Short Report: A Cross-Sectional Survey of *Plasmodium falciparum* pfcrt Mutant Haplotypes in the Democratic Republic of Congo

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Abstract.   In the Democratic Republic of the Congo (DRC), artesunate-amodiaquine is first-line therapy for falciparum malaria; little is known about the prevalence of molecular markers of parasite drug resistance. Across the DRC, we genotyped 166 parasites in *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) using polymerase chain reaction (PCR) and sequencing. Of these parasites, 73 (44%) parasites were pure wild-type CVMNK, 55 (31%) parasites were chloroquine-resistant CV**IET**, 35 (21.1%) parasites were mixed CVMNK and CV**IET**, and 3 parasites were other genotypes. Ninety-two infections (55.4%) harbored the *pfcrt* K76**T** substitution that is highly correlated with chloroquine failure. The amodiaquine-resistant **S**VMN**T** haplotype was absent. Geographically, *pfcrt* haplotypes were not clearly clustered. Chloroquine accounted for 19.4% of antimalarial use, and amodiaquine accounted for 15.3% of antimalarial use; there were no associations between drug use and mutant haplotype prevalence. In the DRC, our molecular survey indicates that resistance to chloroquine is substantial but that resistance to amodiaquine is absent. These contrasting findings highlight the need for molecular surveillance of drug resistance to inform malaria control policies.

Chloroquine (CQ)-resistant *Plasmodium falciparum* parasites are widespread and undermined prior efforts to control falciparum malaria. Resistance is conferred by polymorphisms in the *P. falciparum* CQ resistance transporter (PfCRT)[1] encoded by the *pfcrt* gene. Clinically, the K76**T** substitution elevates the risk of treatment failure sevenfold.[2] Although *pfcrt* was originally associated with CQ susceptibility, a recent chemical genomic screen identified mutations in *pfcrt* as key mediators of response to a diverse array of drugs,[3] suggesting that the biological relevance of *pfcrt* extends far beyond simply CQ.

Two observations suggest that CQ may be redeployed for malaria control, despite widespread resistance. First, in Malawi, CQ susceptibility returned over time after CQ use was curtailed[4] owing to the removal of selective pressure and the reexpansion of CQ-susceptible parasites.[5] Additionally, even in the presence of the K76**T** mutation, the administration of higher doses of CQ can achieve cure rates similar to the rates of artemether-lumefantrine.[6] Because of these data, interest persists in possibly redeploying CQ in certain epidemiologic and clinical settings.

Central Africa harbors diverse *pfcrt* haplotypes that are germane for clinical therapy. The Democratic Republic of the Congo (DRC) adopted artesunate-amodiaquine (ASAQ) as its first-line antimalarial in 2005, and neighboring Burundi, Republic of the Congo, and South Sudan have also adopted ASAQ. In the DRC, ASAQ is highly efficacious: the cure rate of uncomplicated falciparum malaria was > 98% in Katanga province in 2008 and 2009.[7] Recent data confirm the appearance in neighboring Tanzania[8] and Angola[9] of the *pfcrt* haplotype **S**VMN**T**, which may confer resistance to amodiaquine[1,10] and herald failures of ASAQ.[11] Additionally, the haplotype **S**V**I**ET has been reported from Kinshasa in 2000, although it is of unclear biological significance.[12]

To inform current and future guidelines on antimalarial use in Central Africa, we conducted a cross-sectional survey of *pfcrt* haplotypes in the DRC.

Parasites were obtained from respondents to the 2007 Demographic and Health Survey (DHS) in the DRC.[13] Briefly, adults over 15 years old were selected for participation within 300 random population-representative clusters identified across the DRC. Data about household antimalarial use were collected by asking female respondents if children under the age of 5 years in the household with fever during the preceding 2 weeks had received an antimalarial drug; data about drug use by individual respondents who contributed parasite specimens were not collected. Verbal informed consent for the collection of blood spots was obtained from all survey participants in one of the five main languages used in the DRC. The Ethics Committees of Macro International and the School of Public Health of the University of Kinshasa approved the consent procedures, survey administration, and blood sample collection. The Institutional Review Board of the University of North Carolina approved the testing of malaria parasites.

Finger-prick blood samples from the adult survey participants were stored on filter paper as dried blood spots and used to make genomic DNA (gDNA). *P. falciparum* was detected using real-time polymerase chain reaction (PCR).[13] Of 8,838 adult respondents, 2,435 respondents harbored only *P. falciparum* parasites. Of this subset, 180 *P. falciparum* parasitemias were randomly selected for *pfcrt* genotyping.

