

1 **Shifts in antimalarial drug policy since 2006 have rapidly selected *P.***
2 ***falciparum* resistance alleles in Angola**

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15 **ABSTRACT**

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17 **BACKGROUND:** *Plasmodium falciparum* resistance to chloroquine (CQ), the most
18 widely used antimalarial drug, has historically posed a major threat to malaria control in
19 Angola and throughout the world. Although Angola replaced CQ with artemisinin
20 combination therapy (ACT) as a frontline treatment in 2006, malaria cases and deaths
21 have recently been rising. CQ-resistance mutations may still be a contributing factor,
22 given that (1) some also modulate resistance to ACT partner drugs and (2) ACT is not yet
23 consistently implemented across Angola. It is important to continue monitoring all known
24 resistance alleles in *P. falciparum*, but no studies have done so in Angola since 2012.

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26 **METHODS:** We sampled *P. falciparum* DNA from the blood of 50 hospital patients in
27 Cabinda, Angola in 2018. Each infection was genotyped for 13 alleles in the genes *crt*,
28 *mdr1*, *dhps*, *dhfr*, and *kelch13*, which collectively confer resistance to six common drugs.
29 To analyze frequency trajectories over time, we also collated *P. falciparum* genotype data
30 published from across Angola in the last two decades.

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32 **RESULTS:** The two most important alleles for CQ resistance, *crt* 72-76CVIET and
33 *mdr1* 86Y, have both declined in frequency from respective highs of 98% in 1999 and
34 73% in 2003. However, the former remains at 71% frequency in this sample while the
35 latter has dropped to just 7%. Of seven possible alleles for sulfadoxine-pyrimethamine
36 (SP) resistance in *dhps* and *dhfr*, the average total number per isolate increased from 2.9
37 in 2004 to 4.4 in 2018. Finally, we detected no non-synonymous polymorphisms in
38 *kelch13*, which is involved in artemisinin resistance in Southeast Asia.

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40 **CONCLUSIONS:** Changes in drug policy in Angola since 2006 appear to have
41 exerted strong selection on *P. falciparum* drug resistance alleles. Resistance to CQ is
42 declining, but due to functional tradeoffs and novel selection at *mdr1* loci, resistance to
43 ACT partner drugs appears to be rising. More haplotype-based studies at *mdr1* will be
44 needed to understand the changing efficacy of multiple drugs. Finally, SP resistance has
45 jumped rapidly since 2014, consistent with widespread use of intermittent SP treatment
46 during pregnancy. These data can be used to support effective drug policy decisions in
47 Angola.

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50 **KEYWORDS**

51 *Plasmodium falciparum*, Angola, chloroquine, lumefantrine, drug resistance, selection

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54 **BACKGROUND**

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56 Antimalarial drugs have long been important tools for malaria control¹. However, their
57 efficacy is constantly threatened by the evolution of drug resistance in *Plasmodium falciparum*².
58 Multiple *P. falciparum* genes are involved in drug resistance, and selection on them varies by
59 allele, genetic background, and drug environment³⁻⁵. Therefore, frequent monitoring of resistance
60 alleles is crucial to predicting the spread of drug resistance. This is especially true in the West
61 African country of Angola, where malaria cases and deaths are on the rise⁶.

62 The first anti-malarial drug to enjoy widespread use in Angola was chloroquine (CQ) in
63 the 1950s⁷. CQ resistance was first confirmed in Angola in the 1980s, and by the early 2000s,
64 CQ failure rates exceeded 80%^{8,9}. As a result, CQ was discontinued in Angola in favor of
65 artemisinin-based combination therapy (ACT) starting in 2006¹⁰. To discourage the evolution of
66 artemisinin resistance, artemisinin is used in combination with the longer-acting partner drugs
67 lumefantrine (LMF) or amodiaquine (AQ), which is chemically related to CQ¹¹. Artemisinin
68 resistance has not yet appeared in Angola, although many resistant *kelch13* mutations have
69 emerged in Southeast Asia^{5,12}. Nonetheless, occasional ACT treatment failures have been
70 reported in Angola due to partner drug resistance¹⁰.

