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MAJOR ARTICLE

Screening for Mutations Related to Atovaquone/Proguanil Resistance in Treatment Failures and Other Imported Isolates of *Plasmodium falciparum* in Europe

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Background. Two single-point mutations of the *Plasmodium falciparum* cytochrome b gene (Tyr268Asn and Tyr268Ser) were recently reported in cases of atovaquone/proguanil (Malarone) treatment failure. However, little is known about the prevalence of codon-268 mutations and their quantitative association with treatment failure.

Methods. We set out to assess the prevalence of codon-268 mutations in *P. falciparum* isolates imported into Europe and to quantify their association with atovaquone/proguanil treatment failure. Isolates of *P. falciparum* collected by the European Network on Imported Infectious Disease Surveillance between April 2000 and August 2003 were analyzed for codon-268 mutations, by use of polymerase chain reaction–restriction fragment–length polymorphism.

Results. We successfully screened 504 samples for the presence of either Tyr268Ser or Tyr268Asn. One case of Ser268 and no cases of Asn268 were detected. Therefore, we can be 95% confident that the prevalence of Ser268 in the European patient pool does not exceed 0.96% and that Asn268 is less frequent than 0.77%. In 58 patients treated with atovaquone/proguanil, Tyr268Ser was present in 1 of 5 patients with treatment failure but in 0 of 53 successfully treated patients.

Conclusions. Tyr268Ser seems to be a sufficient, but not a necessary, cause for atovaquone/proguanil treatment failure. The prevalence of both codon-268 mutations is currently unlikely to be >1% in the European patient pool.

Infected European travelers and immigrants carry a wide variety of *Plasmodium falciparum* strains from all endemic areas into the continent. Thus, if properly done, data and parasite material from this population can be used to monitor the development of drug resistance in endemic areas, especially in sub-Saharan Africa [1].

A fixed combination of atovaquone and proguanil (Malarone; GlaxoSmithKline) is a drug that has been

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recently introduced for the treatment and prophylaxis of multidrug-resistant *P. falciparum* malaria. Early evidence showed that parasites may quickly develop resistance to atovaquone. One study showed that, when treated with atovaquone alone, 33% of patients had recrudescence of parasitemia [2]. It has been proposed that, because atovaquone inhibits electron transport and collapses mitochondrial membrane potential at similar concentrations [3], this might lead to the formation of oxygen radicals, which could act as locally active mutagens [4].

When atovaquone is administered in combination with proguanil, cure rates of 99%–100% are achieved [5–10]. It has been shown that the biguanide itself, not the metabolic conversion cycloguanil as an inhibitor of dihydrofolate reductase, synergizes with atovaquone by specifically lowering the concentrations at which atovaquone is able to collapse the mitochondrial membrane electropotential [11]. As a result, the inclusion of proguanil leads to an enhancement of atovaquone's activity and reduces the chance of mutations arising in the mtDNA of the malaria parasite [4].

There is evidence that atovaquone, on the basis of its structural similarity to ubiquinol, binds to the parasitic cytochrome bc_1 (cyt b) complex [12], and mutations in the cyt b gene of the parasite mitochondrial genome have been described that confer atovaquone resistance. Two mutations in *Pneumocystis carinii* at the ubichinol-binding pocket (the Q_0 domain) have been shown to be associated with the failure of atovaquone prophylaxis [13]. Atovaquone-resistant *Plasmodium yoelii* lines have been derived by subtherapeutic treatment of infected mice. Five mutations near the putative atovaquone-binding pocket have been identified at codons 258–272 [14]. In a similar study, 3 mutations at the cyt b gene of atovaquone-resistant *Plasmodium berghei* lines were found to be associated with resistance to atovaquone. In that study, mutations at codon 133 or 144, in addition to an amino acid change at codon 284, led to increased resistance levels [15].

In studies with *P. falciparum,* atovaquone-resistant lines have been derived in vitro by incubation at various concentrations [16]. An initial mutation at codon 133 was found to confer a low resistance level that could be increased by additional mutations in the codon 272–280 domain. In vivo, a *P. falciparum* isolate from a Thai patient with recrudescence after atovaquone and pyrimethamine treatment showed a mutation at codon 268 (Tyr268Ser) of the cyt b gene [2, 16]. A different amino acid change at the same codon (Tyr268Asn) was described in an English patient traveling to Nigeria who had failed atovaquone/proguanil therapy [17].