Samples were genotyped at codons 72–76 by amplifying *pfcrt* across the codons of interest and Sanger sequencing the product. The 25 µL PCR reaction contained 12.5 µL HotStarTaq Master Mix (Qiagen, Valencia, CA), 4 mM MgCl_2, and 800 nM each of primers CRTFwd (GGAAATGGCTCACGTTT-AGG) and CRTRev (TGTGAGTTTCGGATGTTACAA-AA; adapted from a previous study).[14] PCR products were bidirectionally sequenced using ABI BigDye Terminator chemistry, and reads were aligned to National Center for Biotechnology Information reference sequence XM_001348968 using Sequencer (v4.8; Gene Codes, Ann Arbor, MI). All

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sequences were manually inspected at the loci of interest. We defined mixed alleles as those alleles at which a secondary peak was ≥ 10% of the height of the primary peak.

Of 180 randomly selected parasite isolates, 166 isolates were successfully genotyped across *pfcrt* codons 72–76. Of these isolates, the wild-type CVMNK haplotype was solely present in 73 (44.0%) isolates, the CQ-resistant haplotype CV**IET** was solely present in 55 (33.1%) isolates, and 35 (21.1%) isolates harbored parasites with both CVMNK and CV**IET** haplotypes. Additionally, two isolates bore a CVMN**T** haplotype, and one isolate had a CVM**D**K. Therefore, 92 of 166 samples that were successfully genotyped (55.4%) harbored the K76**T** mutation that most clearly confers CQ resistance.

The **S**VMN**T** haplotype reported previously from Angola and Tanzania was absent, and the **S**V**IET** that had been reported from Kinshasa was also absent. There were no novel substitutions identified in the sequenced fragment of *pfcrt*.

Geographically, the most common wild-type (CVMNK) and mutant (CV**IET**) haplotypes were widely distributed across the DRC without apparent geographic clustering (Figure 1A). The prevalence of the K76**T** mutation was not significantly different between 11 DRC provinces.

Of 790 DHS respondents with children under 5 years who received antimalarials in the preceding 2 weeks for fever, the most commonly administered antimalarials were quinine (49.6%), CQ (19.4%), amodiaquine (15.3%), and sulfadoxine-pyrimethamine (9.7%). On a provincial level, there were no significant differences in antimalarial use (Figure 1B).

Overall, we report a higher prevalence of wild-type *pfcrt* haplotypes than prior studies in the region: 44% of isolates in our study harbored the CVMNK haplotype alone. In three studies in Kinshasa or neighboring Republic of the Congo, wild-type CVMNK haplotype prevalence was 4–12%,[15–17] and only 3%

of isolates collected in Luanda, Angola in 2007 harbored wild-type *pfcrt*.[9] We note that, consistent with these studies, prevalence of wild-type parasites was lower than the national average in Kinshasa and Bas-Congo provinces (Figure 1A).

What might these data mean for CQ redeployment? Currently, ASAQ is effective therapy for malaria in the DRC, but the recent reports of reduced efficacy of artemisinin therapies for malaria in Asia highlight the need to continually evaluate the efficacy of alternative drugs. The correlation between prevailing K76**T** mutation frequency and the efficacy of CQ is unknown. In Malawi, 25 mg/kg CQ were highly effective in treating uncomplicated childhood malaria in a setting where the prevalence of the K76**T** mutation was < 0.2%.[18] More surprisingly, in a Guinea-Bissau study in which 31% of parasites harbored K76**T**, 50 mg/kg CQ were 94% effective as treatment of uncomplicated malaria.[6] Given these data, it seems plausible that a threshold of prevailing K76**T** frequency may define locations where it is likely to be effective. Areas with moderate but heterogeneous mutation prevalence, like the DRC, can provide settings in which to test CQ efficacy and its correlation with molecular markers of resistance. Nonetheless, redeploying CQ in an area with parasites bearing K76**T** mutations will promote the spread of resistant parasites and ultimately, reduce drug efficacy. For this reason, clinical and molecular monitoring of drug resistance will be critical if CQ is deployed in any limited setting.

