Comparative Prevalence of *Plasmodium falciparum* Resistance-Associated Genetic Polymorphisms in Parasites Infecting Humans and Mosquitoes in Uganda

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Abstract. Controlling malaria in high transmission areas, such as much of sub-Saharan Africa, will require concerted efforts to slow the spread of drug resistance and to impede malaria transmission. Understanding the fitness costs associated with the development of drug resistance, particularly within the context of transmission, can help guide policy decisions to accomplish these goals, as fitness constraints might lead to decreased transmission of drug-resistant strains. To determine if *Plasmodium falciparum* resistance-mediating polymorphisms impact on development at different parasite stages, we compared the genotypes of parasites infecting humans and mosquitoes from households in Uganda. Genotypes at 14 polymorphic loci in genes encoding putative transporters (*pfcr*t and *pfmdr*1) and folate pathway enzymes (*pfdhfr* and *pfdhps*) were characterized using ligase detection reaction-fluorescent microsphere assays. In paired analysis using the Wilcoxon signed-rank test, prevalences of mutations at 12 loci did not differ significantly between parasites infecting humans and mosquitoes. However, compared with parasites infecting humans, those infecting mosquitoes were enriched for the *pfmdr*1 86Y mutant allele ($P = 0.0001$) and those infecting *Anopheles gambiae* s.s. were enriched for the *pfmdr*1 86Y ($P = 0.0001$) and *pfcr*t 76T ($P = 0.0412$) mutant alleles. Our results suggest modest directional selection resulting from varied fitness costs during the *P. falciparum* life cycle. Better appreciation of the fitness implications of drug resistance mediating mutations can inform optimal malaria treatment and prevention strategies.

INTRODUCTION

Effective antimalarial drugs are among our best tools for the control and eventual elimination of malaria. In sub-Saharan Africa, artemisinin-based combination therapies (ACTs) have replaced chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) as the standard treatments for malaria because of widespread resistance to the older drugs. In Uganda, artemether-lumefantrine (AL) became the first-line treatment of uncomplicated malaria in 2004, with gradual implementation thereafter. Currently, AL remains highly efficacious,^{1,2} but increased use of AL and decreased use of CQ has been accompanied by marked changes in the prevalence of *Plasmodium falciparum* genetic polymorphisms associated with drug resistance,^{3,4} and modest shifts in ex vivo drug sensitivities.⁵ CQ and related drugs select for mutations in two putative transporters (in particular *pfcr*t K76T and *pfmdr*1 N86Y and D1246Y) that are associated with reduced sensitivity to aminoquinolines, but increased sensitivity to lumefantrine and some other antimalarials.^{6,7} AL selects for wild-type sequences at these alleles.^{3,8–10} SP remains the standard of care in Africa for intermittent preventive treatment to prevent malaria in pregnancy (IPTp), but resistance is common, mediated by mutations in the enzyme targets dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS).¹¹ In Africa, *P. falciparum* commonly demonstrates five DHFR/DHPS mutations that confer an intermediate level of resistance to SP, leading to poor treatment outcomes and modest efficacy for IPTp.¹²

Resistance to antimicrobial drugs is often accompanied by some loss of fitness.^{13–15} Fitness consequences of antimalarial drug resistance have been demonstrated using cultured malaria parasites, animal models, and clinical samples.¹³ It

has generally been seen that resistance-mediating polymorphisms lead to malaria parasites that are outcompeted by wild type in culture,^{16–19} in laboratory animals,^{20,21} and in humans^{22–24} when drug pressure is diminished. However, the relative impacts of drug resistance on the fitness of different parasite stages are poorly understood. Of particular interest is whether drug resistance impacts on transmission of parasites to mosquitoes. Recently, in studies from Zambia, markers of resistance to pyrimethamine (*pfdhfr* 51I, 59R, and 108N)²⁵ and CQ (*pfcr*t 76T)²⁶ were at significantly lower prevalence in parasites infecting mosquitoes compared with those infecting humans in the same households. Similarly, in continental Equatorial Guinea, markers of resistance to CQ (*pfcr*t 76T and *pfmdr*1 86Y) were at lower prevalence in parasites infecting mosquitoes.²⁷ These results suggested that resistance-mediating parasite polymorphisms were of greater detriment in mosquito, compared with human stages of infection. To further assess whether *P. falciparum* resistance-mediating polymorphisms preferentially impact on parasites infecting mosquitoes, we genotyped parasites infecting humans and mosquitoes from households in Uganda.

MATERIALS AND METHODS

Study site. Samples were collected from Nagongera Sub-county in Tororo District in southeastern Uganda as part of an ongoing cohort study that has previously been described.^{28,29} The major mosquito vectors in Tororo District are *Anopheles gambiae* s.s. and *Anopheles arabiensis*, and to a lesser extent *Anopheles funestus*.²⁹ Nagongera had a high transmission intensity, with an annual entomological inoculation rate of 310 infectious bites per year²⁹ and an estimated parasite prevalence in children of 29%²⁸ before the initiation of indoor residual spraying in December 2014, after which transmission dropped significantly.³⁰ A mass, community-based distribution of insecticide-impregnated bed nets was conducted in

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2011 and 2013, and cohort households were provided with additional bed nets.

