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1 Changing antimalarial drug resistance patterns identified by surveillance at
2 three sites in Uganda

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26

27 **Footnote page**

28

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38

39 **Abstract**

40 We assessed *Plasmodium falciparum* drug resistance markers in parasites collected in 2012,
41 2013, and 2015 at 3 sites in Uganda. The prevalence and frequency of parasites with mutations
42 in putative transporters previously associated with resistance to aminoquinolines, but increased
43 sensitivity to lumefantrine (*pfcr1* 76T; *pfmdr1* 86Y and 1246Y), decreased markedly at all sites.
44 Antifolate resistance mutations were common, with apparent emergence of mutations (*pfdhfr*
45 164L; *pfdhps* 581G) associated with high level resistance. K13 mutations linked to artemisinin
46 resistance were uncommon and did not increase over time. Changing malaria treatment
47 practices have been accompanied by profound changes in markers of resistance.

48

49

50 Drug resistance challenges the treatment and control of malaria. In Africa, use of
51 artemisinin-based combination therapies (ACTs) has become standard to treat uncomplicated
52 malaria, accompanied by changes in the selective pressure for resistance. In Uganda, the first
53 line regimen to treat uncomplicated malaria changed from chloroquine to chloroquine +
54 sulfadoxine/pyrimethamine in 2000 and artemether-lumefantrine (AL) in 2004, although
55 implementation was slow. Elsewhere in Africa, artesunate-amodiaquine (AS/AQ) is first line to
56 treat malaria in many countries and dihydroartemisinin-piperaquine (DHA/PQ) is under study for
57 chemoprevention. SP remains the standard-of-care to prevent malaria in pregnant women.

58 Our understanding of antimalarial drug resistance is incomplete, but some *Plasmodium*
59 *falciparum* genetic polymorphisms are clearly important. The 76T mutation in the putative
60 transporter PfCRT is linked to decreased sensitivity to the aminoquinolines chloroquine and
61 amodiaquine, and *pfcr1* mutations are selected in new infections that occur soon after treatment
62 with aminoquinolines [1, 2]. Mutations in *pfmdr1*, which encodes another putative transporter,
63 the p-glycoprotein homologue, are also associated with altered drug sensitivity. In Africa, the
64 *pfmdr1* 86Y and 1246Y mutations are common, associated with decreased sensitivity to

65 aminoquinolines, and selected by prior treatment with AS/AQ and DHA/PQ [1, 3]. Interestingly,
66 wild type sequences at these same alleles are associated with decreased sensitivity to
67 lumefantrine and selected by recent treatment with AL, demonstrating opposite effects of the
68 same polymorphisms on sensitivity to different drugs [1, 2].

69 Resistance to SP is well characterized, with 5 mutations in dihydrofolate reductase (51I,
70 59R, and 108N) and dihydropteroate synthetase (437G and 540E) now common in much of
71 Africa and associated with an intermediate level of resistance [4]. Additional mutations, notably
72 *pfdhfr* 164L and *pfdhps* 581G, lead to high level resistance. These mutations have been rare in
73 African surveys, but recent studies have suggested emergence in some areas [5, 6].

74 Changing malaria treatment practices may lead to changes in drug sensitivity. In Malawi,
75 the replacement of chloroquine with SP was followed by widespread *pfcr* wild type parasites
76 and excellent clinical efficacy of chloroquine [7]. In Uganda, increased prevalence of wild type
77 *pfcr* K76 and *pfmdr1* N86 and D1246 sequences was seen in Tororo from 2003-12 [2, 8],
78 although analyses were complicated by numerous mixed genotypes. In ex vivo studies,
79 increasing sensitivity to chloroquine and decreasing sensitivity to lumefantrine were
80 documented [2]. Consistent with these changes, and in contrast to older studies that showed
81 superiority of AL, in a recent 3-site trial, treatment with AS/AQ was followed by decreased
82 recurrent malaria compared to AL [9]. Thus, parasites in Uganda are changing, and these
83 changes appear to have clinical consequences. However, improved measures of parasite
84 trends will be helpful, in particular utilizing randomly collected samples rather than those
85 available from drug efficacy trials, and including analyses of frequency, which circumvent the
86 complexities of polyclonal infections. We now report the prevalence and frequency of key
87 polymorphisms utilizing samples collected by probability sampling.

88
89
90

91 **METHODS**

92 **Cross-sectional surveys and sample collection.** Cross-sectional surveys were conducted in
93 200 randomly selected households each at 3 sites in 2012, 2013, and 2015 as previously
94 described [10]. Samples were collected during the same period for each year of the study,
95 January-February in Nagongera, Tororo District, in eastern Uganda near Kenya; March-April in
96 Walukuba, Jinja District, in south-central Uganda on Lake Victoria; and May-June in Kihhi,
97 Kanungu District, in southwestern Uganda. The sites varied greatly in malaria transmission
98 intensity (annual entomological inoculation rates 3.8, 26.6, and 125.0 infectious bites per person
99 year for Walukuba, Kihhi, and Nagongera, respectively). Households were randomly selected
100 as described, and finger prick blood samples collected on filter paper from all children under 15
101 years of age and 1 randomly selected adult from each of 5 age categories; samples were
102 collected regardless of whether symptoms were present [10].

