AAC Journals.ASM.org

Molecular Characterization of the Cytochrome *b* Gene and *In Vitro* Atovaquone Susceptibility of *Plasmodium falciparum* Isolates from Kenya

Luicer A. Ingasia,[a] Hoseah M. Akala,[a] Mabel O. Imbuga,[b] Benjamin H. Opot,[a] Fredrick L. Eyase,[a] Jacob D. Johnson,[a] Wallace D. Bulimo,[a] Edwin Kamau[a]

Department of Emerging Infectious Diseases-Global Emerging Infections Surveillance and Response System (DEID-GEIS) Program, U.S. Army Medical Research Unit—Kenya (USAMRU-K), Kenya Medical Research Institute (KEMRI)-Walter Reed Project, Kisumu, Kenya[a]; Department of Biochemistry, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya[b]

The prevalence of a genetic polymorphism(s) at codon 268 in the cytochrome *b* gene, which is associated with failure of atovaquone-proguanil treatment, was analyzed in 227 *Plasmodium falciparum* parasites from western Kenya. The prevalence of the wild-type allele was 63%, and that of the Y268S (denoting a Y-to-S change at position 268) mutant allele was 2%. There were no pure Y268C or Y268N mutant alleles, only mixtures of a mutant allele(s) with the wild type. There was a correlation between parasite 50% inhibitory concentration (IC_{50}) and parasite genetic polymorphism; mutant alleles had higher IC_{50}s than the wild type.

Atovaquone-proguanil (AP) is a fixed-dose combination antimalarial drug, mostly used for treatment and chemoprophylaxis of falciparum malaria for international travelers (1). Use of atovaquone alone leads to high rates of treatment recrudescence (2), which is attributed to mutations in the cytochrome *b* gene (*pfcytb*) (3). *Plasmodium falciparum* atovaquone-resistant isolates have been described following atovaquone or AP treatment failures (4–11) and *in vitro* drug susceptibility testing (5, 8, 10, 12). *In vitro* and *in vivo* resistance to atovaquone has been associated with point mutations at codon 268 in *pfcytb* (5, 9, 10, 13). These mutations include Y268S (denoting the Y-to-S change at position 268), Y268N, and Y268C (5, 9, 11, 13, 14) and can induce a >1,000-fold increase in atovaquone 50% inhibitory concentration (IC_{50}) (13, 15). There are cases of AP treatment failure for travelers returning from Africa (4-6, 9-11, 16-19) and appearance of *pfcytb* mutations following AP treatment (4-6, 9-11, 14). However, treatment failure is not always associated with a known *pfcytb* mutation (17, 18, 20, 21), indicating that other factors such as genetic polymorphisms in other genetic loci play a role in AP resistance.

Kenya has a large number of international travelers and foreign residents who use AP for malaria prophylaxis. Additionally, AP is one of the second-line treatment options for uncomplicated malaria (22). In this study, a baseline epidemiological surveillance study was conducted to determine the prevalence of a genetic polymorphism(s) at codon 268 of *pfcytb* in Kenyan *P. falciparum* parasites. Field clinical isolates from an ongoing approved malaria epidemiological surveillance protocol (KEMRI SSC document 1330 and WRAIR document 1384), collected between 2008 and 2012 from three locations in Kenya (Kisumu, Kisii, and Kericho), were randomly selected for inclusion in the study. Kisumu is a lowland where malaria is endemic, with highly stable transmission, whereas Kisii and Kericho are highlands, with unstable transmission (23). Sample collection and preparation were performed as previously described (24). Genomic DNA from whole blood was extracted using a Qiagen DNA minikit (Qiagen, Valencia, CA) as recommended by the manufacturer. Clinical isolates were culture adapted before being subjected to the SYBR green I assay as previously described (25). A total of 227 (167 from Kisumu, 37 from Kisii, and 23 from Kericho) samples were successfully analyzed by PCR-restriction fragment length polymorphism (RFLP) at codon 268 as previously described (10), and a subset ($n = 68$) of the samples was sequenced to confirm PCR-RFLP results using an ABI Prism 3500xL genetic analyzer (Applied Biosystems, Foster City, CA) as previously described (10). Reference strain sequences were used to score the genotype.