71 Strong *P. falciparum* resistance to CQ and AQ is caused by *crt* K76T, a lysine to
72 threonine substitution at codon 76 of the chloroquine resistance transporter (Table 1). A meta-
73 analysis found this allele to be 7.2-fold overrepresented in CQ treatment failures¹³, reflecting its
74 selection by CQ and AQ in many clinical studies (Table 1). In Angola, K76T is found on the
75 haplotype *crt* 72-76 CVIET, which is of Asian origin¹⁴. CQ resistance has also evolved
76 independently through the haplotype *crt* 72-76 SVMNT in South America and Papua New
77 Guinea¹⁵.

78 The N86Y allele of *mdr1*, or multidrug resistance gene 1, also confers resistance to CQ
79 and AQ¹³. Although this specific polymorphism dominated early studies of *mdr1* and CQ
80 resistance, the evolution of *mdr1* is complicated by linkage between position 86 and other
81 functional polymorphisms¹⁶. Precise *mdr1* haplotypes vary among *P. falciparum* populations and
82 drug settings, but in Angola alone, at least six alleles at three *mdr1* positions have been proposed
83 to modulate resistance to CQ, AQ, and the ACT partner drug lumefantrine (LMF) (Table 1;
84 Table S1).

85 The drug sulfadoxine-pyrimethamine (SP) has also been in widespread use in many
86 African countries since the 1960s¹⁷. *P. falciparum* quickly began evolving partial resistance to
87 SP, mediated by numerous substitutions in *dhps* and *dhfr*¹⁸. The risk of SP treatment failure
88 increases with the number of mutant alleles present, with “quintuple mutants” at codons 437/540
89 of *dhps* and codons 51/59/108 of *dhfr* of particular concern¹⁹⁻²¹. By the early 2000s, these alleles
90 were common in Angola and 25-39% of *P. falciparum* infections failed to respond to SP
91 treatment⁹. SP has since been discontinued as a frontline therapy, but it is still administered to
92 pregnant women to reduce common complications from malaria²². Although this approach is

drug	<i>crt</i>					<i>mdr1</i>		
	72	73	74	75	76	86	184	1246
CQ,AQ (chloroquine, amodiaquine)						Y	-	-
	C	V	I	E	T	-	-	Y
	S	V	M	N	T	Y	Y [†]	Y
						Y	F*	D [†]
						-	F*	-
LMF (lumefantrine)						N	-	-
						-	-	D
						N	-	D
	C	V	M	N	K*	N	F	D
						N	F	-
						N	Y [†]	D
						N	Y [†]	-

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94 **Table 1. Alleles of *P. falciparum* genes *crt* and *mdr1* that are preferred in the presence of frontline**
95 **antimalarial drugs.** Although CQ has been discontinued in Angola, CQ-resistance loci are also involved
96 in resistance to the ACT partner drugs AQ and LMF. Numbers in the header indicate amino acid position.
97 For *mdr1*, incomplete haplotypes are shown as reported in the literature. *These alleles are unlikely to
98 confer resistance directly, but they are less deleterious than the alternate allele in the presence of drug.
99 †This allele is unlikely to confer resistance directly, but it is linked to other functional alleles. Additional
100 details and references are available in Table S1.

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103 generally still useful in Africa^{23,24}, its efficacy is waning as additional *dhps* mutations continue to
104 emerge^{18,25-27}. In one recent example from Tanzania, a novel mutation at *dhps* 581 was both
105 selected by SP treatment and associated with worse pregnancy outcomes²⁸. Because SP is still in
106 widespread use, it is critical to continue monitoring its effectiveness along with variation in its
107 target genes.

108 In this work, 50 *P. falciparum* infections from Cabinda, Angola were genotyped for 13
109 markers of drug resistance in the genes *crt*, *mdr1*, *dhps*, *dhfr*, and *kelch13*. Similar allele
110 frequency data were also gathered from studies published on Angolan *P. falciparum* in the last
111 two decades. For every locus but *kelch13*, we found temporal patterns of allele frequency change
112 that are consistent with changes in drug policy. This work can inform future decisions on drug
113 administration in Angola, particularly given rapid increases in SP resistance.

