Am. J. Trop. Med. Hyg., 80(4), 2009, pp. 555–558
Copyright © 2009 by The American Society of Tropical Medicine and Hygiene

Short Report: Resistance-mediating Polymorphisms in *Plasmodium falciparum* Infections in Kinshasa, Democratic Republic of the Congo

Linda Mobula, Bruce Lilley, Antoinette K. Tshefu, and Philip J. Rosenthal*

Department of Medicine, San Francisco General Hospital, University of California, San Francisco, California; Ecole de Santé Publique, Faculté de Medecine, Université de Kinshasa, Republique Démocratique du Congo

Abstract. Genetic polymorphisms in *Plasmodium falciparum* are associated with resistance to a number of drugs, but data on their prevalence are limited from many areas. We explored the prevalence of key polymorphisms in patients presenting with malaria in Kinshasa. Prevalences of *pfcrt* K76T; *pfmdr1* N86Y; *pfdhfr* N51I, C59R, and S108N; and *pfdhps* A437G were well above 50% and of *pfmdr1* Y184F, N1042D, and D1246Y; *pfdhfr* I164L; and *pfdhps* K540E were low. These results suggest an intermediate level of resistance to aminoquinoline and antifolate antimalarials in Kinshasa compared with other areas of Africa.

Resistance of *Plasmodium falciparum* to commonly used antimalarial drugs is a growing problem in Africa.[1] In general, resistance is most severe in east and southern, compared with west Africa. Resistance to aminoquinolines, especially chloroquine (CQ), is widespread, and CQ is no longer recommended to treat malaria in sub-Saharan Africa. Resistance to amodiaquine (AQ), a related aminoquinoline, is less common, although its antimalarial efficacy is unsatisfactory in many areas. Resistance to antifolates, notably sulfadoxine-pyrimethamine (SP), is increasing. With increasing resistance to older drugs, highly efficacious artemisinin-based combination therapies (ACTs) are now the recommended first-line antimalarials in nearly all countries in sub-Saharan Africa.[2]

Despite the move to newer regimens, it remains important to assess resistance to older antimalarial agents for a number of reasons. First, many episodes of malaria continue to be treated with CQ, AQ, or SP, because transition to ACTs has been slow. Second, AQ and SP are included in ACTs (in both cases combined with artesunate) currently recommended by the World Health Organization (WHO). Third, one older combination regimen, AQ/SP, is recommended by the WHO to treat malaria when ACTs are not available, and this regimen remains highly efficacious in some areas, particularly parts of west Africa.[3] Fourth, use of CQ and related drugs may be indicated in the future if the prevalence of resistance diminishes after removal of selective pressure, as has occurred in Malawi.[4] Fifth, antifolates are widely used in Africa to prevent infections, including intermittent doses of SP to prevent malaria in children and pregnant women[5] and trimethoprim-sulfamethoxazole to prevent opportunistic infections, including malaria, in HIV-infected individuals.[6]

Mechanisms of resistance of *P. falciparum* to aminoquinolines and antifolates are fairly well understood. Resistance to CQ is mediated principally by the 76T mutation in *pfcrt*, which encodes a putative transporter.[7] Mutations in *pfmdr1*, which encodes another putative transporter, may contribute to resistance to CQ and seem to play a greater role in resistance to AQ.[8,9] Resistance to SP is mediated by a series of mutations in *pfdhfr* and *pfdhps*, the genes encoding dihydrofolate reductase and dihydropteroate synthase, the two enzyme targets of this combination, with both stepwise progression of mutations and selective sweeps of resistant parasites contributing to drug resistance.[10]

Recent standardization of methods for the characterization of antimalarial treatment outcomes and identification of resistance-mediating polymorphisms has streamlined the characterization of drug resistance in Africa. However, information on resistance is spotty, with some parts of Africa poorly represented. One such area is the western Democratic Republic of the Congo (DRC), including Kinshasa, the third largest city in Africa, with a population of 8 million, which is the third largest city in Africa. In addition to the obvious importance of characterizing resistance in a major city, an understanding of malaria in Kinshasa will help us to appreciate the geographical flux of resistance between the very high levels recorded across east Africa and much lower levels seen in some areas of west Africa. To this end, we evaluated molecular markers of resistance to aminoquinoline and antifolate antimalarials in Kinshasa.