Persistent CQ use for childhood fever may be sustaining transmission of K76**T**-bearing parasites in the DRC. In Malawi, the decrease in CQ use facilitated a return of CQ-susceptible parasites, but in a meta-analysis of population-level data from several other African countries, continued CQ use was associated with the persistence of CQ resistance.[19] Although parasites bearing mutant *pfcrt* haplotypes
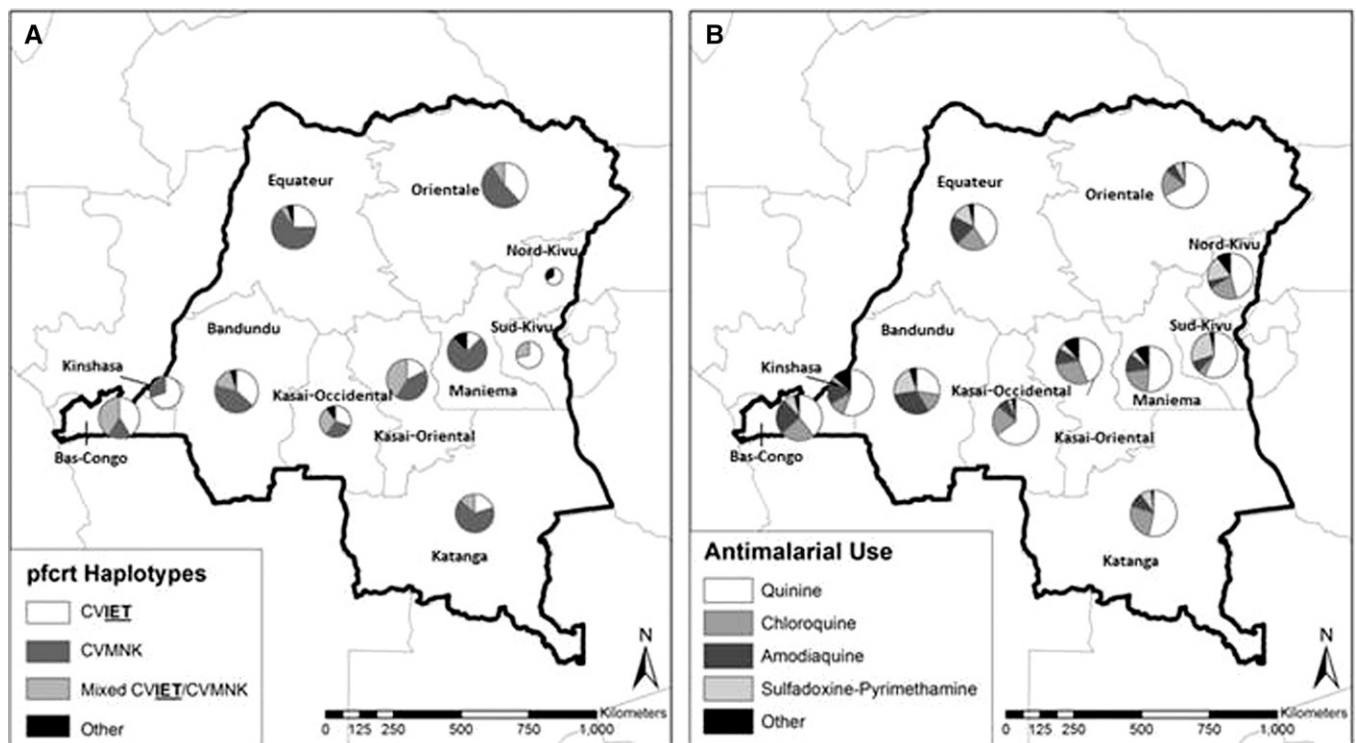

FIGURE 1.   Provincial distributions of (**A**) *pfcrt* haplotypes and (**B**) antimalarial drug use for childhood fever.

are believed to suffer an overall fitness disadvantage, it may be counterbalanced in the presence of continued drug pressure by increased infectivity to mosquitos.[20] The K76**T** mutation may be further sustained by the poor quality of CQ formulations in the DRC,[21] which may simultaneously fail to clear parasites and promote gametocytogenesis. Until 2002, when failure rates reached 45%,[22] CQ was the first-line antimalarial for uncomplicated malaria; our study was conducted in 2007, and its administration for 20% of treated childhood fevers may reflect residual practice patterns, which have since abated.

Notably, no parasites in our study harbored the **S**VMN**T** haplotype. This haplotype has been reported in Angola and Tanzania, and it has been associated with resistance to amodiaquine in a clinical study in Afghanistan[10] and *in vitro* experimentation.[1] Our study was conducted only 2 years after the adoption of ASAQ as first-line antimalarial in the DRC, and respondents endorsed minimal use for childhood antimalarial treatment of either amodiaquine (4.9%) or artesunate (0.6%). Our data suggest that ASAQ may be a durable antimalarial, although ongoing surveillance is necessary.

Our study has several limitations. We sampled only 166 isolates from a large country. Nevertheless, they were randomly sampled from a nationally representative survey, and we believe that they reflect DRC parasite populations in a less-biased fashion than more local studies. Our genotyping method may have missed minority variant subpopulations, although we set a liberal cutoff for secondary peaks in chromatograms, all of which were scored manually. We have no clinical efficacy data with which to correlate our molecular data, and future studies are needed to understand the impact of the *pfcrt* prevalence. Finally, the DHS did not collect data on the recent use of antimalarials by the participants themselves. The *pfcrt* K76**T** mutation decreases susceptibility to both CQ and amodiaquine; if participants had recently received these antimalarials in the period before the survey, they would be expected to inflate the reported mutant allele frequencies above their true values.

Across the DRC, parasites bearing *pfcrt* haplotypes conferring CQ resistance remain prevalent, perhaps owing to ongoing CQ use for childhood fever. A *pfcrt* haplotype associated with amodiaquine resistance was absent, suggesting that first-line antimalarial ASAQ may be a durable therapy. Ongoing population-level molecular surveillance may assist strategies to redeploy CQ in select African settings.

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