103

104 **Assessment of *P. falciparum* polymorphisms.** Parasite DNA was extracted from dried blood
105 spots from samples positive by microscopy, and sequences of alleles of interest in *pfprt*, *pfmdr1*,
106 *pfdhfr*, and *pfdhps* were determined using a ligase detection reaction-fluorescent microsphere
107 assay, as previously described [11], with minor modifications, including nested PCR
108 amplifications of templates, as described [12]. The K13 gene propeller domain was amplified
109 and sequenced as previously described [13].

110

111 **Frequency and linkage analyses.** Parasite population frequencies were estimated using all
112 1466 samples and a previously described model that accounts for mixed infections [14]. Linkage
113 disequilibrium was estimated for all samples, but results from alleles with mixed or missing
114 genotyping outcomes were omitted from the analysis (see supplementary file for details).

115

116

117 **RESULTS**

118 **Prevalence of resistance-mediating polymorphisms in putative transporters.** A total of
119 1486 microscopy-positive samples were collected and analyzed. Sequences at all alleles of
120 interest were classified as wild type (identical in sequence to the reference 3D7 strain), mutant,
121 or mixed for the 1466 samples that yielded data for at least one polymorphism. Considering the
122 key transporter polymorphisms *pfcr1* K76T, *pfmdr1* N86Y, and *pfmdr1* D1246Y, the prevalence
123 of parasites with mutant sequences decreased steadily from 2012 to 2015 (Figure 1A). Results
124 were similar at the 3 sites. For another polymorphic allele, *pfmdr1* Y184F, the prevalence of
125 parasites with mutant alleles increased in Jinja, but was stable at other sites. The *pfmdr1* 1034C
126 and 1042D mutations, generally seen only outside Africa, were identified rarely.

127
128 **Prevalence of resistance-mediating polymorphisms in folate pathway enzymes.** The
129 prevalence of parasites with 5 mutations (*pfdhfr* 51I, 59R, 108N; *pfdhps* 437G, 540E) that were
130 common in prior surveys remained high (Figure 1A). In addition, 2 mutations that have
131 previously been rare in most surveys from Africa were seen, with *pfdhfr* 164L in Kanungu, and
132 *pfdhps* 581G at all 3 sites in in 2015.

133
134 **Frequency of resistance-mediating polymorphisms and haplotype analysis.** Prevalence
135 data did not take into account multiplicity of infection (MOI) and were complicated by many
136 samples containing both wild type and mutant sequences. Therefore, we used a statistical
137 model to estimate parasite population frequencies for the studied polymorphisms. Frequency
138 results were broadly similar to those based on prevalence; point estimates for the mutant
139 transporter alleles *pfcr1* 76T, *pfmdr1* 86Y, and *pfmdr1* 1246Y decreased markedly from 2012 to
140 2015 at all 3 sites (Figure 1B). Estimates of linkage disequilibrium suggested linkage between
141 *pfmdr1* 86, 184, and 1246, and *pfcr1* 76; between *pfmdr1* 1246 and *pfcr1* 76; and between
142 *pfdhps* 437 and 540 (Figure 2). Considering haplotypes, Y184F did not appear to impact trends,

143 with increases in both NYD and NFD haplotypes over time (Supplemental Figure 1); there was a
144 marked increase in frequency of the *pfmdr1* N86/D1246 haplotype, suggesting that parasites
145 with both wild type alleles have a selective advantage (Supplemental Figure 2); and the wild
146 type *pfmdr1* N86/D1246 haplotype was associated with both *pfcr1* K76T alleles, while K76 was
147 associated only with N86/D1246 (Supplemental Figure 3).

148
149 **Prevalence of K13 polymorphisms.** We randomly selected 20 samples from each site and
150 year for analysis. Of the 153 sequences obtained, 4 samples had non-synonymous
151 polymorphisms, with a total of 5 mutations, each identified once (V555A from Kihihi in 2012;
152 M472V from Nagongera in 2013; A569S from Nagongera in 2015; K563E and A578S in a single
153 sample from Walukuba in 2013). K13 mutations were seen in samples from all 3 sites and all 3
154 years of study. Mutations at 4 of the loci were reported previously in Africa, from Niger (M472I),
155 Rwanda (V555A), Kenya and Cameroon (A569S), and 7 different countries, including Uganda
156 (A578S), but to our knowledge K563E has not been reported ([http://www.wwarn.org/molecular-](http://www.wwarn.org/molecular-surveyor-k13)
157 [surveyor-k13](http://www.wwarn.org/molecular-surveyor-k13)).

158

159 **DISCUSSION**

160 We surveyed the prevalence and calculated the frequency of key drug resistance
161 polymorphisms in *P. falciparum* isolates collected at 3 sites in Uganda from 2012 to 2015. This
162 study improved on prior evaluations by studying randomly collected isolates, rather than those
163 collected in the context of a clinical trial, and by evaluating the frequency of polymorphisms,
164 circumventing confounding by varied MOI and prevalence of mixed infections at different sites.
165 We identified important changes over time. Notably, with increasing use of AL to treat malaria,
166 the prevalence and frequency of mutant sequences at 3 key *pfcr1* and *pfmdr1* alleles decreased,
167 consistent with decreasing sensitivity to lumefantrine, but increasing sensitivity to

168 aminoquinolines. In addition, mutations in *pfdhfr* and *pfdhps* that mediate high level antifolate
169 resistance appear to be emerging.