Sample collection. Our cohort consisted of 100 randomly selected households that had at least one resident 0.5–10 years of age.²⁸ All children aged 0.5–10 years and one parent/guardian from each household who met eligibility criteria²⁸ were followed from August 2011 to June 2015. Monthly mosquito collections were performed using Centers for Disease Control and Prevention light traps as previously described.²⁹

Parent/guardians were encouraged to bring their children to the clinic any time they were ill. Children who presented with a documented fever ($\geq 38.0^{\circ}\text{C}$) or history of fever in the previous 24 hours had blood obtained for a thick blood smear. Those with positive blood smears were diagnosed with malaria. Uncomplicated malaria episodes were treated with AL. Episodes of complicated malaria or recurrent malaria occurring within 14 days of prior therapy were treated with quinine. Routine thick blood smears were performed every 3 months. Whenever a blood smear was performed, blood was also collected on filter paper.

Mosquito sample processing. Following collection, mosquitoes were stored on desiccant, and morphological features were used to identify mosquitoes in the *An. gambiae* sensu lato complex (*An. gambiae* s.l.) according to established taxonomic keys.^{31,32} *Plasmodium falciparum*-infected mosquitoes were identified with a loop-mediated isothermal amplification (LAMP) assay targeting mitochondrial DNA, as previously described.³³ The thoraxes of desiccated mosquitoes were homogenized in microcentrifuge tubes with 20 μL of phosphate-buffered saline using tissue grinders, and DNA was extracted by heating the tissue in 150 μL of 7% Chelex 100 (Bio-Rad, Hercules, CA) at 97°C for 10 minutes and centrifugation at $\sim 13,000$ rpm for 5 minutes. The supernatant was centrifuged at $\sim 13,000$ rpm for 10 minutes and then transferred into barcoded tubes for storage and molecular analyses. Molecular assays were used to distinguish species within the *An. gambiae* s.l. complex as *An. gambiae* s.s. or *An. arabiensis*.³⁴ *Plasmodium falciparum* in mosquitoes that were positive for parasite DNA in LAMP assays was genotyped at loci of interest as described below. Those samples for which a minimum of three *P. falciparum*

loci of interest were successfully genotyped were included in our study.

Selection and processing of *P. falciparum*-infected blood samples. For each *P. falciparum*-infected mosquito studied (162 samples, with 70–116 samples genotyped per locus [Table 1]), we selected a paired filter paper blood sample from a cohort participant. Selected samples were from the same or nearest household in which a human *P. falciparum* infection was detected within 40 days of the mosquito collection. When multiple samples were available, the human sample collected closest to the time of the mosquito collection was chosen. Of the 162 paired filter paper samples, 75 were from the same household from which mosquitoes were collected, and 87 were from a different household within 2.7 km (median distance 144 m) of mosquito collection. The median number of days between the collection of mosquito and paired human samples was 12 days. DNA was extracted from filter paper blood spots using Chelex 100 as previously described³⁵ and genotyped as described below.

Genotyping. *Plasmodium falciparum* DNA was genotyped at alleles of interest in *pfcr*, *pfmdr1*, *pfdhfr*, and *pfdhps* using a ligase detection reaction-fluorescent microsphere assay, as previously described,³⁶ with nested PCR reactions. Nested PCRs for *pfcr* and *pfmdr1* were performed as previously described.³ Primers and thermocycler parameters for *pfdhfr* and *pfdhps* are described in Supplemental Table 1.³⁷

Analysis. Data analysis was with Stata version 14 (Stata Corp., College Station, TX). Wilcoxon matched-pairs signed-rank tests were used to compare *P. falciparum* genotypes (pure mutant versus mixed or wild type) between paired infected mosquito and human samples. A *P* value of < 0.05 was considered significant.

RESULTS

Screening of 6,277 *An. gambiae* s.l. mosquitoes by *P. falciparum*-specific LAMP identified 645 (10.3%) infected mosquitoes (522 *An. gambiae* s.s., 102 *An. arabiensis*, 21 species undetermined). Infected mosquito samples were assessed for *P. falciparum* polymorphisms in 14 loci in *pfcr*, *pfmdr1*, *pfdhfr*, and *pfdhps*. Genotyping success was limited in LAMP-positive mosquito samples, presumably due to many

TABLE 1

Presence of *Plasmodium falciparum* drug resistance genotypes in malaria-infected humans and mosquitoes from the same households