Data revealed that none of the samples carried a pure Y268C or Y268N mutant allele. Sixty-three percent of the isolates carried the wild-type (WT) allele, and 2% carried the Y268S mutant allele. Thirty-three percent had a double mixed genotype (WT/Y268S), and 3% had a triple mixed genotype (WT/Y268S/Y268N). Interestingly, 100% (4 of 4) of Y268S mutant alleles, 95% (70 of 74) of samples with the double mixed genotype, and 100% (6 of 6) of samples with the triple mixed genotype were found in Kisumu parasites. The remaining 5% (4 of 74) of samples with the double mixed genotype were from Kisii. None of the samples with mutant or mixed genotypes were from Kericho. The frequency of parasite genotypes in samples collected from 2008 to 2012 was analyzed. There were significant fluctuations ($P < 0.0001$; chi-square test) in frequency of isolates carrying the WT allele from year to year, with the highest frequency of the WT allele present in samples collected in 2010 (82%) and the lowest in samples collected in 2009 (37%). The Y268S mutant allele was not present in samples collected in 2008 and 2009 but emerged in samples collected in 2010 to 2012, albeit at a low frequency. Similarly, the WT/Y268S mixed genotype showed significant fluctuations in frequency

Received 24 July 2014 **Returned for modification** 2 September 2014
Accepted 4 January 2015

Accepted manuscript posted online 12 January 2015

Citation Ingasia LA, Akala HM, Imbuga MO, Opot BH, Eyase FL, Johnson JD, Bulimo WD, Kamau E. 2015. Molecular characterization of the cytochrome *b* gene and *in vitro* atovaquone susceptibility of *Plasmodium falciparum* isolates from Kenya. Antimicrob Agents Chemother 59:1818–1821. doi:10.1128/AAC.03956-14.

Address correspondence to Edwin Kamau, edwin.kamau.mil@mail.mil.

doi:10.1128/AAC.03956-14

TABLE 1 Different genotypes of parasites collected over a 5-year period

Genotype[a]	No. (%) of parasites collected in:				
	2008	2009	2010	2011	2012
Y	8 (67)	20 (37)	75 (82)	18 (56)	22 (59)
S	0	0	2 (2)	1 (3)	1 (3)
Y/S	3 (25)	32 (59)	14 (15)	11 (34)	14 (38)
Y/N/S	1 (8)	2 (4)	1 (1)	2 (6)	0
Total	12	54	92	32	37

[a] Y, genotype with tyrosine encoded by codon 268 (wild type); S, genotype with serine encoded by codon 268 (pure mutant); Y/S, double mixed genotype; Y/N/S, triple mixed genotype, where N is asparagine, encoded by a mutant allele. The total number samples analyzed for the study was 227, for both molecular and *in vitro* analyses.

from year to year ($P < 0.0001$; chi-square test), with the highest frequency present in 2009 samples (59%) and the lowest in 2010 samples (15%) (Table 1).

Figure 1 shows the atovaquone IC_{50}s for parasites collected from 2008 to 2012. The median IC_{50} (interquartile range [IQR]) for the control reference strain, 3D7, was 1.68 nM (1.09 to 2.70 nM). The median (IQR) IC_{50} for all isolates in this study was 3.5 nM (1.4 to 8.4 nM). The IC_{50} cutoff points used for sensitive and resistant parasites were set at <30 nM and >1,900 nM, respectively (8). Based on these criteria, only 1.3% isolates had high IC_{50}s, between 1,121 nM and 2,250 nM. Comparison of the median IC_{50}s from year to year revealed that there was a significant difference ($P = 0.0003$; Kruskal-Wallis H statistics) (Fig. 1). Further, Dunn's multiple comparison posttest revealed significant differences in median IC_{50} for 2008 versus 2010 samples, 2008 versus 2011 samples, and 2009 versus 2011 samples. However, these data have to be interpreted with caution because of the small sample size for 2008. Overall, the data showed that there was fluctuation of IC_{50} throughout the study period.

The median (IQR) IC_{50} for parasites carrying the WT allele was 3.0 nM (1.0 to 6.9 nM), whereas the median (IQR) IC_{50} for the parasites carrying the pure Y268S mutant was 5.7 nM (1.7 to 1,216 nM). The median (IQR) IC_{50} for parasites carrying the WT/Y268S mixture was 4.7 nM (2.2 to 11.1 nM), whereas the median (IQR) IC_{50} for parasites carrying the WT/Y268S/Y268N mixed genotype was 5.0 nM (2.0 to 11.8 nM). The differences in median IC_{50} for the different parasite genotypes reached statistical significance ($P = 0.0106$; Kruskal-Wallis H statistics).

