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## Molecular surveillance of drug resistance through imported isolates of *Plasmodium falciparum* in Europe

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### Abstract

**Background:** Results from numerous studies point convincingly to correlations between mutations at selected genes and phenotypic resistance to antimalarials in *Plasmodium falciparum* isolates. In order to move molecular assays for point mutations on resistance-related genes into the realm of applied tools for surveillance, we investigated a selection of *P. falciparum* isolates that were imported during the year 2001 into Europe to study the prevalence of resistance-associated point mutations at relevant codons. In particular, we tested for parasites which were developing resistance to antifolates and chloroquine. The screening results were used to map the prevalence of mutations and, thus, levels of potential drug resistance in endemic areas world-wide.

**Results:** 337 isolates have been tested so far. Prevalence of mutations that are associated with resistance to chloroquine on the *pfprt* and *pfmdr* genes of *P. falciparum* was demonstrated at high

levels. However, the prevalence of mutations associated with resistance to antifolates at the DHFR and DHPS genes was unexpectedly low, rarely exceeding 60% in endemic areas.

**Conclusions:** Constant screening of imported isolates will enable TropNetEurop to establish a screening tool for emerging resistance in endemic areas.

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## Background

In an increasing number of countries where malaria is endemic, the effectiveness of routinely administered antimalarials like chloroquine and sulfadoxine/pyrimethamine is severely reduced because of the multi-drug resistance of *Plasmodium falciparum*. This poses a serious problem in terms of treatment and prophylaxis. Yet, mainly for financial reasons, chloroquine remains the first-line drug for treatment of malaria in these countries.

On the other hand, travelers from Europe and other industrialised countries, who have contracted *Falciparum* malaria, are commonly treated, on their return home, with highly effective antimalarials (usually mefloquine, halofantrine or quinine) that are frequently not freely available for routine treatment in endemic areas. Therefore, drug resistance is as yet not a frequent problem in the treatment of *Falciparum* malaria in hospitals of industrialized countries. Infected European travelers and immigrants carry a wide variety of *P. falciparum* strains from all endemic areas. Data and parasite material gained from this population, if properly used, can help to predict the development of drug resistance in endemic areas.