We evaluated a convenience sample of children 1–10 years of age at five clinics in Kinshasa (Center Hospitalier de Mont Amba, Center de Santé de Kindele, Center Pédiatrique de Kalembelembe, Center Hospitalier de Kingasani and Clinique Riviera) in March–April 2008. Children presenting with acute febrile illnesses received standard evaluations including Giemsa-stained blood smears. When uncomplicated malaria was diagnosed, parents or guardians of patients were asked to participate in this study. Selection criteria for our study were diagnosis of microscopy-proven uncomplicated malaria by the health center and provision of informed consent by the parent or guardian. Exclusion criteria were evidence or clinical suspicion of complicated malaria, as defined by the WHO.[11] Patients were managed for malaria following standard clinic protocols. With enrollment, a short questionnaire concerning prior use of antimalarials was completed, and blood was collected by finger prick for thin and thick blood smear and collection of blood spots on filter paper (Whatman 3MM). Blood smears were stained with 10% Giemsa for 10 minutes and examined by a trained microscopist. Filter paper samples were labeled and stored with desiccant at room temperature. Subsequently, DNA was extracted from filter paper with chelex,[12] and *P. falciparum* polymorphisms of interest were assessed by nested amplification of genes of interest, sequence-specific restriction endonuclease digestion, separation of DNA fragments by agarose gel electrophoresis, and visual characterization of DNA digestion patterns, with minor modifications of methods that have previously been described.[13,14] The study was approved by the Ethics Committee at the Kinshasa School

* Address correspondence to Philip J. Rosenthal, Department of Medicine, Box 0811, University of California, San Francisco, CA 94143. E-mail: prosenthal@medsfgh.ucsf.edu

of Public Health and the Committee for Human Research of the University of California, San Francisco, CA.

A total of 142 children with a mean age of 3.5 years were referred for the study. For 55 of these subjects, prior use of antimalarial treatment was reported by caregivers. This treatment was reported to be quinine in 38 (27% of study children), artesunate plus quinine in 4 (2.8%), other standard therapies in 7 (4.9%), and iron or traditional remedies in 6 (4.2%). Thus, antimalarial treatment before clinic presentation was common, and the drug most frequently reported was quinine, which is a standard therapy for severe, but not uncomplicated, malaria. No subjects reported prior use of artesunate/AQ, the recommended therapy to treat uncomplicated malaria in DRC. However, it is important to note that our findings do not provide a reliable gauge of presumptive antimalarial therapy in Kinshasa, because caretaker reports may have been inaccurate and because those who received effective initial therapy would not be expected to present to clinics with fever. Nonetheless, the results highlight heavy reliance on quinine for the treatment of uncomplicated malaria, a strategy that will likely be limited by poor tolerance and poor compliance with the full 7-day regimen.

The diagnosis of falciparum malaria was confirmed by repeat microscopy and polymerase chain reaction (PCR) in 121 study children. For the other 21 children, in nearly all cases, both the follow-up blood smear and PCR were negative, indicating a false-positive initial smear reading. *P. falciparum* polymorphisms associated with altered responses to aminoquinolines (*pfcrt* and *pfmdr1*) and antifolates (*pfdhfr* and *pfdhps*) were assessed (Table 1). Less then 121 outcomes were available for each polymorphism because of occasional failure of PCR reactions (despite repeat assays with increased template) and because, for the *pfdhfr* 164 polymorphism, analysis was stopped after the first 87 samples were all wild type. These results fill a gap in our appreciation of the map of drug resistance in Africa. In general, the prevalence of multiple resistance-mediating polymorphisms is highest in much of east and southern Africa and lowest in parts of west and central Africa (Figure 1). Consideration of key resistance mediating polymorphisms in Africa can be simplified to four sets of mutations. First, the key marker of CQ resistance is *pfcrt* 76T. This mutation is now common throughout sub-Saharan Africa, except in regions (primarily Malawi) where elimination of CQ use has allowed