The current study describes the presence of the Y268S mutant allele in samples collected in Kenya. None of samples carried the pure Y268C or Y268N mutant allele. Interestingly, however, there was a large number of samples carrying the WT/Y268S mixed genotype. The Y268N mutant allele occurred only in mixed genotypes, with these samples carrying the WT/Y268S/Y268N mixed genotype. To the best of our knowledge, this is the first study to describe the presence of mixed genotypes at codon 268 in the *pfcytb* gene carrying the WT allele and one or two mutant alleles.

Mutations in the *pfcytb* gene have been shown to arise *de novo* due to AP selection (9, 11). However, although low in prevalence, these mutations have been shown to occur in parasite populations even in locations where there is no AP pressure (26). AP is minimally used for control of malaria and for treatment of *Pneumocystis jirovecii* pneumonia in patients with HIV infection who cannot tolerate trimethoprim-sulfamethoxazole (27–29). In the current study, 2% of the sample parasites had the Y268S mutant allele. Interestingly, a large number of samples (35%) were the WT/Y268S or WT/Y268S/Y268N mixed genotype, carrying a mutant allele(s); 95% of these samples were from Kisumu and 5% from Kisii, but none were from Kericho. Studies have demonstrated that parasite genomic polymorphisms result in fitness consequences and that parasites will not maintain any polymorphism that is not beneficial (30–32). Further studies will be required to determine whether these mutations would appear in nature at such high proportions without any selection pressure. Given the overwhelming occurrence of these mutations is in Kisumu, a high-transmission region, it will be interesting to investigate if there is any correlation. It will also be critical to determine if these mutations confer any other benefit(s) to parasite survival to warrant such high prevalence. Interestingly, there seems to be a correlation between HIV prevalence and prevalence of mutations at codon 268; Kisumu has the highest HIV prevalence (19.3% of the population), compared to Kisii (8%) and Kericho (3.4%) (33). It is likely that the use of AP or other drugs to control opportunistic infections in HIV-infected populations exerts pressure on the parasite population.

The current study also describes the temporal trends of mutations at codon 268 in *pfcytb* and the IC_{50}s for the samples collected in Kenya between 2008 and 2012. There were fluctuations of genotype and median IC_{50} throughout the study period. Of interest is the emergence of the Y268S mutant allele in the final 3 years of the study. Also, the frequency of mixed genotypes carrying a mutant allele(s) remained high throughout, indicating that the selection pressure remained sustained throughout the study period.

In a study that analyzed the atovaquone *in vitro* susceptibility of isolates from Africa, one sample carrying Y268S, isolated from a patient for which AP treatment failed, had an IC_{50} of 8,230 nM (8).

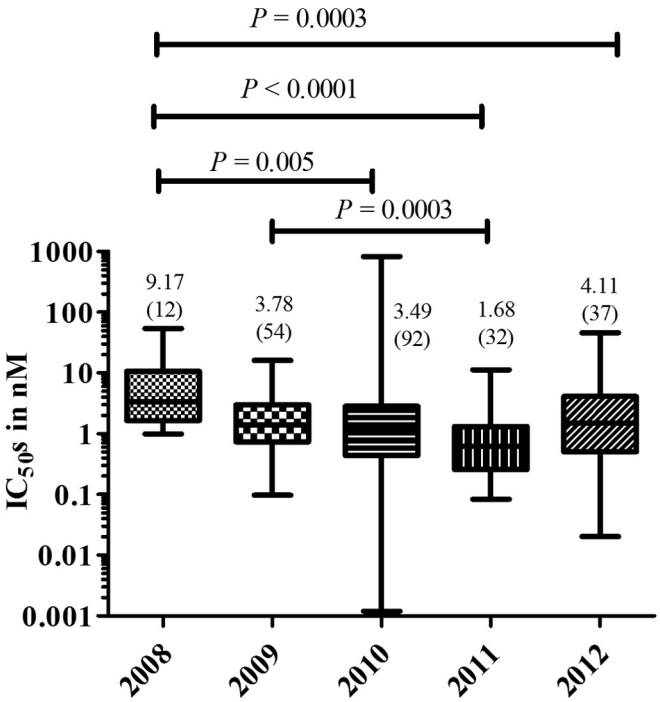

FIG 1 Atovaquone median IC_{50} in nM, indicated above each box plot. The number of isolates analyzed in each year is shown in parentheses. There was a significant decline in median IC_{50}, from 9.17 nM in 2008 to 4.11 nM in 2012.