170 Changes in the prevalence of parasites with drug resistance polymorphisms were not
171 unexpected. In Malawi, discontinuation of chloroquine as the first-line antimalarial was
172 accompanied by loss of the *pfcr1* 76T mutation and regaining of chloroquine antimalarial efficacy
173 [7]. Multiple studies showed selection for mutant genotypes by amodiaquine-containing
174 regimens and for wild type genotypes by AL [1]. In Uganda, wild type sequences at 3 key
175 transporter alleles are increasingly common. Most parasites studied in Tororo were mutant at
176 these 3 alleles through about 2010 [8], but wild type sequences have been increasingly
177 prevalent since that time. The rate of change toward wild type transporter sequences was
178 greater in a cohort treated with AL for every episode of malaria, compared to a cohort treated
179 with DHA/PQ, documenting the contribution of selective pressure from AL to this process [12].

180 Mutations in *pfdhfr* and *pfdhps* have been common in Uganda for at least a decade. Use
181 of SP to treat malaria, WHO-recommended SP for intermittent preventive therapy in pregnant
182 women, antifolates to treat bacterial infections, and trimethoprim-sulfamethoxazole in HIV-
183 infected individuals likely all offer continued selective pressure for antifolate resistance. It was
184 thus of interest to see if additional mutations that have been seen primarily outside Africa [4] are
185 emerging in Uganda. The *pfdhfr* 164L mutation was identified in an earlier survey in
186 southwestern Uganda [5], and it was present in Kanungu, also in southwestern Uganda, over
187 the course of our study. The *pfdhps* 581G mutation, which has been noted in Tanzania [6], was
188 detected at all 3 study sites. These additional mutations will probably render SP useless for the
189 treatment or control of malaria. Consideration of other regimens for the prevention of malaria,
190 notably DHA/PQ, which recently showed outstanding efficacy in children and pregnant women
191 [3, 15], is an urgent priority.

192 Frequency analyses clarified results by accounting for MOI and mixed infections, and
193 were consistent with prevalence results. Linkage analyses demonstrated linkage between the

194 transporter polymorphisms *pfmdr1* 86, 184, 1246, and *pfcr1* 76. Haplotype analyses
195 demonstrated an apparent selective advantage of parasites with the wild type alleles *pfmdr1*
196 N86 and D1246, and that wild type *pfcr1* K76 was present almost exclusively with a background
197 of *pfmdr1* N86/D1246. As *pfcr1* 76T is the main mediator of resistance to chloroquine, but
198 decreased sensitivity to lumefantrine is linked to all 3 of these polymorphisms [1, 2], it seems
199 likely that the evolution of transporter polymorphisms has been driven both by decreasing use of
200 chloroquine and increasing use of AL over time.

201 Resistance to artemisinin, mediated principally by mutations in K13, is of great concern,
202 but resistance does not appear to have yet spread to Africa. We identified a handful of K13
203 propeller domain mutations, but no evidence of geographic differences in prevalence or
204 changes over time. K13 polymorphisms may be under selection from ACT use in Uganda, but
205 they do not yet appear to be mediating artemisinin resistance.

206 Our study had some limitations. First, we studied only 3 sites over 4 years; important
207 additional trends may be underway, but not evident over this short interval. Second, sample
208 sizes were fairly small, especially for Jinja, where decreasing prevalence limited available
209 samples over time. Third, we considered only a small number of well characterized resistance-
210 mediating polymorphisms. Consideration of full sequences of genes of interest or of whole
211 genomes might identify additional important trends in the evolution of drug resistance.

212 In summary, surveillance for *P. falciparum* drug resistance markers in Uganda has
213 demonstrated marked changes in recent years, with a return to wild type transporter sequences
214 that likely mediate decreased sensitivity to AL, the national regimen to treat malaria, emergence
215 of mutations that mediate high level antifolate resistance, but no convincing evidence of
216 artemisinin resistance. Continued surveillance for mediators of antimalarial drug resistance is
217 warranted. Furthermore, as selective pressures of AS/AQ and DHA/PQ differ from those of AL
218 [1-3, 9, 12], we suggest consideration of rotating treatment regimens to delay emergence of
219 resistance.

220

221

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227

228

229 **References**

- 230 1. Rosenthal PJ. The interplay between drug resistance and fitness in malaria parasites. *Mol*
231 *Microbiol* **2013**; 89:1025-38.
- 232 2. Tumwebaze P, Conrad MD, Walakira A, et al. Impact of antimalarial treatment and
233 chemoprevention on the drug sensitivity of malaria parasites isolated from Ugandan children.
234 *Antimicrob Agents Chemother* **2015**; 59:3018-30.
- 235 3. Nankabirwa JI, Conrad MD, Legac J, et al. Intermittent preventive treatment with
236 dihydroartemisinin-piperaquine in Ugandan schoolchildren selects for *Plasmodium falciparum*
237 transporter polymorphisms that modify drug sensitivity. *Antimicrob Agents Chemother* **2016**;
238 60:5649-54.
- 239 4. Gregson A, Plowe CV. Mechanisms of resistance of malaria parasites to antifolates.
240 *Pharmacol Rev* **2005**; 57:117-45.
- 241 5. Lynch C, Pearce R, Pota H, et al. Emergence of a dhfr mutation conferring high-level drug
242 resistance in *Plasmodium falciparum* populations from southwest Uganda. *J Infect Dis* **2008**;
243 197:1598-604.
- 244 6. Kavishe RA, Kaaya RD, Nag S, et al. Molecular monitoring of *Plasmodium falciparum* super-
245 resistance to sulfadoxine-pyrimethamine in Tanzania. *Malar J* **2016**; 15:335.

