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 the 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 (IC50) and parasite genetic polymorphism; mutant alleles had higher IC50s than the wild type.

A tovaquone-proguanil (AP) is a fixed-dose combination antimarial 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 (IC50) (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. Cultural isolates were cultured 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. 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 from 2008 to 2010.
TABLE 1 Different genotypes of parasites collected over a 5-year period

<table>
<thead>
<tr>
<th>Genotype</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>8 (67)</td>
<td>20 (37)</td>
<td>75 (82)</td>
<td>18 (56)</td>
<td>22 (59)</td>
</tr>
<tr>
<td>S</td>
<td>0</td>
<td>0</td>
<td>2 (2)</td>
<td>1 (3)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Y/S</td>
<td>3 (25)</td>
<td>32 (59)</td>
<td>14 (15)</td>
<td>11 (34)</td>
<td>14 (38)</td>
</tr>
<tr>
<td>Y/N/S</td>
<td>1 (8)</td>
<td>2 (4)</td>
<td>1 (1)</td>
<td>2 (6)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>54</td>
<td>92</td>
<td>32</td>
<td>37</td>
</tr>
</tbody>
</table>

*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 of samples analyzed for the study was 227, for both molecular and in vitro analyses.

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} 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 prevalence 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.

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