Molecular characterization of the cytochrome b gene and in vitro atovaquone susceptibility of *Plasmodium falciparum* isolates from Kenya

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Running Head: Polymorphisms Associated with Atovaquone Resistance

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Competing Interests:
The authors declare that there are no competing interests
Abstract

The prevalence of genetic polymorphism(s) at codon 268 in cytochrome b gene which is 24 associated with Atovaquone-proguanil treatment failure was analyzed in 227 Plasmodium 26 falciparum parasites from western Kenya. Prevalence of wild type allele was 63% and Y268S 27 mutant allele was 2%. There were no pure Y268C or Y268N mutant alleles, but mixture with 28 wild type. There was a correlation between parasite IC50 and parasite genetic polymorphism; 29 mutant alleles had higher IC50s than the wild type.
Introduction

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 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, Y268N or Y268C (5, 9, 11, 13, 14), and can induce more than 1000-fold increase in atovaquone IC50 (13, 15). There are cases of AP treatment failure for travelers returning from Africa (4-6, 9-11, 16-19), and the appearance of pf cyt b mutations following AP treatment (4-6, 9-11, 14). However, treatment failure is not always associated with any known pf cyt b mutation (17, 18, 20, 21), indicating 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 residences 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 genetic polymorphism(s) at codon 268 of Pfcytb in Kenyan P. falciparum parasites. Field clinical isolates from an on-going approved malaria epidemiological surveillance protocol (KEMRI SSC# 1330, WRAIR #1384), collected 2008-2012 from 3 locations; Kisumu, Kisii and Kericho were randomly selected for inclusion in the study. Kisumu is malaria endemic lowland, with high stable transmission whereas Kisii and Kericho are highlands, with unstable transmission (23). Sample collection and preparation was
performed as previously described (24). Genomic DNA from whole blood was extracted using Qiagen DNA mini kit (Qiagen, Valencia, CA) as recommended by the manufacturer. Clinical isolates were culture-adapted before subjecting to the SYBR Green I assay as previously described (25). A total of 227 (167 Kisumu, 37 Kisii and 23 Kericho) samples were successfully analyzed by PCR-RFLP at codon 268 as previously described (10), and a subset (n = 68) of the samples sequenced to confirm PCR-RFLP results using the ABI Prism 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA) as previously described (10). Reference strain sequences were used to score the genotype. All sequences were deposited in GenBank under the following accession numbers; KP293776-KP293843.

Data revealed that none of the samples carried pure mutant Y268C or Y268N alleles. 63% isolates carried wild-type (WT) allele and 2% Y268S mutant allele. 33% had double mixed genotype (WT/Y268S) and 3% had triple mixed genotype (WT/Y268S/Y268N). Interestingly, 100% (4 of 4) of Y268S mutant alleles, 95% (70 of 74) of double and 100% (6 of 6) of triple mixed genotypes were found in Kisumu parasites. The remaining 5% (4 of 74) of double mixed genotype were from Kisii. Kericho did not have any mutant or mixed genotypes. Frequency of parasite genotype collected from 2008 to 2012 was analyzed. There was 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 WT allele present in samples collected in 2010 (82%) and lowest in 2009 (37%). Y268S mutant allele was not present in samples collected in 2008 and 2009, but emerged in 2010-2012 albeit at a low frequency. Similarly, the mixed genotype of WT/Y268S showed significant fluctuations in frequency from year to year (P < 0.0001, chi-square test), with the highest frequency present in 2009 samples at 59% and lowest in 2010 samples at 15% (Table).
Fig. 1 shows the atovaquone IC\textsubscript{50} values for parasites collected from 2008 to 2012. The median IC\textsubscript{50} (Interquartile range [IQR]) for the control reference 3D7 strain was 1.68 nM (1.09 – 2.70). The median IC\textsubscript{50} for all isolates in this study was 3.5 nM (1.4 - 8.4). The IC\textsubscript{50} cut-off point used for sensitive and resistance parasites was set at < 30 nM and > 1900 nM (8). Based on this criterion, only 1.3% isolates had high IC\textsubscript{50} values of between 1121 nM and 2250 nM.

Comparison of the median IC\textsubscript{50}s from year to year revealed there was significant difference ($P = 0.0003$; Kruskal-Wallis H statistics; Figure 1). Further, Dunn’s multiple comparison post-test revealed significant difference in the median IC\textsubscript{50}s of samples collected in 2008 vs. 2010, 2008 vs. 2011 and 2009 vs. 2011. However, this data has to be interpreted with caution because of the small samples size in 2008. Overall, the data showed there was fluctuation of IC\textsubscript{50} throughout the study period.