- 246 7. Laufer MK, Thesing PC, Eddington ND, et al. Return of chloroquine antimalarial efficacy in
247 Malawi. *N Engl J Med* **2006**; 355:1959-66.
- 248 8. Mbogo GW, Nankoberanyi S, Tukwasibwe S, et al. Temporal changes in prevalence of
249 molecular markers mediating antimalarial drug resistance in a high malaria transmission setting
250 in Uganda. *Am J Trop Med Hyg* **2014**; 91:54-61.
- 251 9. Yeka A, Kigozi R, Conrad MD, et al. Artesunate/amodiaquine versus artemether/lumefantrine
252 for the treatment of uncomplicated malaria in Uganda: a randomized trial. *J Infect Dis* **2016**;
253 213:1134-42.
- 254 10. Yeka A, Nankabirwa J, Mpimbaza A, et al. Factors associated with malaria parasitemia,
255 anemia and serological responses in a spectrum of epidemiological settings in Uganda. *PLoS*
256 *One* **2015**; 10:e0118901.
- 257 11. Leclair NP, Conrad MD, Baliraine FN, Nsanzabana C, Nsobya SL, Rosenthal PJ.
258 Optimization of a ligase detection reaction fluorescent microsphere assay for the
259 characterization of resistance-mediating polymorphisms in African samples of *Plasmodium*
260 *falciparum*. *J Clin Microbiol* **2013**; 51:2564-70.
- 261 12. Conrad MD, LeClair N, Arinaitwe E, et al. Comparative impacts over 5 years of artemisinin-
262 based combination therapies on *Plasmodium falciparum* polymorphisms that modulate drug
263 sensitivity in Ugandan children. *J Infect Dis* **2014**; 210:344-53.
- 264 13. Arie F, Witkowski B, Amaratunga C, et al. A molecular marker of artemisinin-resistant
265 *Plasmodium falciparum* malaria. *Nature* **2014**; 505:50-5.
- 266 14. Taylor AR, Flegg JA, Nsobya SL, et al. Estimation of malaria haplotype and genotype
267 frequencies: a statistical approach to overcome the challenge associated with multiclonal
268 infections. *Malar J* **2014**; 13:102.
- 269 15. Kakuru A, Jagannathan P, Muhindo MK, et al. Dihydroartemisinin-piperaquine for the
270 prevention of malaria in pregnancy. *N Engl J Med* **2016**; 374:928-39.

271

Figure 2.

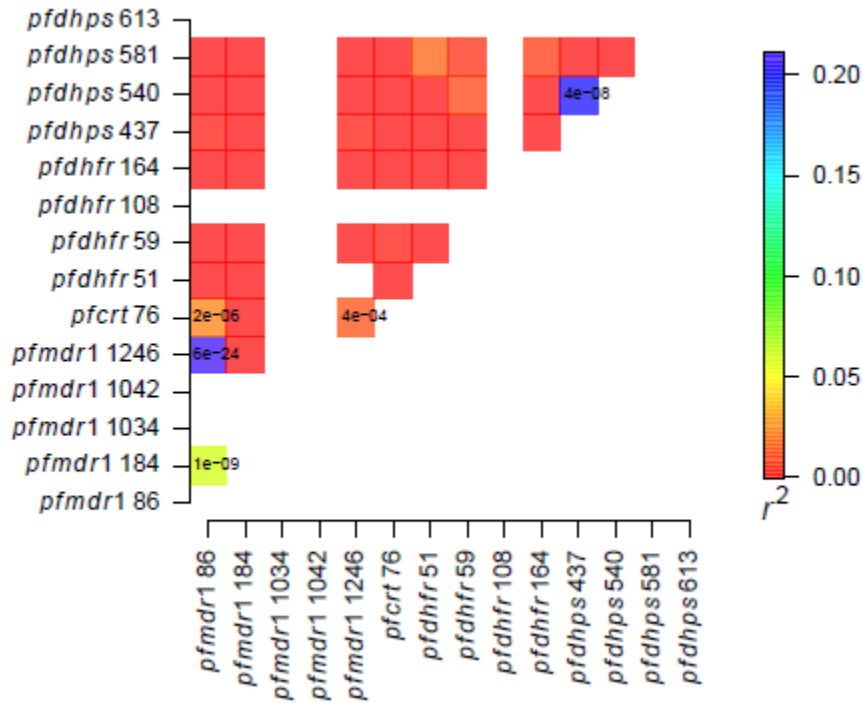


Figure 2. Plot of linkage disequilibrium for pairwise comparisons of all studied *pfcr1*, *pfmdr1*, *pfdhfr*, and *pfdhps* polymorphisms. Linkage disequilibrium, r^2 , captures the non-random association of alleles within one gene or in different genes, with values ranging from 0 (no correlation) to 1 (perfect correlation). Colors indicate r^2 values (see supplementary file for details), calculable for polymorphic markers only; white denotes non-polymorphic markers. P-values of statistically significant r^2 values are shown. These were calculated using Fisher's exact test, with a Bonferroni correction for 45 tests over 10 polymorphic markers, placing significance at 0.001.

