MAJOR ARTICLE

Novel Mutation in Cytochrome B of *Plasmodium falciparum* in One of Two Atovaquone-Proguanil Treatment Failures in Travelers Returning From Same Site in Nigeria

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Background. Atovaquone-proguanil (AP) is the most commonly used treatment for uncomplicated *Plasmodium falciparum* malaria in the United States. Apparent AP treatment failures were reported 7 months apart in 2 American travelers who stayed in the same compound for foreign workers in Rivers State, Nigeria.

Methods. We analyzed pretreatment (day 0) and day of failure samples from both travelers for mutations in the *P falciparum* cytochrome B (*pfcytb*) and dihydrofolate reductase (*pfdhfr*) genes associated with resistance to atovaquone and cycloguanil, the active metabolite of proguanil, respectively. We genotyped the parasites and sequenced their mitochondrial genomes.

Results. On day 0, both travelers had proguanil-resistant genotypes but atovaquone-sensitive *cytb* sequences. Day of failure samples exhibited mutations in *cytb* for both travelers. One traveler had the common Y268S mutation, whereas the other traveler had a previously unreported mutation, I258M. The travelers had unrelated parasite genotypes and different mitochondrial genomes.

Conclusions. Despite the infections likely having been contracted in the same site, there is no evidence that the cases were related. The mutations likely arose independently during the acute infection or treatment. Our results highlight the importance of genotyping parasites and sequencing the full *cytb* and *dhfr* genes in AP failures to rule out transmission of AP-resistant strains and identify novel mechanisms of AP resistance.

Keywords. dihydrofolate reductase; drug resistance; malaria; Malarone; *Plasmodium falciparum*.

The drug combination atovaquone-proguanil (AP), trade name Malarone (GlaxoSmithKline, Brentford, UK), was the most commonly prescribed drug for malaria prophylaxis in US travelers in 2011 [1], and it was the most frequently used drug for the treatment of uncomplicated malaria in the United States in 2011 [2]. Atovaquone-proguanil is also the drug most frequently used for acute uncomplicated *Plasmodium falciparum* malaria in France and other countries in Europe [3, 4]. Despite high efficacy, there have been sporadic reports of AP treatment failures since AP was first introduced for malaria prophylaxis and treatment in 2000 [5–15].

Resistance to cycloguanil, the active metabolite of proguanil, is conferred by the accumulation of multiple mutations in the *P falciparum* dihydrofolate reductase (*pfdhfr*) gene. These mutations also confer resistance to pyrimethamine and have become fixed in many malaria-endemic areas worldwide. In contrast, resistance to atovaquone can be conferred by just 1 single-nucleotide polymorphism (SNP) in the *P falciparum* cytochrome B gene (*Pfcytb*), encoding the molecular target of atovaquone. Unlike *pfdhfr* mutations, *Pfcytb* mutations that are associated with AP treatment failures are rarely found in pretreatment isolates [16–21], but they can arise after treatment with AP. Monotherapy

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with atovaquone has one of the highest rates of inducing resistant parasites during therapy [22], with up to 30%–40% of *P falciparum* infections exhibiting resistant recrudescent parasites after treatment [23, 24]. Surprisingly, AP has remained highly effective, even in areas with a high prevalence of proguanil-resistant genotypes. This result is probably due to proguanil acting as a possible synergistic potentiator of atovaquone activity [25].

Reported AP treatment failures therefore tend to follow a specific pattern, in which some parasites that are originally proguanil-resistant but atovaquone-sensitive develop *de novo* mutations that confer resistance during the acute infection, leading to late recrudescence after treatment. To date, all reported mutations directly associated with resistance to the atovaquone in AP treatment failures have occurred in codon 268 of the *pfcytb* gene [5–12]. Most commonly, the mutations replace a tyrosine with a serine (Y268S) or a cysteine (Y268C), but they can also substitute an asparagine (Y268N).

In this study, we present 2 cases of AP treatment failures in US travelers who stayed in the same compound for foreign workers near Port Harcourt in Nigeria, 7 months apart. Although the parasites from 1 recrudescent infection exhibited the previously described Y268S mutation in *Pfcytb*, the parasites from the other infection exhibited a novel mutation in *cytb* (I258M).