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116 **RESULTS**

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118 Genotyping success and MOI

119 Each sample was successfully genotyped at an average of 12 out of 13 loci (Table S3).
120 The *kelch13* locus had the highest success rate (100%), while *crt* had the lowest success rate
121 (78%). Although the *crt* primers have performed well on other Angolan samples²⁹, in this cohort,
122 even the nested protocol amplified products of multiple sizes (Fig S1).

123 Fifteen of 50 samples had sequence diversity (i.e., peaks of two bases) in at least one
124 resistance marker site. Assuming that double peaks indicated the presence of two strains, the
125 overall multiplicity of infection (MOI) was 1.3.

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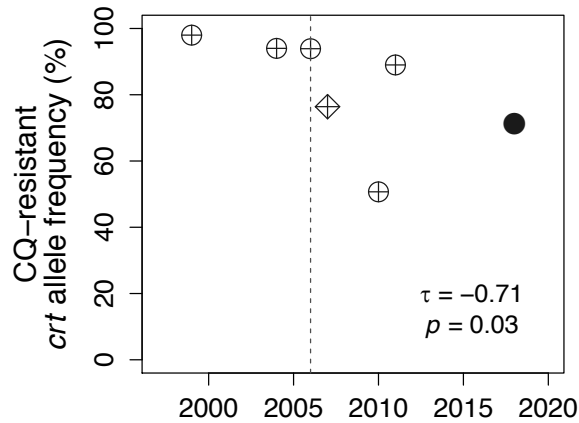
127 Very little polymorphism in *kelch13*

128 No *kelch13* polymorphisms were observed at codons 578-580, which have been
129 associated with ACT resistance in Southeast Asia and Uganda¹². Moreover, with the exception of
130 one synonymous variant in one sample, no polymorphism was observed across all 261 *kelch13*
131 codons sequenced in this study.

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133 Markers of CQ resistance and LMF susceptibility are declining

134 The CVIET haplotype at *crt* codons 72-76, which confers strong resistance to CQ, was
135 detected at 71% frequency in this study (Fig 1). This represents a significant decline from a peak
136 of 98% in 1999 ($p = 0.03$), although individual estimates have been noisy over time.



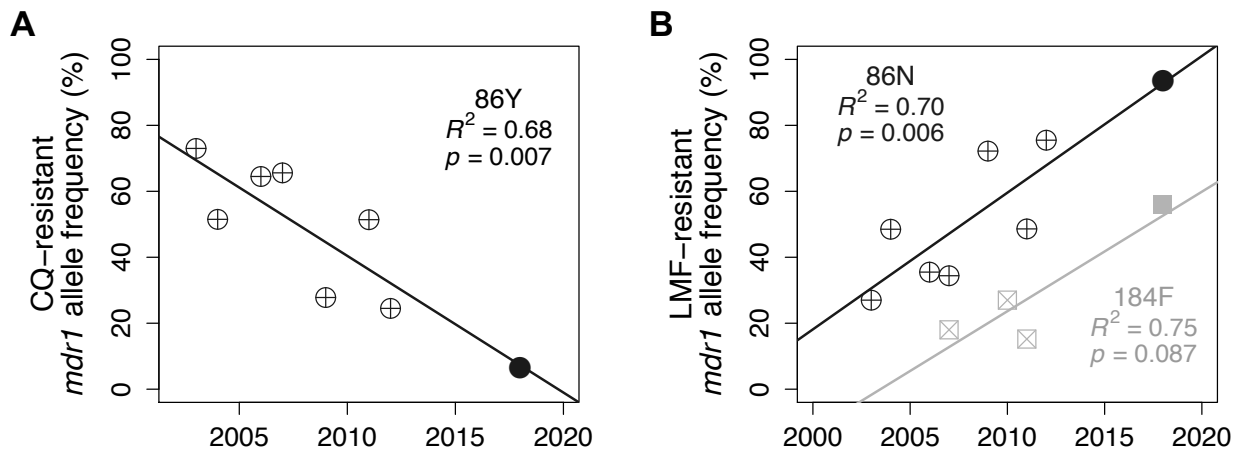
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138 **Figure 1. The frequency of CQ (chloroquine)-resistant alleles in *crt* has declined in Angola since the**
139 **late 1990s.** The solid circle indicates new data from this study. Historical data were obtained from ^{29,31-35}.
140 The diamond indicates the combined presence of two resistant haplotypes (CVIET and SVMNK) at *crt*
141 72-76 in ²⁹; all other points represent CVIET only. τ is the Kendall rank correlation between time of
142 sampling and frequency of resistance. The dashed line shows the year that CQ was officially discontinued
143 in Angola.