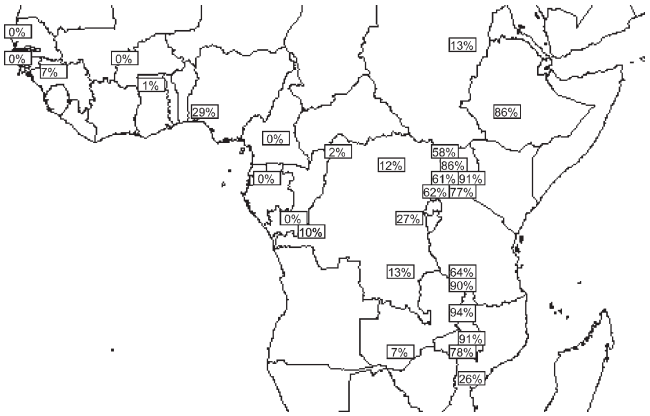

FIGURE 1. Map of prevalence of *pfdhfr/pfdhps* quintuple mutation in Africa. Results from representative evaluable studies performed since 2000 are shown. Results are from our study (10%) and for one or more sites in Burkina Faso,[3] Cameroon,[22] Republic of the Congo,[23] DRC,[24,25] Ethiopia,[26] Gabon,[27,28] Ghana,[29] Guinea,[30] Guinea-Bissau,[31] Malawi,[32,33] Mozambique,[34] Nigeria,[35] Senegal,[36] Sudan,[37] Tanzania,[38] Uganda,[39] and Zambia.[40]

wild-type parasites to replace resistant mutants.[4] Mutant parasites were common in Kinshasa, although prevalence of the 76T mutation was below that seen in east Africa, where it is commonly 100%. Second, the 86Y mutation in *pfmdr1* mediates decreased sensitivity to aminoquinolines, but interestingly, increased sensitivity to mefloquine, halofantrine, and quinine.[15] Other *pfmdr1* polymorphisms, including 184F, 1034C, 1042D, and 1246Y, may contribute to altered drug sensitivity.[16] Parasites from Kinshasa showed intermediate prevalence of *pfmdr1* 86Y, 184F, and 1246Y compared with sites with higher prevalence in east Africa and generally lower prevalence in west Africa. Third, considering antifolate resistance, two *pfdhfr* mutations (108N and 51I) and one *pfdhps* mutation (437G) are common in most areas, but not predictive of SP treatment outcomes. The key mediators of resistance seem to be *pfdhfr* 59R and *pfdhps* 540E, with both of these mutations needed for significant loss of SP treatment efficacy.[10,17] This conclusion is supported by the poor efficacy of SP in recent years at many locations in east Africa,[18] where prevalence of all five relevant mutations (the quintuple mutation) is common, and by continued good efficacy of SP in parts of west Africa where one relevant mutation, *pfdhps* 540E, is generally absent.[19] In Kinshasa, four of the five relevant mutations are very common, but *pfdhps* 540E is uncommon, although more prevalent than in countries farther to the west. Fourth, high-level antifolate resistance is mediated in Asia and South America by an additional mutation, *pfdhfr* 164L. This mutation has generally been rare in Africa, although modest prevalence has been noted recently in a few areas.[20,21] The *pfdhfr* 164L mutation was not identified in any parasites from Kinshasa.

In summary, *P. falciparum* causing symptomatic malaria in Kinshasa commonly contained mutations that mediate resistance to aminoquinoline and antifolate antimalarials. The results predict an intermediate level of drug resistance between the very high levels seen in east Africa and lower levels in parts of west Africa. Specifically, the results suggest poor antimalarial activity of CQ, uncertain efficacy of AQ, but fairly good efficacy for SP. The moderate prevalence of key *pfmdr1* polymorphisms might suggest concern regarding the efficacy of aminoquinoline-containing ACTs (artesunate/AQ and

TABLE 1

P. falciparum genetic polymorphisms identified in samples from Kinshasa

Gene	Polymorphism	Number studied*	Wild type†	Mixed†	Mutant†	Mixed and mutant (%)
pfcrt	K76T	105	17	11	77	88 (83.8)
pfmdr1	N86Y	102	34	21	47	68 (66.7)
	Y184F	102	66	9	27	36 (35.3)
	S1034C	104	104	0	0	0
	N1042D	104	104	0	0	0
	D1246Y	103	79	8	16	24 (23.3)
pfdhfr	S108N	113	1	0	112	112 (99.1)
	N51I	96	2	1	93	94 (97.9)
	C59R	114	22	20	72	92 (80.7)
	I164L	87	87	0	0	0
pfdhps	A437G	102	7	4	91	95 (93.1)
	K540E	105	95	6	4	10 (9.5)

* Numbers studied varied, because some reactions did not lead to evaluable products.

† Sequences were categorized as wild type, mutant, or mixed (both wild-type and mutant digestion products seen) based on comparison of DNA digestion patterns with control DNA from strains with known sequences.

dihydroartemisinin/piperaquine), although it remains unclear if these polymorphisms will affect ACT treatment outcomes. These results further suggest that SP or trimethoprim-sulfamethoxazole will remain efficacious in Kinshasa to prevent malaria. However, the genetics of parasite populations can change quickly under heavy drug pressure, and therefore continued surveillance of resistance mediating polymorphisms and, ideally, drug efficacy results from clinical trials, will be needed to best assess the utility of different antimalarial treatment and preventive regimens in Kinshasa over time.