Anopheles gambiae s.l.								Anopheles gambiae s.s.					
Gene	Locus	N	Presence of pure mutant genotype				P value	N	Presence of pure mutant genotype				P value
			Only mosquito	Both	Neither	Only human			Only mosquito	Both	Neither	Only human	
pfcr	K76T	78	17	44	9	8	0.07	72	17	40	8	7	0.04
	N86Y	77	16	0	61	0	0.0001	69	16	0	53	0	0.0001
	Y184F	77	17	12	40	8	0.07	69	15	10	36	8	0.14
	S1034C	115	0	0	115	0	NA	100	0	0	100	0	NA
	D1042Y	115	0	0	115	0	NA	100	0	0	100	0	NA
pmdr1	D1246Y	95	11	0	76	8	0.49	86	8	0	70	8	1.00
	N51I	116	3	105	0	8	0.13	100	3	89	0	8	0.13
	C59R	116	11	96	0	10	0.83	100	11	79	0	10	0.83
	S108N	116	1	113	0	2	0.56	100	1	97	0	2	0.56
	I164L	116	6	0	109	1	0.56	100	6	0	93	1	0.06
pfdhfr	A437G	70	2	67	0	1	0.06	62	2	59	0	1	0.56
	K540E	70	12	51	1	6	0.16	62	11	44	1	6	0.23
	A581G	70	0	0	70	0	NA	62	0	0	62	0	NA
	A613S	70	0	0	70	0	NA	62	0	0	62	0	NA

low-density infections. One hundred and sixty-two mosquito samples were successfully genotyped at a minimum of three loci (136 *An. gambiae* s.s., 21 *An. arabiensis*, and four undetermined). Prevalences of polymorphisms of interest were generally similar between human and mosquito infections (Figure 1), but in a paired analysis using the Wilcoxon matched-pairs signed-rank test the only significant difference between human and mosquito infections was that, compared with parasites infecting humans, those infecting mosquitoes were enriched for the *pfmdr1* 86Y mutant allele ($P = 0.0001$) (Table 1). Limiting the analysis to the 136 mosquitoes molecularly determined to be *An. gambiae* s.s., parasites with *pfmdr1* 86Y ($P = 0.0001$) and *pfcr1* 76T ($P = 0.0412$) mutations were significantly enriched in mosquito infections (Table 1). No differences were found between parasites infecting humans and the limited number of *An. arabiensis* ($N = 21$) collected.

DISCUSSION

Surveillance of resistance-conferring mutations has been an important tool in understanding *P. falciparum* drug resistance and associated fitness costs, but most studies have focused only on asexual parasites. An understanding of effects on other stages is critical, as without transmission to mosquitoes, resistance will not be maintained. To determine if resistance-mediating polymorphisms differentially impact on development at different parasite stages, we genotyped *P. falciparum* infecting humans and mosquitoes at 14 loci of interest in *pfcr1*, *pfmdr1*, *pfdhfr*, and *pfdhps*. In paired analysis, parasites with *pfmdr1* 86Y and *pfcr1* 76T mutant alleles were enriched in parasites infecting mosquitoes in comparison to those infecting humans. Overall, prevalences of studied polymorphisms differed only modestly between human and

mosquito infections, and we did not see evidence that stage-specific fitness constraints play a major role in limiting the spread of antimalarial drug resistance in Uganda.

Our results contrast with reports of marked differences in the prevalence of antifolate²³ and CQ²⁶ resistance-mediating mutations between human and *An. arabiensis* infections in Zambia and with a report from Equatorial Guinea, where wild-type alleles were more prevalent in mosquito infections at *pfcr1* K76T, *pfmdr1* N86Y, and *pfdhfr* I164L, although statistical significance was not reported. Differences between results from sites across Africa might be explained by different malaria vectors (primarily *An. gambiae* s.s. in Uganda versus *An. arabiensis* in Zambia), different levels of vector competence, differences in complexities of infection at sites with varied levels of transmission intensity, different parasite backgrounds that impact on transmissibility independent of drug resistance-mediating polymorphisms, and random effects due to bottlenecks rather than fitness differences.

Our study had some limitations. First, the high prevalence of multiple-genotype infections complicated the interpretation of trends, and mixed infections may have confounded results. Second, mosquito infections were often of low parasite density, preventing us from successfully genotyping parasites from all mosquitoes shown to be infected by LAMP. Third, limited numbers of infected mosquitoes necessitated that comparisons were between human and mosquito infections located up to a few kilometers from each other, rather than only within the same household. Despite these limitations, we quite clearly saw less marked differences between human and mosquito infections than reported previously from Zambia.

Our results suggest that determining how fitness costs of resistance impact on different stages of *P. falciparum* will be challenging, as findings may not be applicable across different

FIGURE 1. Prevalence of resistance-associated polymorphisms in *Plasmodium falciparum* infecting humans and *Anopheles gambiae* s.l. mosquitoes. This figure appears in color at www.ajtmh.org.

populations or different mosquito vectors. As a result, controlled laboratory experiments rather than observational studies will be required to test for causality, and additional field studies will be needed to determine the generalizability of reported findings.

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