Protocols for the detection of relevant mutations have been developed and evaluated with in vitro isolates plus a few samples from patients with documented in vivo resistance. The results point convincingly toward correlations between the detection of point mutations and phenotypic resistance, in particular for correlation to the codon-268 mutations and in vivo resistance. The cyt b gene mutations Tyr268Asn and Tyr268Ser have been linked to cases of atovaquone/proguanil treatment failure [17–21]. To move molecular assays for point mutations on resistance-related genes into the realm of applied tools for surveillance, we investigated a series of *P. falciparum* isolates that had been imported into Europe between April 2000 and August 2003 for the prevalence of point mutations associated with atovaquone/proguanil resistance.

SUBJECTS, MATERIALS, AND METHODS

Sampling. The study was established within the infrastructure of the European Network on Imported Infectious Disease Surveillance (TropNetEurop), which has been successfully providing surveillance data on imported malaria since 1999 [22]. The network covers ~12% of all imported cases of malaria in western and central Europe. Sentinel surveillance reporting is currently done by 46 participating clinical sites throughout 16 European countries by use of a standardized and computerized reporting system. Although the organization of the network does not guarantee a representative data collection for Europe, most referral centers in Europe are represented. A total of 18 centers sent in malaria isolates with their case notifications. During standard malaria testing by thick and thin blood film, 10 μL of full blood was dotted on Whatman 3MM chromatography paper and air-dried at room temperature before the initiation of treatment. DNA was prepared from the dried blood spots, as described elsewhere [23].

Polymerase chain reaction–restriction fragment–length polymorphism (PCR-RFLP) for codon-268 mutations. The detection of resistance-related point mutations on the cyt b gene was done according to protocols established elsewhere. For detection of the codon-268 mutations on cyt b, a PCR-RFLP method was used. Details have been published elsewhere [18]. A nested PCR was designed that used CYTb1 and CYTb2 as outer primers and 3 different pairs of nested primers to distinguish the 3 known polymorphisms at codon 268. For the primary amplification reaction, a mix that contained 0.125 μmol/L each outer primer, 0.2 mmol/L dNTPs, 1.5 mmol/L Mg^{2+}, and 0.5 U Taq polymerase (Qbiogene) was initially heated at 94°C for 5 min and then cycled at 94°C for 50 s, 50°C for 50 s, and 70°C for 1 min for 35 cycles, with a final extension at 70°C for 5 min. For the secondary amplification, 1 μL of PCR product was added to the master mix that contained 0.5 μmol/L primers and dNTP, $MgCl_2$, and Taq polymerase, as described above. PCR conditions were 94°C for 5 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min for the primer pairs CYTb3/CYTb5 and CYTb2/CYTb6. CYTb2/CYTb7 was annealed at 45°C. The products of the second round were confirmed by electrophoresis in ethidium bromide–stained agarose gel.

For RFLP analysis, 5 μL of PCR product was mixed with 1 U of the appropriate enzyme and its specific buffer in a total volume of 22 μL and incubated overnight at 37°C. The result was detected by electrophoresis in ethidium bromide–stained agarose gel. The primer pair CYTb3/CYTb5, when used in combination with the enzyme *Nsi*I, cuts the wild type *(wt)* and the Asn268 mutation but not the Ser268 mutation. The primer pair CYTb2/CYTb6, used in combination with the enzyme *Alw*NI, cuts the Ser268 mutation but not the *wt* and the Asn268 mutation. The primer pair CYTb2/CYTb7, used in combination with the enzyme *Ssp*I, cuts the *wt* and the Ser268 mutation but not the Asn268 mutation.

The established *P. falciparum* laboratory clones K1 and FCR3, as well as our own in vitro isolates that are resistant to atovaquone/proguanil, were used as representative controls [18]. In cases where the PCR testing of samples did not reveal any result (neither *wt* nor mutation), the testing was repeated at least once. If the PCR result remained inconclusive, the testing was defined as unsuccessful, and the sample was excluded from further analysis.

Statistical analysis. Results of the PCR testing of the *P. falciparum* isolates were individually matched with epidemiological and clinical data from the TropNetEurop surveillance database and were analyzed by use of the statistical software SAS (release 8.01; SAS Institute). To be able to present statistically ascertained estimates for mutation prevalence, even if no mutations were observed, maximum mutation prevalences were calculated that, given the study power, one could be 95% sure they were not surpassed. This may be interpreted as a 1-sided 95% confidence interval (CI). To derive the estimate, the number of to-be-expected mutations that, under the assumption that the estimate was true, would make it <5% likely to find the observed number of mutations or fewer in the sample had to be determined. Under the assumption of Poisson distribution of the mutation data, the probability could be derived from the distribution, which was defined as the number of expected cases. Dividing the determined number of expected cases by sample size yielded the maximum mutation prevalence.