In another study, the IC_{50} for a parasite with Y268N, isolated from a patient who recrudesced after AP treatment, was 1,888 nM (5). The median IC_{50} for parasites in the current study was 3.5 nM, well within the range previously shown (8, 32). The median IC_{50} for samples carrying the Y268S genotype was 5.7 nM, whereas that for samples carrying the WT/Y268S or WT/Y268S/Y268N mixed genotype was 4.7 nM or 5.0 nM, respectively. Two of the samples with the highest IC_{50}s, 1,618 nM and 2,251 nM, carried the Y268S and WT/Y268S alleles, respectively. Although we did not have patient treatment information for these parasites, high IC_{50} of parasite coupled with mutations in *pfcytb* strongly suggest that these parasites might be resistant to AP.

In conclusion, we have shown that AP resistance-associated mutations are present in Kenyan parasites. The pure mutant (Y268S) exists at a low prevalence, but interestingly, the prevalences of double and triple mixed genotypes are relatively high. These mutations are overwhelmingly prevalent in Kisumu, a high-transmission region. These data are puzzling, given that AP has not been widely used in Kenya for treatment of malaria. More studies are required to further elucidate our findings.

Nucleotide sequence accession numbers. All sequences were deposited in GenBank under accession numbers KP293776 to KP293843.

ACKNOWLEDGMENTS

We thank Duke Omariba, our study coordinator, and all the staff members at the MDR sentinel sites for their contribution in participant recruitment and sample collection. We also thank the director of the Kenya Medical Research Institute for permission to publish this work.

This work was supported by the Global Emerging Infections Surveillance and Response System (GEIS), a division at the Armed Forces Health Surveillance Center (AFHSC).

The opinions and assertions contained herein are the private opinions of the authors and are not to be construed as reflecting the views of the U.S. Army Medical Research Unit—Kenya (USAMRU-K), the Department of the Army, the Department of Defense, or the U.S. Government.

We declare that there are no competing interests.