Supplement to Tumwebaze, et al.: Changing antimalarial drug resistance patterns identified by surveillance at three sites in Uganda

SUPPLEMENTAL METHODS

Frequency analyses

Frequencies were estimated using a previously published model [1] applied to data from different sites and years separately. We used a Dirichlet prior with concentration parameter equal to 0.1 over frequencies and a truncated geometric prior over each multiplicity of infection

(MOI), $\rho(MOI) = \frac{\lambda (1-\lambda)^{MOI}}{\sum_{MOI=MOI_{min}}^{MOI_{max}} \lambda (1-\lambda)^{MOI}}$, where $MOI_{min} = 1$, or 2 if the sample was discernibly

multiclonal, $MOI_{max} = 20$, and λ was based on experimentally derived MOI estimates by genotyping the *msh2* locus via nested PCR and capillary electrophoresis [2]. To account for ambiguity in MOI estimates greater than or equal to 5, the frequency results were generated by combining a posteriori samples from two separate runs of the model, one with λ equal to the reciprocal of the sample mean MOI per site and year, and one with λ equal to the reciprocal of the mean MOI over samples with MOI less than or equal to 5 per site and year.

Linkage analyses

Linkage disequilibrium, r^2 [3], was estimated for all studied *pfcr1*, *pfmdr1*, *pfdhfr*, and *pfdhps* polymorphisms, using data from all 1466 samples yielding results, but discarding counts with one or more mixed or missing genotyping outcomes from within-calculation quotients. P-values were calculated using Fisher's exact test. For 45 tests over 10 polymorphic markers, a Bonferroni correction on uncorrected significance at 0.05 placed significance at 0.001.

Supplemental references

1. Taylor AR, Flegg JA, Nsohya SL, et al. Estimation of malaria haplotype and genotype frequencies: a statistical approach to overcome the challenge associated with multiclonal infections. *Malar J* **2014**; 13:102.
2. Gupta, V., Dorsey, G., Hubbard, A.E., et al. Gel versus capillary electrophoresis genotyping for categorizing treatment outcomes in two anti-malarial trials in Uganda. *Malar J* **2010** 9, 19
3. Slatkin, M. Linkage disequilibrium--understanding the evolutionary past and mapping the medical future. *Nat Rev Genet* **2008**; 9:477-85.

272 **Supplemental Table 1. Prevalence of wild type, mixed, or mutant sequences at**
 273 **transporter alleles of interest at the indicated sites and years. N = number of samples.**
 274

Locus	Site	Year	N	Wild type	Mixed	Mutant
<i>Pfmdr1</i> N86Y	Jinja	2012	61	11 (18.0%)	27 (44.3%)	23 (37.7%)
	Jinja	2013	45	30 (66.7%)	13 (28.9%)	2 (4.4%)
	Jinja	2015	36	31 (86.1%)	5 (13.9%)	0
	Kanungu	2012	77	15 (19.5%)	34 (44.2%)	28 (36.4%)
	Kanungu	2013	60	36 (60.0%)	16 (26.7%)	8 (13.3%)
	Kanungu	2015	114	105 (92.1%)	7 (6.1%)	2 (1.8%)
	Tororo	2012	317	128 (40.4%)	163 (51.4%)	26 (8.2%)
	Tororo	2013	363	170 (46.8%)	151 (41.6%)	42 (11.6%)
	Tororo	2015	282	229 (81.2%)	51 (18.1%)	2 (0.7%)
<i>Pfmdr1</i> Y184F	Jinja	2012	64	34 (53.1%)	21 (32.8%)	9 (14.1%)
	Jinja	2013	47	18 (38.3%)	23 (48.9%)	6 (12.8%)
	Jinja	2015	32	9 (28.1%)	14 (43.8%)	9 (28.1%)
	Kanungu	2012	80	37 (46.3%)	27 (33.8%)	16 (20.0%)
	Kanungu	2013	59	27 (45.8%)	22 (37.3%)	10 (17.0%)
	Kanungu	2015	109	40 (36.7%)	40 (36.7%)	29 (26.6%)
	Tororo	2012	316	99 (31.3%)	147 (46.5%)	70 (22.2%)
	Tororo	2013	361	184 (51.0%)	124 (34.4%)	53 (14.7%)
	Tororo	2015	274	88 (32.1%)	112 (40.9%)	74 (27.0%)
<i>Pfmdr1</i> S1034C	Jinja	2012	72	72 (100%)	0	0
	Jinja	2013	47	47 (100%)	0	0
	Jinja	2015	15	15 (100%)	0	0
	Kanungu	2012	81	81 (100%)	0	0
	Kanungu	2013	67	67 (100%)	0	0
	Kanungu	2015	59	59 (100%)	0	0
	Tororo	2012	174	174 (100%)	0	0
	Tororo	2013	374	374 (100%)	0	0
<i>Pfmdr1</i> N1042D	Jinja	2012	72	72 (100%)	0	0
	Jinja	2013	47	46 (97.9%)	1 (2.1%)	0
	Jinja	2015	15	15 (100%)	0	0
	Kanungu	2012	81	81 (100%)	0	0
	Kanungu	2013	55	54 (98.2%)	1 (1.8%)	0
	Kanungu	2015	65	65 (100%)	0	0
	Tororo	2012	167	167 (100%)	0	0
	Tororo	2013	363	361 (99.5%)	2 (0.6%)	0
<i>Pfmdr1</i> D1246Y	Jinja	2012	62	24 (38.7%)	24 (38.7%)	14 (22.6%)
	Jinja	2013	45	33 (73.3%)	9 (20.0%)	3 (6.7%)
	Jinja	2015	35	28 (80.0%)	6 (17.1%)	1 (2.9%)
	Kanungu	2012	79	28 (35.4%)	35 (44.3%)	16 (20.3%)
	Kanungu	2013	61	47 (77.1%)	6 (9.8%)	8 (13.1%)
	Kanungu	2015	116	92 (79.3%)	18 (15.5%)	6 (5.2%)
	Tororo	2012	300	149 (49.7%)	107 (35.7%)	44 (14.7%)
	Tororo	2013	362	233 (64.4%)	103 (28.5%)	26 (7.2%)
	Tororo	2015	286	205 (71.7%)	65 (22.7%)	16 (5.6%)
<i>Pfcrt</i> K76T	Jinja	2012	70	3 (4.3%)	0	67 (95.7%)
	Jinja	2013	50	2 (4.0%)	5 (10.0%)	43 (86.0%)
	Jinja	2015	35	5 (14.3%)	6 (17.1%)	24 (68.6%)