The median IC\textsubscript{50} for parasite carrying the WT allele was 3.0 nM (1.0 – 6.9), whereas the median IC\textsubscript{50} for the parasites carrying pure mutant Y268S was 5.7 nM (1.7 – 1216). The median IC\textsubscript{50} for parasite carrying a mixture of WT/Y268S was 4.7 nM (2.2 – 11.1), whereas the median IC\textsubscript{50} for parasites carrying a mixture of WT/Y268S/Y268N was 5.0 nM (2.0 – 11.8). The differences in median IC\textsubscript{50} of the different parasite genotype reached a statistical significance ($P = 0.0106$; Kruskal-Wallis H statistics).

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

Mutations in *Pfcytb* gene have been shown to arise by *de novo* due to AP selection (9, 11). However, although in low prevalence, these mutations have been shown to occur in parasite population even in locations where there is no AP pressure (26). AP is minimally used for control of malaria and for treatment of *Pneumocystis carinii* pneumonia in patients with HIV infection who cannot tolerate trimethoprim-sulphamethoxazole (27-29). In the current study, 2% of the sample parasites had Y268S mutant allele. Interestingly, a large number of samples (35%) carried mixed genotype of the WT/Y268S or WT/Y268S/Y268N allele; 95% of these samples (carrying mutant allele) were from Kisumu and 5% from Kisii but none from Kericho. Studies have demonstrated parasite genomic polymorphisms do result in fitness consequences, and parasites will not maintain any polymorphism that is not beneficial (30-32). Whether these mutations would appear in nature at such high proportion without any selection pressure will require further studies. Given the overwhelmingly occurrence of these mutations is in Kisumu, a high transmission region, it will be interestingly to investigate if there is any correlation. It will also be critical to determine if these mutations confer any other benefit(s) to the parasite survival to warrant such high prevalence. Interestingly, there seem to be a correlation between HIV prevalence and the prevalence of mutations at codon 268; Kisumu has the highest HIV prevalence at 19.3% compared to Kisii at 8% and Kericho at 3.4% (33). It is likely that the use of AP or other drugs to control opportunistic infections in HIV population 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 of the samples collected in Kenya between 2008 and 2012. There was fluctuation...
of the genotypes and the median IC_{50}s throughout the study period. Of interest is the emergence of Y268S mutant allele in the final three years of the study. Also, the frequency of mixed genotypes carrying the mutant alleles 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 AP treatment failure which carried Y268S had IC_{50} of 8230 nM (8). In another study, the IC_{50} of parasite with Y268N from a patient who recrudesced after AP treatment was 1888 nM (5). The median IC_{50} of parasite in the current study was 3.5 nM, well within the range as previously shown (8, 32). The median IC_{50} of Y268S genotype was 5.7 nM whereas for mixed genotype carrying WT/Y268S or WT/Y268S/Y268N was 4.7 nM and 5.0 nM respectively. Two of the samples with the highest IC_{50} of 1618 nM and 2251 nM carried Y268S and WT/Y268S alleles respectively. Although we did not have patient treatment information of these parasites, high IC_{50} of parasite coupled with mutations in Pfcytb strongly suggest these parasites might be resistant to AP.

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

Acknowledgements

We thank Duke Omariba, our study coordinator and all the staff members at the MDR sentinel sites for their contribution in recruitment of participants 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 US Army Medical Research Unit-Kenya (USAMRU-K), Department of the Army, the Department of Defense or the U.S. Government.
Reference


Figure Legends

FIG 1. Atovaquone median IC$_{50}$ values in nM, indicated above each box plot. The number of isolates analyzed in each year is shown in brackets. There was a significant decline in median IC$_{50}$ from 9.17 nM in 2008 to 4.11 nM in 2012.
Table showing different parasite genotypes collected over 5 year period. In each row, the number and percentage (in bracket) of parasite collected is shown per genotype. Y is the tyrosine (wild-type), S is serine (pure mutant), Y/S is double mixed genotype, and Y/N/S is triple mixed genotype, where N is asparagine, a mutant allele. The total number of parasites collected each year is shown as total. The total number samples analyzed for the study was 227, for both molecular and in vitro analysis.

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