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148 **Figure 2. *mdr1* allele frequencies have changed steadily in Angola since the early 2000s.** Solid points
149 indicate new data from this study. Historical data were obtained from ^{29,32-37}. Lines of best fit, variance
150 explained (R^2), and p -values from linear regression are shown for each allele.

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153 The *mdr1* allele 86Y, which also confers resistance to CQ, was detected at just 6.5%
154 frequency in this study (Fig 2A). This marker has declined rapidly and steadily from ~80%
155 frequency in 2003 ($p = 0.007$). Accordingly, the alternate allele 86N—which is both CQ-
156 sensitive and LMF-resistant (Table 1)—has increased in frequency to 93.5% (Fig 2B, $p = 0.006$).
157 The linked polymorphism *mdr1* 184F, which is also preferred in the presence of LMF (Table 1),
158 has been rising in frequency at a similar rate (Fig 2B, $p = 0.087$), although it remains less
159 common than 86N. A single sample contained the additional CQ-resistance allele *mdr1* 1246Y
160 (Table 1), which occurred on an 86Y/184Y background (Table S3).

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162 Markers of SP resistance markers have become more common

163 One third (33%) of *P. falciparum* isolates sampled here were “quintuple mutants” for five
164 *dhfr* and *dhps* alleles that confer strong SP resistance (Fig 3). Compared to samples from migrant
165 workers collected around 2014³⁸, this represents a 2.8X increase of quintuple mutants in Angola
166 in less than five years. Three “sextuple mutants” were also observed for the first time in Angola,
167 including resistance alleles at *dhps* codons 436 (22% frequency) and 581 (8.2% frequency). The
168 average number of combined *dhfr/dhps* resistance alleles per isolate has increased sharply over
169 time, from 2.9/7 in 2004 to 4.4/7 in this study ($t = -9.71, p < 2.2 \times 10^{-16}$).

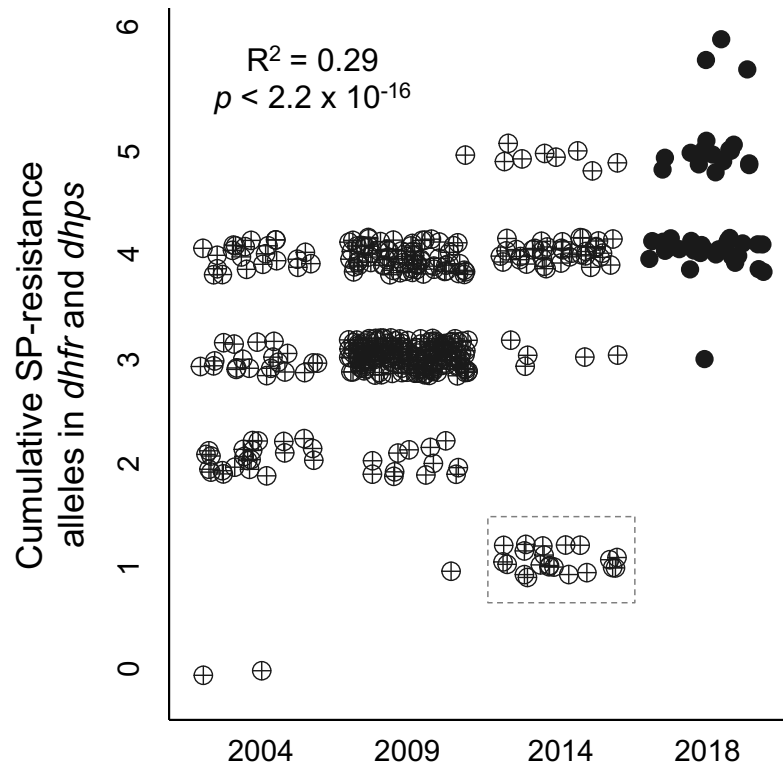
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172 **DISCUSSION**