CASE 1 PRESENTATION

A 31-year-old American traveled to Rivers State in Nigeria in September of 2012 for a 4-week trip. The traveler worked in a shipyard and lived in a gated community for foreign workers. He began taking doxycycline for malaria prophylaxis on the first day of travel and stopped upon returning rather than continuing for 4 weeks as was prescribed. One week after returning, the traveler developed fever, followed by loss of appetite and diarrhea. Three days later, the traveler presented to a Louisiana hospital, where he was diagnosed with *P falciparum* infection by blood smear. The patient's parasitemia was reported as ~5% at this time. The traveler, weighing 110 kg, was admitted and treated with a standard 3-day course of AP (1000 mg atovaquone, 400 mg proguanil once per day). The traveler's diarrhea resolved on the second day of treatment. On the third day of treatment, his parasitemia was 0.3%. The day after finishing treatment, the traveler was discharged from the hospital. He returned for a scheduled follow-up visit 8 days later. After analysis of his blood smear showed *P falciparum* gametocytes, the traveler was treated with another 3-day course of AP. This treatment was taken at home, and the traveler did not report diarrhea or vomiting.

Thirty-one days after finishing his first course of AP, without having had any subsequent travel, the traveler developed fever and loss of appetite and reported to the ER. His blood smear was again positive for *P falciparum*, with a parasitemia of 2%. The traveler was treated with intravenous quinidine and clindamycin and recovered without complication.

Fortunately, ethylenediaminetetraacetic acid (EDTA) whole-blood samples from throughout his 2 hospitalizations were saved in the refrigerator of the hospital laboratory. The traveler's blood specimens from the initial diagnosis (D0), the follow-up visit in which gametocytes were detected (D11), the day when the patient presented to the ER for the second time (D34), and the following day (D35) were collected and sent to the laboratory unit of the Malaria Branch at the Centers for Disease Control and Prevention (CDC) for molecular analyses.

CASE 2 PRESENTATION

A 54-year-old American, working for a different company than Traveler 1, traveled to the same part of Nigeria in April of 2013 for a 1-month-long trip. Traveler 2 worked repairing air-conditioning units in ships and stayed in the same gated compound as Traveler 1. He was also on doxycycline prophylaxis, which he stopped upon leaving Nigeria. Two days after returning home, the patient had onset of fever, presented to the ER at the same hospital as Traveler 1, and was diagnosed with *P falciparum* malaria. His parasitemia was stated to be <5%, and he was admitted for treatment. He received the same 3-day course of AP (1000 mg atovaquone, 400 mg proguanil once per day). He was discharged on the third day, and returned 1 week later (10 days after starting treatment) for a follow-up visit; his smear was negative. Three weeks later, 31 days after first starting treatment and also without any subsequent travel, the patient began to feel unwell. Three days after the onset of symptoms, he presented to the ER and was diagnosed with *P falciparum* malaria after a blood smear showing 3% parasitemia. The traveler weighing 58 kg recovered without complication after treatment with intravenous quinidine and clindamycin. Stored blood specimens from this traveler were available from the day of the first (D0) and second (D34) malaria diagnoses and were sent to the malaria laboratory unit at the CDC for molecular analyses.

LABORATORY METHODS

The 6 blood specimens (4 from case 1 and 2 from case 2) were extracted for DNA purification using a QIAamp Blood DNA kit (Qiagen) and analyzed by polymerase chain reaction (PCR) amplification of DNA, sequencing, and genetic analysis [26, 27]. Fragments of genes encoding the molecular targets of atovaquone (*pfcytb*), proguanil (*pfdhfr*), chloroquine (*P falciparum* chloroquine resistance transporter, *pfcrt*), and sulfadoxine (*P falciparum* dihydropteroate synthase, *pfdhps*) and encompassing all the known drug resistance mutations were amplified twice by PCR using high-fidelity polymerase and Sanger

sequencing of both DNA strands using an ABI PRISM 3130xl Genetic Analyzer (PerkinElmer Applied Biosystems). The genetic signature of the parasites in each specimen was evaluated by fragment size analysis on an ABI PRISM 3130xl, Genetic Analyzer, and parasite genotypes were determined by amplifying 7 neutral *P falciparum* microsatellite loci: TA1, Polyα, PK2, TA109, 2490 [28], C2M34, and C3M69 [29]. The entire 6 kb mitochondrial genomes from 2 specimens (D0 and D35) from case 1 and the D0 and D34 specimens from case 2 were also amplified by PCR and sequenced using the Sanger method. The respective GenBank accession numbers are as follows: KM065497, KM065498, KM065499, and KM065500.