RESULTS

A total of 504 isolates of *P. falciparum* were screened for 2 different mutations on codon 268 of the parasite's cyt b gene, which had been previously associated with atovaquone/proguanil treatment failure. Combining the results of the PCRs with primer pairs CTYb3/CYTb5 and CYTb2/CYTb6, both of which focus on the detection of the Ser268 mutation, a total of 495 samples were successfully screened for this specific mutation. With the PCR using primer pair CYTb2/CYTb7, which focuses on the detection of the Asn268 mutation, 391 samples were tested successfully.

Characteristics of the total study population. Table 1 summarizes the characteristics of the 504 patients. The majority of infections (54.2%) were acquired in West Africa, and most patients did not receive any malaria prophylaxis (82.0% of cases with available data). The ratio of non-Europeans (immigrants or foreign visitors) to Europeans (either living in Europe or expatriates) was 1.06:1.

Table 1. Characteristics of the total study population.

Characteristic	Total study population (n = 504)
Male/female/no data	244/114/146
Median age (range), years	36 (0–83)
European/non-European/no data	167/177/160
Region of infection	
West Africa	273 (54.2)
Central Africa	85 (16.9)
East Africa	69 (13.7)
South Africa and Madagascar	31 (6.2)
Southeast Asia	18 (3.6)
Indian subcontinent	7 (1.4)
South and Central America	5 (1.0)
Other	2 (0.4)
No data	14 (2.8)
Malaria prophylaxis	
None	260 (51.6)
Mefloquine	18 (3.6)
Chloroquine/proguanil	18 (3.6)
Chloroquine only	12 (2.4)
Proguanil only	1 (0.2)
Atovaquone/proguanil	1 (0.2)
Other	7 (1.4)
No data	187 (37.1)

NOTE. Data are no. (%) of subjects, unless otherwise noted.

Characteristics of the treatment failures. Treatment information was available for 329 of 504 patients. Of these, 253 received drugs other than atovaquone/proguanil, 18 received atovaquone/proguanil in combination with other drugs, and 58 received atovaquone/proguanil monotherapy. In the latter group, 5 treatment failures were reported. Of these, 3 cases of malaria had been acquired in West Africa, 1 in East Africa, and 1 in Central Africa (table 2).

Patient A, a 30-year-old Gambian who is a resident of Germany, was diagnosed with *P. falciparum* monoinfection (3% parasitemia) in September 2001 after returning from Gambia. He did not receive any malaria prophylaxis during the visit. The patient was treated with atovaquone/proguanil for 3 days; no parasites were detected in the thick blood film 8 days after the initiation of therapy. Three weeks after therapy, the patient had a parasitological recrudescence without any symptoms.

Patient B, a 28-year-old male German, was diagnosed with malaria in February 2002 in Mali, 8 days before he returned. He received chloroquine and proguanil as malaria prophylaxis. Treatment was provided with atovaquone/proguanil in ade-

Table 2. Subsample of 58 travelers treated for malaria with atovaquone/proguanil after returning to Europe.

Characteristic	Atovaquone/proguanil treatment		
	Failure (n = 5)	Success (n = 53)	Total (n = 58)
Male/female/no data	4/1/0	43/10/0	47/11/0
Median age (range), years	34 (28–57)	36 (16–83)	35.5 (16–83)
European/non-European/no data	2/3/0	22/31/0	24/34/0
Region of infection			
West Africa	3 (60)	30 (56.6)	33 (56.9)
Central Africa	1 (20)	4 (7.5)	5 (8.6)
East Africa	1 (20)	11 (20.8)	12 (20.7)
South Africa and Madagascar	…	6 (11.3)	6 (10.3)
Southeast Asia	…	2 (3.8)	2 (3.4)
Malaria prophylaxis			
None	2 (40)	41 (77.4)	43 (74.1)
Mefloquine	…	1 (1.9)	1 (1.7)
Chloroquine/proguanil	1 (20)	5 (9.4)	6 (10.3)
Chloroquine only	1 (20)	3 (5.7)	4 (6.9)
Other	…	2 (3.8)	2 (3.4)
No data	1 (20)	1 (1.9)	2 (3.4)
Prevalence of codon-268 mutations	1 (20)	0 (0)	1 (1.7)

NOTE. Data are no. (%) of subjects, unless otherwise noted.

quate doses. Four weeks later, the patient developed a symptomatic recrudescence with a density of 1.5% *P. falciparum*. PCR-RFLP and sequencing revealed a mutation (Tyr268Ser) in the parasite's cyt b gene [18].