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174 The official withdrawal of CQ in Angola since 2006 has likely contributed to the decline
175 of CQ-resistance alleles in *crt* (Fig 1) and *mdr1* (Fig 2A). This result is similar to other African
176 countries that have discontinued CQ, including Malawi, the Gambia, Kenya, Ethiopia, Tanzania,
177 and Grand Comore³⁹. In Malawi, clinical CQ sensitivity largely returned after the prevalence of
178 *crt* K76T declined from 85% in 1992 to 13% in 2000⁴⁰. In Angola, however, the prevalence of
179 the CVIET haplotype in Angola remains high at 71% (Fig 1). Although the exact fraction of
180 resistant parasites may vary by locality (Fig 1), these results imply that CQ resistance via *crt* is
181 still standard in Angola. In contrast, the rate of decline of *mdr1* 86Y—the second-most important
182 CQ-resistance allele—is sharp enough to suggest its disappearance from Angola within a few
183 years (Fig 2A). This stark difference between the evolution of *crt* and *mdr1* may be best



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185 **Figure 3. SP-resistant alleles in *dhfr* and *dhps* have become more common in Angola since**
 186 **the early 2000s.** Each point represents the total number of resistance alleles in both genes from a
 187 single isolate. Solid points indicate new data from this study; historical data are from ^{32,38,41}.
 188 Points are jittered horizontally and vertically for clarity. Resistance alleles were counted at *dhfr*
 189 codons 51, 59, and 108 and *dhps* codons 436, 437, 540, and 581. The dashed box surrounds a
 190 subset of 2014 isolates that carried 0-3 mutant alleles, but for which more precise data were not
 191 available. Variance explained (R^2) and p -value are shown are from a linear regression excluding
 192 all 2014 data.

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195 explained by the differential effects of LMF, an ACT partner drug, on their CQ-sensitive alleles
 196 (Table 1). Specifically, wild-type *crt* K76 is only passively selected in the absence of CQ, while
 197 wild type *mdr1* N86 directly confers LMF resistance (Table 1). LMF resistance is highest when
 198 *mdr1* N86 co-occurs with *mdr1* 184F, which is rapidly spreading in Angola (Fig 2B), and *mdr1*
 199 1246D, which is nearly fixed (Table S3). Consequently, the rise of LMF resistance could soon
 200 challenge the success of ACT as currently implemented in Angola.

201 The most rapid change observed in this study was the increase in total SP-resistance alleles
 202 per isolate (Fig 3). Similar increases over time have been reported in a number of other African

203 countries⁴²⁻⁴⁴, likely in response to the implementation of WHO recommendations for SP during
204 pregnancy. It is now clear that more than five mutations in *dhfr/dhps* contribute to SP resistance:
205 in our sample, seven such mutations were present, and three infections (7.0%) carried six of them.
206 Because intermittent SP treatment currently recommended by the WHO does not eliminate
207 parasitemia⁴⁵, it is strongly expected to select for additional SP resistance. The benefits of SP in
208 pregnancy have outweighed these costs in the past, but the present rate of resistance evolution
209 implies that these benefits may be eroding^{27,28}. Further research will be required to weigh the
210 impact of novel resistance haplotypes against other factors impacting SP treatment efficacy. To
211 help accomplish this goal, we emphasize the importance of reporting complete haplotype
212 information for all combined *dhfr/dhps* alleles in each sampled infection.

213 Finally, we detected no signs of artemisinin-resistant alleles in *kelch13*. This result is
214 consistent with the high efficacy of ACT in Angola¹⁰, and overall, there is little evidence that
215 artemisinin resistance alleles are spreading in Africa⁴⁶. Monitoring of *kelch13* in Africa is
216 nonetheless important, as artemisinin is the only drug for which resistance alleles are not already
217 widespread.

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220 **CONCLUSIONS**

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222 Changes in drug policy since 2006 have had clear impacts on the frequencies of several
223 drug resistance alleles in Angola. Markers of SP resistance are rapidly becoming more common,
224 which endangers the efficacy of intermittent treatment during pregnancy. Resistance to CQ is
225 declining, but resistance to LMF appears to be rising. More frequent monitoring and drug policy
226 adjustments will likely be necessary to regain control of *P. falciparum* malaria in Angola.