Received October 31, 2008. Accepted for publication December 21, 2008.

Acknowledgments: The authors thank Pius Mafuta, Dr. Edouard Mayimbi, Dr. Jack Kokolomami, Dr. Michel Kaya, Bicko Makubikwa Mamengi, Grec Kiloto Kakoma, and laboratory technicians at Clinic Riviera and at Kalembelembe. We also thank the children and their parents or guardians who participated in this study.

Financial support: This work was supported by the Doris Duke Charitable Foundation, of which PJR is a Distinguished Clinical Scientist.

Authors' addresses: Linda Mobula, Bruce Lilley, and Philip J. Rosenthal, Department of Medicine, Box 0811, University of California, San Francisco, CA 94143. Antoinette K. Tshefu, Ecole de Santé Publique, Faculté de Medecine, Université de Kinshasa, B.P. 11850 KIN I, Kinshasa, République Democratique du Congo.

Reprint requests: Philip J. Rosenthal, Department of Medicine, Box 0811, University of California, San Francisco, CA 94143.

REFERENCES

1. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR, 2002. Epidemiology of drug-resistant malaria. *Lancet Infect Dis 2:* 209–218.
2. Nosten F, White NJ, 2007. Artemisinin-based combination treatment of falciparum malaria. *Am J Trop Med Hyg 77:* 181–192.
3. Zongo I, Dorsey G, Rouamba N, Tinto H, Dokomajilar C, Guiguemde RT, Rosenthal PJ, Ouedraogo JB, 2007. Artemether-lumefantrine versus amodiaquine plus sulfadoxine-pyrimethamine for uncomplicated falciparum malaria in Burkina Faso: a randomised non-inferiority trial. *Lancet 369:* 491–498.
4. Laufer MK, Thesing PC, Eddington ND, Masonga R, Dzinjalamala FK, Takala SL, Taylor TE, Plowe CV, 2006. Return of chloroquine antimalarial efficacy in Malawi. *N Engl J Med 355:* 1959–1966.
5. ter Kuile FO, van Eijk AM, Filler SJ, 2007. Effect of sulfadoxine-pyrimethamine resistance on the efficacy of intermittent preventive therapy for malaria control during pregnancy: a systematic review. *JAMA 297:* 2603–2616.
6. Kamya MR, Gasasira AF, Achan J, Mebrahtu T, Ruel T, Kekitiinwa A, Charlebois ED, Rosenthal PJ, Havlir D, Dorsey G, 2007. Effects of trimethoprim-sulfamethoxazole and insecticide-treated bednets on malaria among HIV-infected Ugandan children. *AIDS 21:* 2059–2066.
7. Sidhu AB, Verdier-Pinard D, Fidock DA, 2002. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfcrt mutations. *Science 298:* 210–213.
8. Happi CT, Gbotosho GO, Folarin OA, Bolaji OM, Sowunmi A, Kyle DE, Milhous W, Wirth DF, Oduola AM, 2006. Association between mutations in *Plasmodium falciparum* chloroquine resistance transporter and *P. falciparum* multidrug resistance 1 genes and *in vivo* amodiaquine resistance in *P. falciparum* malaria-infected children in Nigeria. *Am J Trop Med Hyg 75:* 155–161.
9. Dokomajilar C, Lankoande ZM, Dorsey G, Zongo I, Ouedraogo JB, Rosenthal PJ, 2006. Roles of specific *Plasmodium falciparum* mutations in resistance to amodiaquine and sulfadoxine-pyrimethamine in Burkina Faso. *Am J Trop Med Hyg 75:* 162–165.
10. Gregson A, Plowe CV, 2005. Mechanisms of resistance of malaria parasites to antifolates. *Pharmacol Rev 57:* 117–145.
11. World Health Organization, 2000. Severe falciparum malaria. World Health Organization, Communicable Diseases Cluster. *Trans R Soc Trop Med Hyg 94 (Suppl 1):* S1–S90.
12. 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.