	Kanungu	2012	86	3 (3.5%)	2 (2.3%)	81 (94.2%)
	Kanungu	2013	66	10 (15.2%)	13 (19.7%)	43 (65.2%)
	Kanungu	2015	117	35 (29.9%)	13 (11.1%)	69 (59.0%)
	Tororo	2012	342	9 (2.6%)	45 (13.2%)	288 (84.2%)
	Tororo	2013	378	39 (10.3%)	51 (13.5%)	288 (76.2%)
	Tororo	2015	285	85 (29.8%)	54 (19.0%)	146 (51.2%)

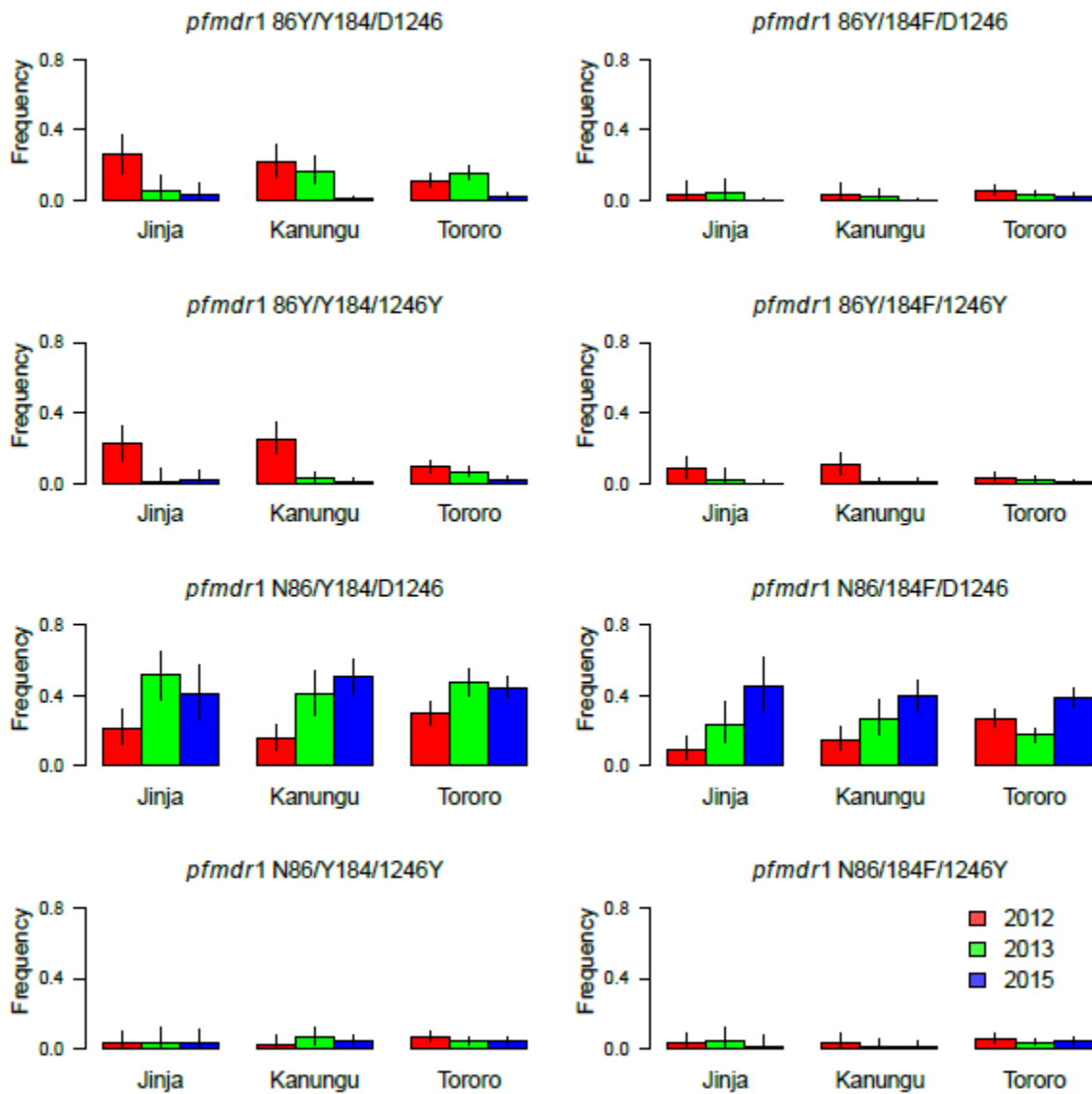
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276