REFERENCES

1. **Sutherland CJ, Laundy M, Price N, Burke M, Fivelman QL, Pasvol G, Klein JL, Chiodini PL.** 2008. Mutations in the Plasmodium falciparum cytochrome b gene are associated with delayed parasite recrudescence in malaria patients treated with atovaquone-proguanil. Malar J **7:**240. http://dx.doi.org/10.1186/1475-2875-7-240.
2. **Chiodini PL, Conlon CP, Hutchinson DB, Farquhar JA, Hall AP, Peto TE, Birley H, Warrell DA.** 1995. Evaluation of atovaquone in the treatment of patients with uncomplicated Plasmodium falciparum malaria. J Antimicrob Chemother **36:**1073–1078. http://dx.doi.org/10.1093/jac/36.6.1073.
3. **Srivastava IK, Morrisey JM, Darrouzet E, Daldal F, Vaidya AB.** 1999. Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. Mol Microbiol **33:**704–711. http://dx.doi.org/10.1046/j.1365-2958.1999.01515.x.
4. **David KP, Alifrangis M, Salanti A, Vestergaard LS, Ronn A, Bygbjerg IB.** 2003. Atovaquone/proguanil resistance in Africa: a case report. Scand J Infect Dis **35:**897–898. http://dx.doi.org/10.1080/00365540310016862.
5. **Fivelman QL, Butcher GA, Adagu IS, Warhurst DC, Pasvol G.** 2002. Malarone treatment failure and in vitro confirmation of resistance of Plasmodium falciparum isolate from Lagos, Nigeria. Malar J **1:**1. http://dx.doi.org/10.1186/1475-2875-1-1.
6. **Kuhn S, Gill MJ, Kain KC.** 2005. Emergence of atovaquone-proguanil resistance during treatment of Plasmodium falciparum malaria acquired by a non-immune north American traveller to west Africa. Am J Trop Med Hyg **72:**407–409.
7. **Looareesuwan S, Viravan C, Webster HK, Kyle DE, Hutchinson DB, Canfield CJ.** 1996. Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand. Am J Trop Med Hyg **54:**62–66.
8. **Musset L, Pradines B, Parzy D, Durand R, Bigot P, Le Bras J.** 2006. Apparent absence of atovaquone/proguanil resistance in 477 Plasmodium falciparum isolates from untreated French travellers. J Antimicrob Chemother **57:**110–115.
9. **Schwartz E, Bujanover S, Kain KC.** 2003. Genetic confirmation of atovaquone-proguanil-resistant Plasmodium falciparum malaria acquired by a nonimmune traveler to East Africa. Clin Infect Dis **37:**450–451. http://dx.doi.org/10.1086/375599.
10. **Schwobel B, Alifrangis M, Salanti A, Jelinek T.** 2003. Different mutation patterns of atovaquone resistance to Plasmodium falciparum in vitro and in vivo: rapid detection of codon 268 polymorphisms in the cytochrome b as potential in vivo resistance marker. Malar J **2:**5. http://dx.doi.org/10.1186/1475-2875-2-5.
11. **Wichmann O, Muehlberger N, Jelinek T, Alifrangis M, Peyerl-Hoffmann G, Muhlen M, Grobusch MP, Gascon J, Matteelli A, Laferl H, Bisoffi Z, Ehrhardt S, Cuadros J, Hatz C, Gjorup I, McWhinney P, Beran J, da Cunha S, Schulze M, Kollaritsch H, Kern P, Fry G, Richter J.** 2004. Screening for mutations related to atovaquone/proguanil resistance in treatment failures and other imported isolates of Plasmodium falciparum in Europe. J Infect Dis **190:**1541–1546. http://dx.doi.org/10.1086/424469.
12. **Khositnithikul R, Tan-Ariya P, Mungthin M.** 2008. In vitro atovaquone/proguanil susceptibility and characterization of the cytochrome b gene of Plasmodium falciparum from different endemic regions of Thailand. Malar J **7:**23. http://dx.doi.org/10.1186/1475-2875-7-23.
13. **Korsinczky M, Chen N, Kotecka B, Saul A, Rieckmann K, Cheng Q.** 2000. Mutations in Plasmodium falciparum cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. Antimicrob Agents Chemother **44:**2100–2108. http://dx.doi.org/10.1128/AAC.44.8.2100-2108.2000.
14. **Musset L, Bouchaud O, Matheron S, Massias L, Le Bras J.** 2006. Clinical atovaquone-proguanil resistance of Plasmodium falciparum associated with cytochrome b codon 268 mutations. Microbes Infect **8:**2599–2604. http://dx.doi.org/10.1016/j.micinf.2006.07.011.
15. **Fisher N, Abd Majid R, Antoine T, Al-Helal M, Warman AJ, Johnson DJ, Lawrenson AS, Ranson H, O'Neill PM, Ward SA, Biagini GA.** 2012. Cytochrome b mutation Y268S conferring atovaquone resistance phenotype in malaria parasite results in reduced parasite bc1 catalytic turnover and protein expression. J Biol Chem **287:**9731–9741. http://dx.doi.org/10.1074/jbc.M111.324319.
16. **Contentin L, Grammatico-Guillon L, Desoubeaux G, Baron S, Thanh HD.** 2011. Atovaquone-proguanil treatment failure in Plasmodium falciparum. Presse Med **40:**1081–1083. (In French.). http://dx.doi.org/10.1016/j.lpm.2011.03.009.
17. **Durand R, Prendki V, Cailhol J, Hubert V, Ralaimazava P, Massias L, Bouchaud O, Le Bras J.** 2008. Plasmodium falciparum malaria and atovaquone-proguanil treatment failure. Emerg Infect Dis **14:**320–322. http://dx.doi.org/10.3201/eid1402.070945.
18. **Farnert A, Lindberg J, Gil P, Swedberg G, Berqvist Y, Thapar MM, Lindegardh N, Berezcky S, Bjorkman A.** 2003. Evidence of Plasmodium falciparum malaria resistant to atovaquone and proguanil hydrochloride: case reports. BMJ **326:**628–629. http://dx.doi.org/10.1136/bmj.326.7390.628.
19. **Rose GW, Suh KN, Kain KC, Le Saux N, McCarthy AE.** 2008. Atovaquone-proguanil resistance in imported falciparum malaria in a young child. Pediatr Infect Dis J **27:**567–569. http://dx.doi.org/10.1097/INF.0b013e318167918d.
20. **Wichmann O, Muehlen M, Gruss H, Mockenhaupt FP, Suttorp N, Jelinek T.** 2004. Malarone treatment failure not associated with previously described mutations in the cytochrome b gene. Malar J **3:**14. http://dx.doi.org/10.1186/1475-2875-3-14.
21. **Wurtz N, Pascual A, Marin-Jauffre A, Bouchiba H, Benoit N, Desbordes M, Martelloni M, Pommier de Santi V, Richa G, Taudon N, Pradines B, Briolant S.** 2012. Early treatment failure during treatment of Plasmodium falciparum malaria with atovaquone-proguanil in the Republic of Ivory Coast. Malar J **11:**146. http://dx.doi.org/10.1186/1475-2875-11-146.
22. **Prescrire International.** 2002. Atovaquone + proguanil: new preparation. Second-line antimalarial combination. Prescrire Int **11:**131–136.
23. **Wanjala CL, Waitumbi J, Zhou G, Githeko AK.** 2011. Identification of malaria transmission and epidemic hotspots in the western Kenya high-

