Supporting Information

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SI Materials and Methods

Area and Population. The study was carried out in 15 representative 25-km^2 geographical grids, selected by simple random procedure. Each selected grid contained one study headman area comprising an average 270 residents primarily belonging to the BaTonga community. In 2003, the first-line drug for uncomplicated malaria was changed from chloroquine to artemether/lumefantrine. Sulfadoxine/pyrimethamine (SP), the second-line treatment during the chloroquine era, remained the drug of choice for uncomplicated malaria in pregnant women and children <5 kg. SP was also the standby treatment during stock-outs of artemether/lumefantrine. Furthermore, SP remains the choice drug for intermittent preventive therapy in pregnancy. In contrast, cycloguanil has not been an approved choice of antimalarials in Zambia.

Study Design and Data Collection. This was a prospective cross-sectional study. *P. falciparum* malaria infections in human and mosquito hosts were genotyped for antifolate drug-resistant polymorphisms in the parasite DHFR gene. DHPS alleles were not evaluated.

Whole mosquitoes were collected by spray catches in sleeping rooms from 6:00 to 10:00 AM. Individual mosquitoes were packed in 0.5-mL microcentrifuge tubes. Blood samples from human subjects were screened for *P. falciparum* the same afternoons by microscopic analysis of Giemsa-stained thick and thin blood smears. Parasite DNA in blood was simultaneously collected by finger-prick onto Whatman filter paper (1). Because parasites in mosquitoes represent human feeding in recent weeks, linking sites of spraying with individual humans was not attempted.

DNA Extraction from Mosquitoes. Each mosquito was dissected to separate the abdominal segment (midgut infection) from the head and thorax (salivary gland infection) section. The abdominal segment for each mosquito was classed as blood meal fed or unfed, but midgut luminal and surface domains were not separated. The mosquito sections were transferred into separate 1.5-mL microcentrifuge tubes and individually subjected to a simplified (2) Chelex protocol for *P. falciparum* DNA extraction. Briefly, each mosquito section was ground in 100 μL of 1× PBS and 1% wt/vol saponin solution, by using a 200-μL pipette tip. After incubating for 20 min at room temperature, the homogenate was spun for 1 min at 20,000 × *g*. The pellet was suspended in 100 μL of PBS, spun for 1 min at 20,000 × *g*, and again pelleted. After adding 75 μL of deionized water and a 25-μL suspension of 20% wt/vol Chelex-100 in deionized water, the pellet was resuspended by gentle vortexing for 3 s and boiled for 13 min. After 2-min spin at 20,000 × *g*, the supernatant was transferred into a sterile-labeled 1.5-mL storage vial and used as template for PCR or stored at −20 °C.

Assays for Antifolate Drug Resistance Alleles. Samples were subjected to two rounds of PCR amplification by using primers flanking antifolate resistance-conferring mutations on the *P. falciparum* DHFR domain, as described (3). Primers M1 and M5,

M1 (5′-TTTATGATGGAACAAGTCTGC-3′)
M5 (5′-AGTATATACATCGCTAACAGA-3′),

were used in the primary round, followed by two separate sets of secondary amplifications with primers:

M3 (5′-TTTATGATGGAACAAGTCTGCGACGTT-3′)
F/ (5′-AAATTCTTGATAAACAACGGAAACCTTTTA-3′)
F (5′-GAAATGTAATTCCCTAGATATGGAATATT-3′)
M4 (5′-TTAATTTCCCAAGTAAAACTATTAGAGCTTC-3′).

Primary round amplifications comprised 2-μL template, 0.25 μM primers, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1× PCR buffer, and 1.0 U of Taq in 25-μL reactions. Secondary amplifications had 4-μL template, 0.25 μM primers, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1× PCR buffer, and 2.0 U of Taq in 50-μL reactions. Amplification cycles for both reactions comprised an initial denaturation step at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 45 seconds, annealing at 43.4 °C for 45 s, and extension at 65 °C for 1 min. A final extension was run at 65 °C for 2 min.

The two sets of secondary (M3-F/ and F-M4) amplicon were digested with 10 different allele-specific restriction enzymes to detect antifolate resistance-associated *Pf*DHFR mutations (3). Restriction fragments were analyzed by electrophoresis on ethidium bromide-stained 2% agarose gels and visualized by UV transillumination. The restriction fragments were sized by using a Kodak EDAS 290D gel documentation system.

1. Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE (1995) Pyrimethamine and proguanil resistance-conferring mutations in Plasmodium falciparum dihydrofolate reductase: Polymerase chain reaction methods for surveillance in Africa. *Am J Trop Med Hyg* 52:565–568.
2. Musapa M, et al. (2011) A simple chelex protocol for DNA extraction from Anopheles spp. *J Visualized Exp Immunol Infect*, in press.
3. Duraisingh MT, Curtis J, Warhurst DC (1998) *Plasmodium falciparum*: Detection of polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes by PCR and restriction digestion. *Exp Parasitol* 89:1–8.