277 **Supplemental Table 2. Prevalence of wild type, mixed, or mutant sequences at antifolate**
 278 **alleles of interest at the indicated sites and years. N = number of samples.**
 279

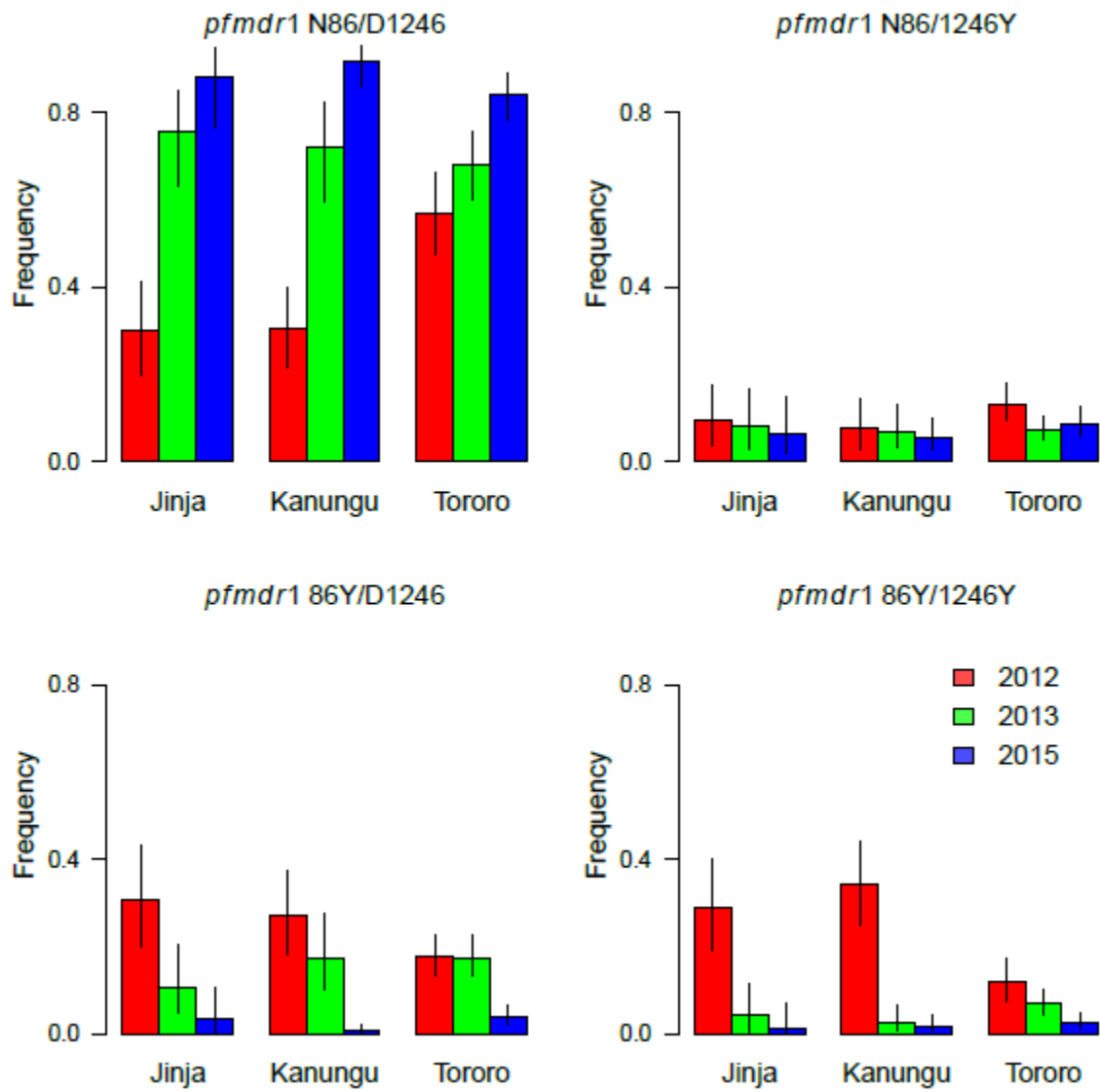
Locus	Site	Year	N	Wild type	Mixed	Mutant
<i>Pfdhfr</i> N51I	Jinja	2012	66	0	0	66 (100%)
	Jinja	2013	49	0	0	49 (100%)
	Jinja	2015	35	0	0	35 (100%)
	Kanungu	2012	72	0	0	72 (100%)
	Kanungu	2013	60	1 (1.7%)	4 (6.7%)	55 (91.7%)
	Kanungu	2015	110	0	0	110 (100%)
	Tororo	2012	258	0	0	258 (100%)
	Tororo	2013	198	0	0	198 (100%)
	Tororo	2015	279	0	0	279 (100%)
<i>Pfdhfr</i> C59R	Jinja	2012	66	1 (1.5%)	2 (3.0%)	63 (95.5%)
	Jinja	2013	49	1 (2.0%)	6 (12.2%)	42 (85.7%)
	Jinja	2015	35	1 (2.9%)	7 (20.0%)	27 (77.1%)
	Kanungu	2012	74	2 (2.7%)	8 (10.8%)	64 (86.5%)
	Kanungu	2013	60	6 (10.0%)	4 (6.7%)	50 (83.3%)
	Kanungu	2015	118	3 (2.5%)	9 (7.6%)	106 (89.8%)
	Tororo	2012	254	2 (0.8%)	22 (8.7%)	230 (90.6%)
	Tororo	2013	198	3 (1.5%)	19 (9.6%)	176 (88.9%)
	Tororo	2015	293	3 (1.0%)	29 (9.9%)	261 (89.1%)
<i>Pfdhfr</i> S108T/N	Jinja	2012	66	0	0	66 (100%)
	Jinja	2013	49	0	0	49 (100%)
	Jinja	2015	36	0	0	36 (100%)
	Kanungu	2012	72	0	0	72 (100%)
	Kanungu	2013	60	0	0	60 (100%)
	Kanungu	2015	110	0	0	110 (100%)
	Tororo	2012	255	0	0	255 (100%)
	Tororo	2013	198	0	0	198 (100%)
	Tororo	2015	282	0	0	282(100%)
<i>Pfdhfr</i> I164L	Jinja	2012	65	65 (100%)	0	0
	Jinja	2013	46	46 (100%)	0	0
	Jinja	2015	35	35 (100%)	0	0
	Kanungu	2012	69	64 (92.8%)	2 (2.9%)	3 (4.4%)
	Kanungu	2013	55	48 (87.3%)	2 (3.6%)	5 (9.1%)
	Kanungu	2015	115	103 (89.6%)	5 (4.4%)	7 (6.1%)
	Tororo	2012	253	253 (100%)	0	0
	Tororo	2013	196	196 (100%)	0	0
	Tororo	2015	294	294 (100%)	0	0
<i>Pfdhps</i> A437G	Jinja	2012	35	0	0	35 (100%)
	Jinja	2013	41	0	0	41 (100%)
	Jinja	2015	33	0	3 (9.1%)	30 (90.9%)
	Kanungu	2012	56	0	0	56 (100%)
	Kanungu	2013	50	0	0	50 (100%)
	Kanungu	2015	117	7 (6.0%)	6 (5.1%)	104 (88.9%)
	Tororo	2012	263	4 (1.5%)	18 (6.8%)	241 (91.6%)
	Tororo	2013	185	0	2 (1.1%)	183 (98.9%)
	Tororo	2015	268	5 (1.9%)	26 (9.7%)	237 (88.4%)
<i>Pfdhps</i> K540E	Jinja	2012	37	0	0	37 (100%)
	Jinja	2013	44	0	2 (4.6%)	42 (95.5%)
	Jinja	2015	33	0	0	33 (100%)