lands: its application to malaria epidemic prediction. Parasit Vectors **4:**81. http://dx.doi.org/10.1186/1756-3305-4-81.

24. **Eyase FL, Akala HM, Ingasia L, Cheruiyot A, Omondi A, Okudo C, Juma D, Yeda R, Andagalu B, Wanja E, Kamau E, Schnabel D, Bulimo W, Waters NC, Walsh DS, Johnson JD.** 2013. The role of Pfmdr1 and Pfcrt in changing chloroquine, amodiaquine, mefloquine and lumefantrine susceptibility in western-Kenya P. falciparum samples during 2008-2011. PLoS One **8:**e64299. http://dx.doi.org/10.1371/journal.pone.0064299.
25. **Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M.** 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob Agents Chemother **48:**1803–1806. http://dx.doi.org/10.1128/AAC.48.5.1803-1806.2004.
26. **Happi CT, Gbotosho GO, Folarin OA, Milner D, Sarr O, Sowunmi A, Kyle DE, Milhous WK, Wirth DF, Oduola AM.** 2006. Confirmation of emergence of mutations associated with atovaquone-proguanil resistance in unexposed Plasmodium falciparum isolates from Africa. Malar J **5:**82. http://dx.doi.org/10.1186/1475-2875-5-82.
27. **Centers for Disease Control and Prevention.** 1997. 1997 USPHS/IDSA guidelines for the prevention of opportunistic infections in persons infected with human immunodeficiency virus. MMWR Morb Mortal Wkly Rep **46:**1–46.
28. **Spencer CM, Goa KL.** 1995. Atovaquone. A review of its pharmacological properties and therapeutic efficacy in opportunistic infections. Drugs **50:** 176–196.
29. **Walker DJ, Wakefield AE, Dohn MN, Miller RF, Baughman RP, Hossler PA, Bartlett MS, Smith JW, Kazanjian P, Meshnick SR.** 1998. Sequence polymorphisms in the Pneumocystis carinii cytochrome b gene and their association with atovaquone prophylaxis failure. J Infect Dis **178:**1767–1775. http://dx.doi.org/10.1086/314509.
30. **Bjorkman J, Andersson DI.** 2000. The cost of antibiotic resistance from a bacterial perspective. Drug Resist Updat **3:**237–245. http://dx.doi.org/10.1054/drup.2000.0147.
31. **Fohl LM, Roos DS.** 2003. Fitness effects of DHFR-TS mutations associated with pyrimethamine resistance in apicomplexan parasites. Mol Microbiol **50:** 1319–1327. http://dx.doi.org/10.1046/j.1365-2958.2003.03756.x.
32. **Froberg G, Ferreira PE, Martensson A, Ali A, Bjorkman A, Gil JP.** 2013. Assessing the cost-benefit effect of a Plasmodium falciparum drug resistance mutation on parasite growth in vitro. Antimicrob Agents Chemother **57:**887–892. http://dx.doi.org/10.1128/AAC.00950-12.
33. **National AIDS and STI Control Programme.** 2014. Kenya HIV estimates. Ministry of Health, Nairobi, Kenya. http://www.nacc.or.ke/attachments/article/428/HIV%20estimates%20report%20Kenya%202014_print.pdf.