13. 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.
14. Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC, 2000. The tyrosine-86 allele of the pfmdr1 gene of *Plasmodium falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. *Mol Biochem Parasitol 108:* 13–23.
15. Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, Kawamoto F, Miller RS, Meshnick SR, 2003. Resistance to antimalarials in southeast Asia and genetic polymorphisms in pfmdr1. *Antimicrob Agents Chemother 47:* 2418–2423.
16. Woodrow CJ, Krishna S, 2006. Antimalarial drugs: recent advances in molecular determinants of resistance and their clinical significance. *Cell Mol Life Sci 63:* 1586–1596.
17. Dorsey G, Dokomajilar C, Kiggundu M, Staedke SG, Kamya MR, Rosenthal PJ, 2004. Principal role of dihydropteroate synthase mutations in mediating resistance to sulfadoxine-pyrimethamine in single-drug and combination therapy of uncomplicated malaria in Uganda. *Am J Trop Med Hyg 71:* 758–763.
18. Staedke SG, Mpimbaza A, Kamya MR, Nzarubara BK, Dorsey G, Rosenthal PJ, 2004. Combination treatments for uncomplicated falciparum malaria in Kampala, Uganda: randomised clinical trial. *Lancet 364:* 1950–1957.
19. Zongo I, Dorsey G, Rouamba N, Dokomajilar C, Lankoande M, Ouedraogo JB, Rosenthal PJ, 2005. Amodiaquine, sulfadoxine-pyrimethamine, and combination therapy for uncomplicated falciparum malaria: a randomized controlled trial from Burkina Faso. *Am J Trop Med Hyg 73:* 826–832.
20. Lynch C, Pearce R, Pota H, Cox J, Abeku TA, Rwakimari J, Naidoo I, Tibenderana J, Roper C, 2008. Emergence of a dhfr mutation conferring high-level drug resistance in *Plasmodium falciparum* populations from southwest Uganda. *J Infect Dis 197:* 1598–1604.
21. Hamel MJ, Poe A, Bloland P, McCollum A, Zhou Z, Shi YP, Ouma P, Otieno K, Vulule J, Escalante A, Udhayakumar V, Slutsker L, 2008. Dihydrofolate reductase I164L mutations in *Plasmodium falciparum* isolates: clinical outcome of 14 Kenyan adults infected with parasites harbouring the I164L mutation. *Trans R Soc Trop Med Hyg 102:* 338–345.
22. Tahar R, Basco LK, 2007. Molecular epidemiology of malaria in Cameroon. XXVII. Clinical and parasitological response to sulfadoxine-pyrimethamine treatment and *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase alleles in Cameroonian children. *Acta Trop 103:* 81–89.
23. Ndounga M, Tahar R, Basco LK, Casimiro PN, Malonga DA, Ntoumi F, 2007. Therapeutic efficacy of sulfadoxine-pyrimethamine and the prevalence of molecular markers of resistance in under 5-year olds in Brazzaville, Congo. *Trop Med Int Health 12:* 1164–1171.
24. Cohuet S, Bonnet M, Van Herp M, Van Overmeir C, D'Alessandro U, Guthmann JP, 2006. Short report: molecular markers associated with *Plasmodium falciparum* resistance to sulfadoxine-pyrimethamine in the Democratic Republic of Congo. *Am J Trop Med Hyg 75:* 152–154.
25. Swarthout TD, van den Broek IV, Kayembe G, Montgomery J, Pota H, Roper C, 2006. Artesunate + amodiaquine and artesunate + sulphadoxine-pyrimethamine for treatment of uncomplicated malaria in Democratic Republic of Congo: a clinical trial with determination of sulphadoxine and pyrimethamine-resistant haplotypes. *Trop Med Int Health 11:* 1503–1511.
26. Schunk M, Kumma WP, Miranda IB, Osman ME, Roewer S, Alano A, Loscher T, Bienzle U, Mockenhaupt FP, 2006. High