	Kanungu	2012	61	1 (1.6%)	0	60 (98.4%)
	Kanungu	2013	51	0	2 (3.9%)	49 (96.1%)
	Kanungu	2015	105	4 (3.9%)	3 (2.9%)	98 (93.3%)
	Tororo	2012	265	2 (0.8%)	4 (1.5%)	259 (97.7%)
	Tororo	2013	187	1 (0.5%)	4 (2.1%)	182 (97.3%)
	Tororo	2015	256	7 (2.7%)	9 (3.5%)	240 (93.8%)
Pfdhps A581G	Jinja	2012	46	45 (97.8%)	1 (2.2%)	0
	Jinja	2013	44	43 (97.7%)	1 (2.3%)	0
	Jinja	2015	38	30 (79.0%)	1 (2.6%)	7 (18.4%)
	Kanungu	2012	61	42 (68.9%)	8 (13.1%)	11 (18.0%)
	Kanungu	2013	50	31 (62.0%)	6 (12.0%)	13 (26.0%)
	Kanungu	2015	117	77 (65.8%)	31 (26.5%)	9 (7.7%)
	Tororo	2012	268	254 (94.8%)	13 (4.9%)	1 (0.4%)
	Tororo	2013	188	179 (95.2%)	7 (3.7%)	2 (1.1%)
	Tororo	2015	261	226 (86.6%)	24 (9.2%)	11 (4.2%)
Pfdhps A613S	Jinja	2012	31	31 (100%)	0	0
	Jinja	2013	44	44 (100%)	0	0
	Jinja	2015	35	35 (100%)	0	0
	Kanungu	2012	58	58 (100%)	0	0
	Kanungu	2013	45	45 (100%)	0	0
	Kanungu	2015	110	110 (100%)	0	0
	Tororo	2012	260	259 (99.6%)	1 (0.4%)	0
	Tororo	2013	181	181 (100%)	0	0

Supplemental Figure 1. Frequency point estimates of *pfmdr1* N86Y/Y184F/D1246Y haplotypes. Error bars represent 95% credible intervals.



Supplemental Figure 2. Frequency point estimates of *pfmdr1* N86Y/D1246Y haplotypes. Error bars represent 95% credible intervals.



Supplemental Figure 3. Frequency point estimates of *pfmdr1* N86Y/D1246Y haplotypes with *pfcr1* K76T. Error bars represent 95% credible interval.