Patient C, a 33-year-old male German, was diagnosed with *P. falciparum* malaria after returning from a holiday in Kenya and Tanzania in February 2003. He did not receive any malaria prophylaxis. The patient developed a febrile recrudescence 3 weeks after directly observed treatment with atovaquone/proguanil in standard doses.

Patient D, a 56-year-old male Nigerian who is a resident of Spain, was diagnosed with *P. falciparum* malaria after returning from Nigeria in March 2003. The patient was treated with atovaquone/proguanil and developed an early treatment failure with no negativity in the thick blood film.

Patient E, a 38-year-old Congolese woman who is a resident of Germany, was diagnosed with *P. falciparum* monoinfection after returning from a trip to Kinshasa. She received only chloroquine for prophylaxis. Parasites recrudesced 3 weeks after a directly observed standard treatment course of atovaquone/proguanil. High-performance liquid chromatography (Shimatzu) on day 2 of treatment confirmed a drug concentration of atovaquone above the required therapeutic plasma concentration (17.2 μg/mL).

All patients were retreated successfully either with coartemether (artemether and lumefantrine; patients B, C, and D), other artemisinin-based combinations (patient E), or mefloquine (patient A). Except for patient B, PCR-RFLP revealed *wt* in codon 268 of all isolates. In 4 of 5 isolates, PCR results were additionally confirmed by sequencing a 716-bp fragment of the cyt b gene [24], and no other variants were found.

Prevalence of codon-268 mutations. In our study population, 1 case of Tyr268Ser (of 495) and 0 cases of Tyr268Asn (of 391) were detected. The prevalence of Tyr268Ser in patients treated in Europe for falciparum malaria between 2001 and 2003 can therefore be calculated with 95% confidence to be 0.01%–1.12%. The maximum prevalence that will not be surpassed with 5% probability of error can be calculated as 0.96%. For Tyr-268Asn, we can be 95% confident that the prevalence of this mutation does not exceed 0.77% in the European patient pool.

Association of codon-268 mutations with treatment failure. In the subsample of 58 patients treated with atovaquone/proguanil, Tyr268Ser was present in 1 of 5 with treatment failure but in 0 of 53 successfully treated patients (table 2), which indicates that this mutation is not a necessary, but may be a sufficient, cause of atovaquone/proguanil treatment failure. Its presence was associated with a 14.3-times higher risk of atovaquone/proguanil treatment failure (relative risk [RR], 14.3 [95% CI, 5.5–36.7]).

Tyr268Asn was detected in none of the samples; however, in 6 of 58 samples, the testing for this specific mutation was not successful, and the status therefore remained unknown. Excluding these 6 samples from the analysis revealed a 12.8-times higher risk for the occurrence of atovaquone/proguanil treatment failure in the presence of either of the 2 described codon-268 mutations (RR, 12.75 [95% CI, 5.0–32.7]).

DISCUSSION

With growing international travel and the continued spread of antimalarial drug resistance, the new fixed-dose combination of atovaquone and proguanil was most warranted as an agent that is not only highly effective for the prophylaxis and treatment of multidrug-resistant *P. falciparum* malaria but is also well tolerated, especially when it is used for prophylaxis [25, 26]. However, early after its introduction, it was predicted that the combination of these 2 agents, which had already been in use for some time, would be vulnerable to resistance in the near future [27]. This is even more the case in drugs in which single- or double-point mutations confer high levels of resistance.

An atovaquone/proguanil donation program in Kenya and Uganda implemented in 1999 (and discontinued in 2001) [28] gave rise to concerns that atovaquone/proguanil needs to be protected, because new, safe, and affordable antimalarial drugs are unlikely to be developed in the near future [29]. Protection can be achieved by judicious use and by combining atovaquone/proguanil with artemisinin derivates, as is practiced in Thailand [30]. But the combination of atovaquone/proguanil with artemisinin derivates will be largely unaffordable in the developing world.

