 software (available at <http://pritchardlab.stanford.edu/structure.html>) to test whether a particular clustering of cases was observed in the 98 isolates.

RESULTS

A mutation at amino acid position 580 (C/Y) was found in the K13 propeller domain of 5 of 98 (5.1%) *P. falciparum*-positive samples. Three of the 5 samples (GUG-9, GUG-11, and GUG-95) were found to have only the C580Y allele, and 2 contained both the C580Y and wild-type alleles (GUG-13 and GUG-71). On closer examination of the recent travel history of the 5 patients from Guyana, we found that they reported travels to Oko (a gold mining area), Million Mountain area (a mining camp in lower Puruni), Aranka (a gold field), and Mabaruma (close to the Aruka River, along the Venezuelan border). Figure 1 shows the drug resistance profile of the 5 C580Y mutant samples.

In addition, 4 of the 5 K13 mutants had an identical K13-linked microsatellite profile (when considering the predominant alleles for those samples with mixed infections). The fifth sample

(GUG-95) had a very similar haplotype with variations at loci 3.7 and 72.3. The microsatellite haplotypes differed between the Cambodian and Guyanan specimens, suggesting their independent origins (Figure 2; Supplementary Table 2). Moreover, the Guyanan microsatellite profiles were also different from those previously reported in Thailand [11], which were similar to the ones observed in Cambodia.

We identified at least 39 different haplotypes in the Guyanan isolates, using allele data obtained from analyzing 7 neutral microsatellite loci (Supplementary Table 3). Population analysis using Structure v2.3 software revealed no substructure within this population. All 98 samples analyzed had the *Pfcr* SVMNT genotype (codons 72–76) typically found in this region and the *Pfdhps* triple mutant A437G/K540E/A581G. We found 2 *Pfdhfr* genotypes in all samples: the triple-mutant C50R/N51I/S108N (98%) and the double-mutant N51I/S108N (2%). *Pfmdr1* copy number analysis and genotyping revealed that 94 samples had a single copy and 4 had 2 copies of the gene (mean number of *Pfmdr1* copies, 1.06, standard deviation, 0.24; values >1.5 were considered 2 copies). We found only 2 *Pfmdr1* mutant genotypes, Y184F/N1042D/D1246Y (triple-mutant) and Y184F/S1034C/N1042D/D1246Y (quadruple-mutant) at frequencies of 37% and 63%, respectively.

DISCUSSION

Given potential changes in the sensitivity of *P. falciparum* parasites to artemisinin in the Guiana Shield, we sought to investigate the polymorphisms in the propeller domain of the *PfK13* gene in isolates from patients with malaria in Guyana. We found the C580Y mutation in 5.1% of the samples. This mutation has been identified with a high prevalence in western Cambodia, and the World Health Organization has designated it as a confirmed artemisinin resistance marker [2].

The K13-flanking microsatellite haplotypes indicate that the C580Y mutant allele found in Guyana emerged independently in South America because the profile is different from that found in Southeast Asia. The introduction of ACT in South America occurred in the early 2000s, whereas in Southeast Asia, artemisinins have been deployed in the form of ACTs since the 1990s, before the official treatment was implemented

South America	-31.9	-6.36	-3.74	-0.15	K13	3.4	3.7	8.6	15.1	72.3
Guyana A	204	277	171	206	580Y	139	296	262	144	244
Guyana B	204	277	171	206	580Y	139	300	262	144	240

Southeast Asia	-31.9	-6.36	-3.74	-0.15	K13	3.4	3.7	8.6	15.1	72.3
Cambodia 1	201	282	148	194	580Y	131	295	286	138	238
Cambodia 2	231	274	150	198	493H	122	295	286	138	248
Cambodia 3	201	282	148	194	539T	122	295	265	138	240
Cambodia 4	201	282	148	194	543T	131	295	290	138	238

Figure 2. K13-linked microsatellite haplotypes from Guyana and Cambodia.

in 2000 [13]. The prevalence of the C580Y allele was about 40% in Cambodia in 2000, and it took at least 8 years to detect evidence for decreased sensitivity to ACT treatment [14]. On the other hand, Guyana completed a therapeutic efficacy study of artemisinin in 2014, and the results showed a 100% efficacy at day 28, whereas only 2% of the patients had persistent parasitaemia at day 3 after treatment [2]. Sanger sequencing of samples collected during this therapeutic efficacy study did not detect any mutations in the K13 propeller domain, therefore confirming the continued artemisinin sensitivity in Guyana [2]. However, failures might occur when the efficacy of both the artemisinin and the partner drug decline.

In Guyana, artemether plus lumefantrine is currently used as first-line treatment. The testing for other well-characterized drug resistance genes carried out in the current study indicated fixation of resistance alleles to previously used drugs. In particular, the mutant *Pfmdr1*-184F has been associated with parasite resistance to lumefantrine [15]. The presence of mutant *Pfmdr1* alleles and the detection of 4 isolates with multiple copies of this gene in our study highlight the importance of monitoring all relevant partner drug resistance markers. Collectively, these findings suggest a vulnerability of partner drugs to resistance pressure in the Guyana region.

Moreover, the problem of mobile populations engaged in gold mining, logging, or illegal activities with high risk of malaria transmission, raises the potential of the spread of artemisinin resistance alleles in neighboring countries of the Guiana Shield and in South America. Besides artemisinin resistance status, the partner drug also plays a crucial role in the overall efficacy of ACT. Molecular data combined with therapeutic efficacy studies, wherever feasible, will be a rational approach to continuously monitor evolving patterns of resistance. There might also be other mutations associated with artemisinin drug resistance that need to be further explored.

In conclusion, the presence of the *PfK13* C580Y allele among highly mobile patients with malaria in Guyana raises the concern of K13 mutants emerging locally and independently from Southeast Asia that could spread to neighboring countries. This finding, along with the presence of fixed resistance alleles for other antimalarial drugs, has serious implications for *P. falciparum* surveillance, malaria control, and elimination efforts in South America. Moreover, the results from the current investigation underscore the importance of undertaking follow-up clinical, molecular, and in vitro drug resistance studies in order to associate the *Plasmodium* genetic background with a clear drug resistance phenotype and monitor changes in prevalence of artemisinin resistant alleles.

Supplementary Data

Supplementary materials are available at <http://jid.oxfordjournals.org>. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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Potential conflicts of interests. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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