prevalence of drug-resistance mutations in *Plasmodium falciparum* and *Plasmodium vivax* in southern Ethiopia. *Malar J 5:* 54.
27. Aubouy A, Jafari S, Huart V, Migot-Nabias F, Mayombo J, Durand R, Bakary M, Le Bras J, Deloron P, 2003. DHFR and DHPS genotypes of *Plasmodium falciparum* isolates from Gabon correlate with *in vitro* activity of pyrimethamine and cycloguanil, but not with sulfadoxine-pyrimethamine treatment efficacy. *J Antimicrob Chemother 52:* 43–49.
28. Nsimba B, Guiyedi V, Mabika-Mamfoumbi M, Mourou-Mbina JR, Ngoungou E, Bouyou-Akotet M, Loembet R, Durand R, Le Bras J, Kombila M, 2008. Sulphadoxine/pyrimethamine versus amodiaquine for treating uncomplicated childhood malaria in Gabon: a randomized trial to guide national policy. *Malar J 7:* 31.
29. Mockenhaupt FP, Teun Bousema J, Eggelte TA, Schreiber J, Ehrhardt S, Wassilew N, Otchwemah RN, Sauerwein RW, Bienzle U, 2005. *Plasmodium falciparum* dhfr but not dhps mutations associated with sulphadoxine-pyrimethamine treatment failure and gametocyte carriage in northern Ghana. *Trop Med Int Health 10:* 901–908.
30. Bonnet M, Roper C, Felix M, Coulibaly L, Kankolongo GM, Guthmann JP, 2007. Efficacy of antimalarial treatment in Guinea: *in vivo* study of two artemisinin combination therapies in Dabola and molecular markers of resistance to sulphadoxine-pyrimethamine in N'Zerekore. *Malar J 6:* 54.
31. Kofoed PE, Alfrangis M, Poulsen A, Rodrigues A, Gjedde SB, Ronn A, Rombo L, 2004. Genetic markers of resistance to pyrimethamine and sulfonamides in *Plasmodium falciparum* parasites compared with the resistance patterns in isolates of *Escherichia coli* from the same children in Guinea-Bissau. *Trop Med Int Health 9:* 171–177.
32. Nkhoma S, Molyneux M, Ward S, 2007. Molecular surveillance for drug-resistant *Plasmodium falciparum* malaria in Malawi. *Acta Trop 102:* 138–142.
33. Bwijo B, Kaneko A, Takechi M, Zungu IL, Moriyama Y, Lum JK, Tsukahara T, Mita T, Takahashi N, Bergqvist Y, Bjorkman A, Kobayakawa T, 2003. High prevalence of quintuple mutant dhps/dhfr genes in *Plasmodium falciparum* infections seven years after introduction of sulfadoxine and pyrimethamine as first line treatment in Malawi. *Acta Trop 85:* 363–373.
34. Enosse S, Magnussen P, Abacassamo F, Gomez-Olive X, Ronn AM, Thompson R, Alifrangis M, 2008. Rapid increase of *Plasmodium falciparum* dhfr/dhps resistant haplotypes, after the adoption of sulphadoxine-pyrimethamine as first line treatment in 2002, in southern Mozambique. *Malar J 7:* 115.
35. Happi CT, Gbotosho GO, Folarin OA, Akinboye DO, Yusuf BO, Ebong OO, Sowunmi A, Kyle DE, Milhous W, Wirth DF, Oduola AM, 2005. Polymorphisms in *Plasmodium falciparum* dhfr and dhps genes and age related *in vivo* sulfadoxine-pyrimethamine resistance in malaria-infected patients from Nigeria. *Acta Trop 95:* 183–193.
36. Henry M, Diallo I, Bordes J, Ka S, Pradines B, Diatta B, M'Baye PS, Sane M, Thiam M, Gueye PM, Wade B, Touze JE, Debonne JM, Rogier C, Fusai T, 2006. Urban malaria in Dakar, Senegal: chemosusceptibility and genetic diversity of *Plasmodium falciparum* isolates. *Am J Trop Med Hyg 75:* 146–151.
37. A-Elbasit IE, Alifrangis M, Khalil IF, Bygbjerg IC, Masuadi EM, Elbashir MI, Giha HA, 2007. The implication of dihydrofolate reductase and dihydropteroate synthetase gene mutations in modification of *Plasmodium falciparum* characteristics. *Malar J 6:* 108.
38. Schonfeld M, Barreto Miranda I, Schunk M, Maduhu I, Maboko L, Hoelscher M, Berens-Riha N, Kitua A, Loscher T, 2007. Molecular surveillance of drug-resistance associated mutations of *Plasmodium falciparum* in south-west Tanzania. *Malar J 6:* 2.
39. Francis D, Nsobya SL, Talisuna A, Yeka A, Kamya MR, Machekano R, Dokomajilar C, Rosenthal PJ, Dorsey G, 2006. Geographic differences in antimalarial drug efficacy in Uganda are explained by differences in endemicity and not by known molecular markers of drug resistance. *J Infect Dis 193:* 978–986.
40. Mkulama MA, Chishimba S, Sikalima J, Rouse P, Thuma PE, Mharakurwa S, 2008. Escalating *Plasmodium falciparum* antifolate drug resistance mutations in Macha, rural Zambia. *Malar J 7:* 87.