LABORATORY RESULTS

Case 1

The *pfcytb* gene on D0 and D11 had wild-type sequence, but on D34 and D35 a nonsynonymous mutation at nucleotide 774 (T to G), corresponding to a change in codon 258 from isoleucine to methionine (I258M), was present in the parasite population on those days (Table 1). Three SNPs in *pfdhfr* (N51I, C59R, S108N) associated with strong resistance to pyrimethamine and proguanil were found in the parasites in all 4 blood specimens. The parasites from all 4 time points also contained 3 SNPS in the *crt* gene associated with chloroquine resistance, forming the CVIET haplotype common to African parasites. In addition, 2 SNPs in *pfdhps* associated with strong resistance to sulfadoxine (S436A, A437G) were found in all 4 blood specimens.

Microsatellite analysis showed that this traveler had an infection with at least 2 detectable genotypes at D0 (Table 2). On D11, because parasites were very few and only gametocytes were present, analysis of all 7 microsatellites was not possible. Nevertheless, the gametocytes appeared to be mostly derived from 1 of the genotypes present on D0, although a minor allele was noticed at 1 locus, indicating possible presence of a second strain at low level. The same major genotype present on D0 and D11 was apparently the source for the recrudescent parasites present on D34 and D35. Recrudescent parasites observed on D34 and D35 had a single detectable genotype suggesting clearance of other genotypes that were noticed on day 0 and selection of this particular strain. The amplification of the mitochondrial genome was possible only from the D0 and D35 blood specimens, and the 6 kb mitochondrial genome sequences from both time points were identical.

Case 2

The parasites from both time points available for Traveler 2 had the same pattern of mutations in *crt*, *pfdhps*, and *pfdhfr* as seen in Traveler 1 (Table 1). The *pfcytb* gene was wild-type before treatment on D0, but on D34 the parasite population had a mutation at nucleotide 803 (A to C) that resulted in the substitution of a tyrosine with a serine in codon 268 (Y268S), the SNP most commonly found in parasites resistant to atovaquone. The parasites from Traveler 2 had the same 7 microsatellite alleles at both time points, and there was no evidence of mixed infection. Both mitochondrial genomes at D0 and D35 were identical in Traveler 2, but they were different from the sequences in the parasites from Traveler 1 by 2 SNPs, in addition to the mutations already noted in the *cytb* genes.

DISCUSSION

The mutation in codon 258 documented here in Traveler 1, resulting in the replacement of an isoleucine with methionine in *pfcytb*, does not appear in any *pfcytb* sequences deposited in GenBank. To our knowledge, this is the first time a mutation in *cytb* other than the mutations in codon 268 has been reported to be associated with AP treatment failure. We were not able to perform in vitro susceptibility testing or confirm adequate blood levels of atovaquone, so we cannot definitively show that the I258M mutation was causally associated with the treatment failure in Traveler 1. Nevertheless, it is likely that the I258M mutation confers high-level resistance to atovaquone. The 258 codon, like the 268 codon, is situated in the putative binding site for atovaquone [30,31], and this isoleucine interacts

Table 1. Drug Resistance Mutations in *pfdhfr, pfdhps, pfcrt,* and *pfcytb*

	pfdhfr	*pfdhps*	*pfcrt*	*pfcytb*
Traveler 1				
D0	N51I, C59R, S108N	S436A, A437G	M74I, N75E, K76T	wt
D11	N51I, C59R, S108N	S436A, A437G	M74I, N75E, K76T	wt
D34	N51I, C59R, S108N	S436A, A437G	M74I, N75E, K76T	I258M
D35	N51I, C59R, S108N	S436A, A437G	M74I, N75E, K76T	I258M
Traveler 2				
D0	N51I, C59R, S108N	S436A, A437G	M74I, N75E, K76T	wt
D34	N51I, C59R, S108N	S436A, A437G	M74I, N75E, K76T	Y268S

Abbreviation: wt, wild-type.

Table 2. Genetic Typing of Patient Parasite Populations by Microsatellite Analysis

	Fragment Length						
	TA1 (Chr6)	Polyα (Chr4)	PfPK2 (Chr12)	TA109 (Chr6)	2490 (Chr10)	C2M34 (Chr2)	C3M69 (Chr3)
Traveler 1							
D0	166*, 178, 190	145, 162	163, 172, 181	173, 197	84, 74	211, 268	124, 136
D11	*NA*	162	163, 172	173	84	NA	124
D34	166	162	172	173	84	268	124
D35	166	162	172	173	84	268	124
Traveler 2							
D0	187	158	166	164	84	255	126
D34	187	158	166	164	84	255	126

* Underlining indicates the predominant microsatellite allele population in the overall parasite population. Abbreviation: NA, not applicable.

directly with the naphthoquinone ring of atovaquone [32]. Moreover, the I258M substitution has previously been noted in the *cytb* gene of *Plasmodium yoelii* parasites that are highly resistant to atovaquone, with an 800-fold increase in EC_{50} concentrations affecting mitochondrial membrane potential [28]. Although there are probably other mechanisms for atovaquone resistance in addition to mutations in *cytb*, because there have been cases of malaria treatment failure with adequate dosing in infections with recrudescent parasites with wild-type *pfcytb* [13, 14], the observation of this novel mutation further highlights the crucial role of *pfcytb* in atovaquone resistance.