The present study was performed to screen imported isolates of *P. falciparum* for mutations previously reported to be associated with in vivo atovaquone/proguanil resistance. In 504 samples, 495 were successfully tested for Tyr268Ser and 391 for Tyr268Asn on codon 268 of the parasite's cyt b gene. One of these mutations (Tyr268Ser) was detected. The fact that 2 independent PCRs were used to reveal the presence of the Tyr268Ser mutation, whereas only 1 focused on the Asn268 mutation, explains the different success rates of the testing. Technical aspects of the PCR protocols might also be responsible for this discrepancy. Given the size of our sample, it can be derived with a 5% probability of error that the prevalence of Tyr268Ser in the European patient pool is <0.96% and that the prevalence of Tyr268Asn is <0.77%.

In our subsample, 5 (8.62%) of 58 patients had atovaquone/proguanil treatment failure. Compared with a large study performed in Thailand ($n = 530$), where failures occurred in 2.8% of patients treated with atovaquone/proguanil alone, this is an unexpectedly high number [31]. Because the reporting within our network is self-selected, it might be assumed that treatment failures are more likely to be reported (and subsequently overrepresented) in our population. Therefore, this number must be interpreted with caution. Poor compliance with the treatment regimen can be excluded in all 5 cases. Impaired bioavailability of the drug could serve as another explanation of recrudescence. For atovaquone, concomitant food intake plays an important role, because of a food-induced increase in drug solubility and, hence, bioavailability [32]. The drug concentration of atovaquone has been measured (and was sufficient) in only 1 of the patients (patient E). In this case, the *P. falciparum* isolate did not carry any codon-268 mutation. According to our data, Tyr268Ser seems to be a sufficient, but not a necessary, cause for atovaquone/proguanil treatment failure—it was present in only 1 of 5 patients in the atovaquone/proguanil failure group but in none of the successfully treated patients. Because Tyr268Asn was ruled out as a cause of the observed atovaquone/proguanil failures, it might be assumed that other mechanisms or other mutations on the cyt b gene might contribute to the development of resistance, in addition to the previously described codon-268 mutations. However, in 4 of 5 isolates, all mutations previously described as involved in atovaquone resistance in vivo and in vitro [16] were ruled out by sequencing a 716-bp fragment of the cyt b gene [24].

It is important to stress that atovaquone/proguanil is a slow-acting antimalarial compound. In the above-mentioned study from Thailand, 36 (6.8%) of 530 patients in the atovaquone/proguanil group still had a positive blood film on day 3, compared with 2 (0.4%) of 533 artesunate-mefloquine recipients [31]. Therefore, late parasite clearance might be misinterpreted as early treatment failure. In our study population, 4 of 5 patients with treatment failure developed the recrudescence ⩾3 weeks after atovaquone/proguanil therapy, with several negative blood smears in between. Reinfection can be excluded in 4 cases, because therapy was initiated in a European country. The other patient left the endemic area 8 days after the last dose of atovaquone/proguanil was received. Considering the intrahepatic efficacy of atovaquone, it does not seem likely that the patient was reinfected during that time.

CONCLUSION

This type of molecular surveillance has little effect on treatment decisions for the individual travelers returning from regions where falciparum malaria is endemic. The high failure rate of atovaquone/proguanil in our population may be attributed to overreporting. Nevertheless, careful treatment follow-ups are recommended after therapy with all antimalarial drugs. Codon-268 mutations located on the parasite's cyt b gene seem to be a sufficient, but not a necessary, cause for atovaquone/proguanil treatment failure. Further studies are recommended to elucidate the mechanisms of resistance development and drug failure.

When used within a large clinical network, screening for molecular resistance markers in travelers with falciparum malaria has an unsurpassed advantage. When efficient methods and reliable molecular markers are available, data on their prevalence can be used as an early warning system for changes occurring in endemic areas, thus providing additional information that may be crucial for regional and international drug-policy changes. With regard to the 2 codon-268 mutations, Tyr268Ser and Tyr268Asn, which have previously been described in patients with atovaquone/proguanil treatment failure, we can be 95% confident that the current prevalence in the European patient pool is <0.96% and <0.77%, respectively. To the best of our knowledge, this is the first large-scale study on the prevalence of codon-268 mutations in a series of imported *P. falciparum* isolates and is the first attempt to quantify the association between the prevalence of these mutations and atovaquone/proguanil treatment failure.

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