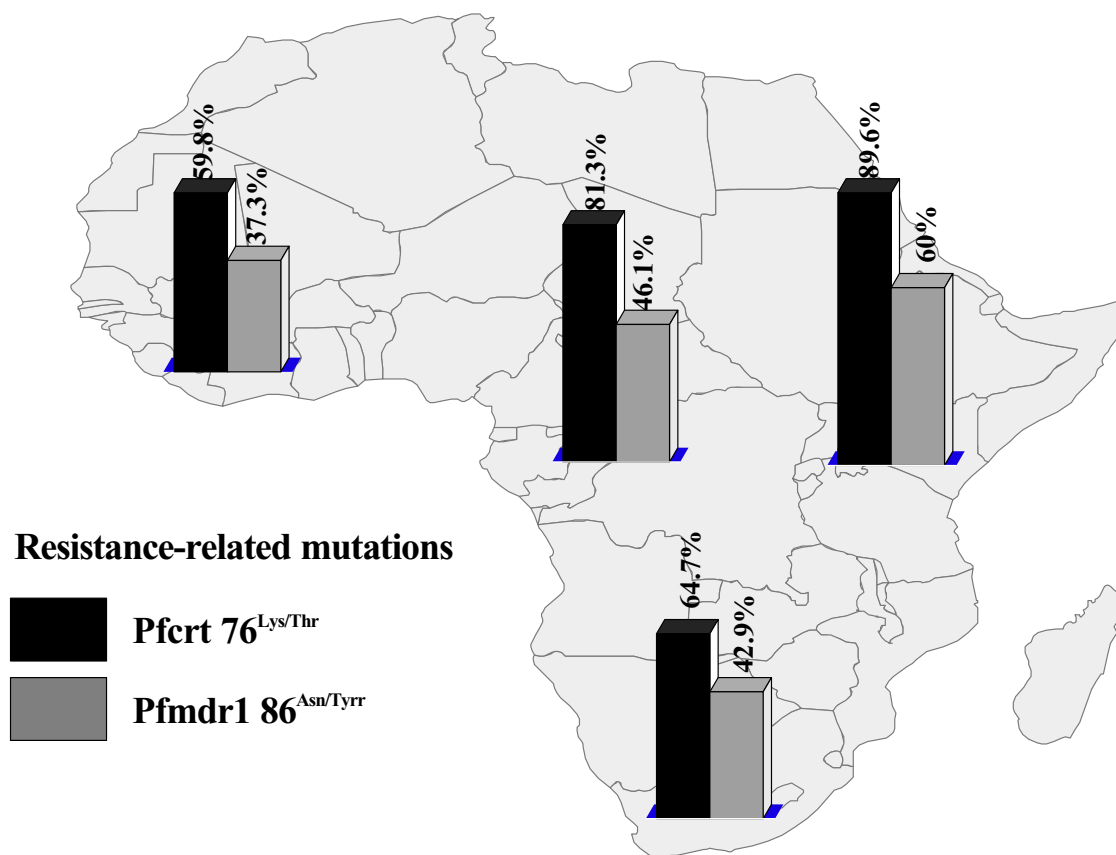
Point mutations at several codons of *Plasmodium falciparum* genes have been associated with emergence of drug resistance to commonly used antimalarial drugs. In particular, resistance to antifolates (e.g. pyrimethamine/sulfadoxine) and chloroquine depends on point mutations at the Dihydrofolate Reductase (DHFR) and Dihydropterolate Synthetase (DHPS) or PfCRT and PfMDR1 genes, respectively [1–3]. Several protocols for the detection of relevant mutations have been developed and have been evaluated with samples from endemic areas. Although a clear correlation between parasite genotype and *in vivo* outcome could not be documented in all field trials, the results show convincingly that a selected parasite population, if properly used, can help to correlations between point mutations and phenotypic resistance distinguish [4–9]. In order to move molecular assays for point mutations on resistance-related genes into the realm of applied tools for surveillance, we investigated a selection of *P. falciparum* isolates that were imported during the year 2001 into Europe to study the prevalence of resistance-associated point mutations at relevant codons. In particular, we tested isolates which were developing resistance to antifolates and chloroquine. The screening results were used

for mapping prevalence of mutations and, thus, levels of potential drug resistance in endemic areas.

## Methods

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 [10]. The network covers approximately 12% of all imported malaria cases in Western and Central Europe. TropNetEurop is designed to effectively detect emerging infections of potential regional, national or global impact at their point of entry into the domestic population. Sentinel Surveillance reporting is carried out by participating sites using a standardized and computerized reporting system. Immediate transmission of anonymized patient and laboratory data to the central database assures timely detection of sentinel events. The comprehensive collection of data on notifiable and non-notifiable infectious diseases in travelers makes it possible to identify needs for further surveillance and investigation and provides the potential for future case-control studies by identification of specific risk factors. The primary objectives of TropNetEurop are a) to construct and maintain a collaborative research network of clinical sites in Europe dealing with imported infectious diseases and b) to establish and maintain a clinical network for effective sentinel surveillance of imported infectious diseases in Europe. Membership is self-selected by participating centers and monitored by the steering committee of the network. Although the organization of the network does not guarantee a representative data collection for Europe, most referral centers in Europe are represented. From the beginning, malaria has been one of the major targets within this network of 38 clinical sites throughout 15 European countries.

Detection of resistance-related point mutation followed established protocols. During standard malaria testing by thick and thin blood film, 10 µl of full blood from each patient were dotted on Whatman 3 MM<sup>®</sup> chromatography paper and air-dried at room temperature before initiation of treatment. DNA was prepared from the dried blood spots as previously described [11]. For detection of the single base change at codon 86 of PfMDR1, a 330-basepair DNA fragment was amplified and followed by restriction-fragment-length-polymorphism (RFLP)-analysis [12,13]. A nested PCR protocol was used to identify the K76T in

**Figure 1**

Proportion of point mutations related to chloroquine resistance in different regions of sub-Saharan Africa (percentage showing proportion of mutation-positive samples from every geographical region)

the *Pfcr* gene [8,14]. After amplification of a 145 bp fragment around the codon 76, alleles carrying the K76 or T76 codon were discriminated by *ApoI*-restriction. For the detection of polymorphisms on the DHFR and DHPS genes, a previously described nested PCR method was used for all samples [6,15]. A volume of 4  $\mu$ l of PCR product was incubated with mutation specific restriction enzymes according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA).