Several factors might have contributed to treatment failure in Traveler 1. The traveler's initial high-density parasitemia (reported as >5%) meant a high parasite burden and thus a greater chance of emergence of AP-resistance genotypes, particularly because proguanil/cycloguanil-resistant *pfdhfr* genotypes were present in these parasites. Moreover, AP is not indicated for severe malaria and he should have been treated with an intravenous antimalarial. The traveler's diarrhea during the first day of treatment could have led to malabsorption resulting in underdosing of the drug. The first traveler's weight also raises the possibility that he did not receive an adequate dose of the drug. Overrepresentation of patients weighing more than 100 kg among AP treatment failures has been previously noted [33]. Although inadequate dosing can lead to treatment failure in infections with susceptible parasites, it may also increase the risk of resistant parasites developing during treatment. Finally, the decision to re-treat Traveler 1 with AP was inappropriate both because the microscopic presence of only gametocytes should not have resulted in antimalarial treatment and because a supposed treatment failure should not have been re-treated with the same drug. This decision could have increased the selection pressure that resulted in the eventual recrudescent infection with a resistant parasite.

The mutation observed in the second treatment failure, Y268S, was the most *cytb* common mutation associated with AP treatment failure. Despite the travelers having contracted the infection in the same site in Nigeria, raising the concern that atovaquone-resistant malaria was being transmitted locally at their shared worksite, the genetic analysis demonstrated that the 2 patients' parasites were unrelated. In both patients, the parasite genotype that eventually exhibited AP resistance and corresponding atovaquone mutations was present at the beginning of infection. This observation is consistent with the hypothesis that the mutations likely arose *de novo* in both travelers during the course of the primary acute infection. Support for this hypothesis is bolstered by the fact that in both patients, the mitochondrial genomes on D0 and D35 were identical except for the respective *cytb* gene mutations present in the D35 population but not the D0 populations. Finally, the D0 mitochondrial genomes differed between the 2 patients, further evidence that the cases were unrelated and that resistance arose during treatment. The independent emergence of resistance during treatment for these 2 travelers is consistent with previous evidence for independent emergence of mutations in other cases of AP treatment failure [5].

In the face of a high prevalence of mutations in *pfdhfr* conferring resistance to proguanil, AP is essentially a monotherapy. The multiple, independent reports of resistance mutations after treatment with AP suggest a certain nonnegligible rate of development of atovaquone resistance during AP treatment. Possibly due to the fitness costs associated with the mutations in *pfcytb* [34, 35] and low selective pressure due to low rates of AP use in endemic areas, there is little evidence that these mutations are spreading, because they are rarely found in general surveys of local and global *P falciparum* isolates [16–21] or in pretreatment isolates from atovaquone refractory cases [5–15]. However, 1 study has reported the Y268N mutation occurring in 4.5% of Nigerian isolates unexposed to AP [36].

Triple mutant *pfdhfr* genotypes, with high pyrimethamine and proguanil resistance, arose independently in Southeast Asia [37] and South America [38], and they are now widespread

throughout *P falciparum* populations worldwide [39], including large swaths of Sub-Saharan Africa [40]. Due to the increasing use of AP for the treatment of *P falciparum* infections, surveillance for AP treatment failures and genetic characterization of malaria parasites should continue. However, because there is little evidence for the presence of atovaquone-resistance mutations in current parasite populations worldwide [22], AP remains a viable choice for prophylaxis and, for now, treatment outside of endemic areas.

CONCLUSIONS

It is worth noting that both cases might have been prevented if the travelers had continued their doxycycline prophylaxis after completing travel. Primary prevention of malaria with insect avoidance and chemoprophylaxis remains an essential strategy for travelers to malaria-endemic areas. The investigation of AP treatment failures should continue in order to identify and monitor the spread of atovaquone-resistant mutations. Our observation of a previously unreported mutation in *pfcytb* highlights the importance of sequencing the entire *pfcytb* gene when searching for mutations associated with atovaquone resistance rather than directly looking for the common 268 mutations [41]. In addition, genotyping analysis makes it possible to rule out transmission of AP-resistant strains, particularly in strains contracted in the same area.

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