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229 **METHODS**

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231 Sample collection and ethics statement

232 Patients reporting to the Hospital Regional de Cabinda in 2018/2019 with fever, chills, or
233 other malaria symptoms were offered the option to be consented to this study. Sample collection

234 followed protocols approved by Stanford University (IRB #39149) and the Medical Ethics
235 Committee of the University 11th of November in Cabinda. Consented participants' blood was
236 drawn from a vein and screened under a microscope for *P. falciparum* parasites. If positive,
237 whole blood was filtered through cellulose columns to remove leukocytes⁴⁷. The filtered red
238 blood cells were spotted on Whatman FTA cards (Sigma Aldrich), dried, and stored for at least 6
239 months.

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241 DNA extraction and genotyping

242 To elute DNA, saturated circles were cut out of the Whatman FTA cards and incubated in
243 800 uL TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) with 20 uL Proteinase K (Invitrogen)
244 for 2 hours at 65°C. DNA was extracted from the liquid supernatant using a phenol-chloroform
245 protocol with phase-lock gel tubes⁴⁸.

246 PCR amplification of the *P. falciparum* genes *crt*, *mdr1*, *dhfr*, *dphs*, and *kelch13* was
247 performed with previously published primers^{29,49,50}. Cycling protocols were based on
248 manufacturer recommendations for OneTaq Hot Start 2X Master Mix (NEB) and/or Phusion
249 High-Fidelity PCR Master Mix with HF Buffer (NEB) (Table S2). Reactions were visualized in
250 1% agarose gels, and if successful, cleaned with ExoSAP-IT (ThermoFisher) and Sanger
251 sequenced (Elim Bio). Sanger chromatogram data were compared to PlasmoDB reference *P.*
252 *falciparum* sequences using Benchling. Amino acid substitutions were identified in the following
253 positions: *mdr1* 86, 184, and 1246; *crt* 72-76; *dhfr* 50, 51, 59, and 108; *dphs* 436, 437, 540, and
254 681; and *kelch13* 578-580.

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256 MOI and allele frequency calculations

257 For each sample, a double infection was inferred if the sequencing chromatogram showed
258 equally sized, double peaks for any of the 13 analyzed loci. Multiplicity of infection (MOI) was
259 calculated as the total number of infections divided by the total number of samples, as previously
260 described⁵¹. Similarly, the frequency of each allele was determined based on the total number of
261 infections, with double infections at any locus contributing two genotypes at every locus.
262 Samples without missing data at *dhfr* or *dphs* were also assessed for the presence of up to seven
263 SP-resistance alleles (*dhfr*-51I, *dhfr*-59R, *dhfr*-108G, *dphs*-436, *dphs*-437G, *dphs*-540E, *dphs*-
264 581)^{52,53}.

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Collection of historical data

Publications reporting allele frequencies for drug-resistance loci anywhere in Angola since 1995 were gathered from the Worldwide Antimalarial Resistance Network (WWARN) Molecular Surveyor tool (<http://www.wwarn.org/molecularsurveyor/>), facilitated by a recent review⁷. The original data published in these studies were used to calculate allele frequencies as described above. For studies that spanned multiple years, the average year was used for time-course analysis (below). Studies that did not provide linked data for *dhfr* and *dhps* (e.g., reported the two genes separately) could not be included.

Statistical analysis

To evaluate changes in *mdr1* and *dhfr/dhps* alleles over time, linear models were fit to the frequency or count data using the `lm` function in R. To avoid a bias from incomplete data, all 2014 samples were excluded from the *dhfr/dhps* timecourse analysis. For *crt*, the relationship between CVIET frequency and time was not linear; therefore, Kendall's rank correlation was applied using the `cor.test` function in R.

DECLARATIONS

Ethics approval and consent to participate

Ethics approval for this study was obtained from Stanford University IRB (#39149) and the Medical Ethics Committee of the University 11th of November in Cabinda.

Consent for publication

Prior to participation, all study subjects and/or their parents consented in writing to the publication of study results in the scientific literature.

Competing interests

The authors declare that they have no competing interests.

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301 Author's Contributions

302 E.R.E., D.A.P., and S.B. designed the study. F.R. and S.B. supervised the study. E.R.E. collected
303 data, analyzed data, and wrote the manuscript. All authors have approved the final manuscript.

304

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