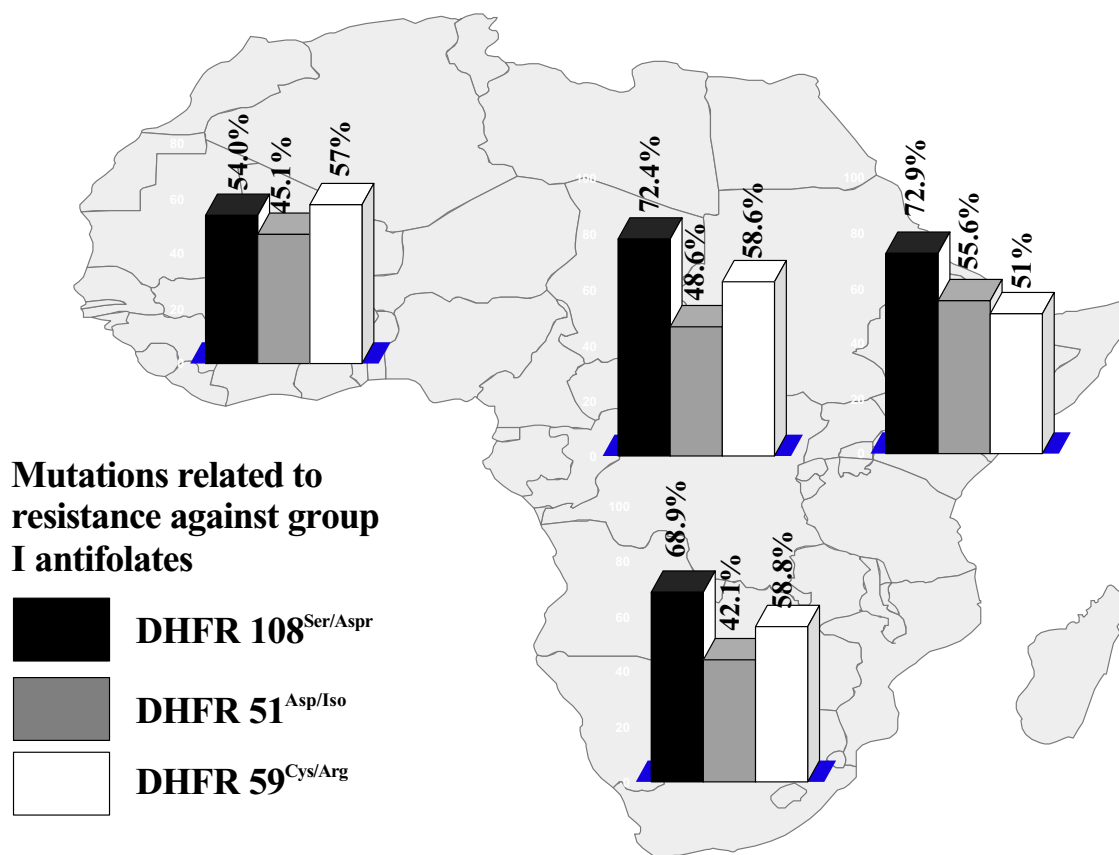
#### DHFR gene digest

To discriminate the three alternatives of codon 108 from each other, the 522 bp PCR product of the primer pair M3-F/ was digested with the following enzymes: *AluI* to detect the serine wild type (327 bp and 203 bp), *BsrI* to detect asparagine (309 bp and 190 bp) and *BstNI* to detect threonine (190 bp and 332 bp). The restriction site for *Tsp509I* was destroyed by an 51-asparagine to 51-isoleu-

cine mutation (215 bp). Another PCR product was digested by *XmnI* to detect the 59-arginine mutation (162 bp).

#### DHPS gene digest

A 438 bp PCR product was digested by *MnII* to detect 436-serine (283 bp), while digestion with *MspAI* identified the 436-alanine mutation (410 bp). Codon 437 was examined by digestion with *AvuII* to detect 437-glycine (402 bp) and *MwoI* to identify 437-alanine (387 bp). *FokI* served to distinguish between 540-lysine (404 bp) and 540-glutamic acid (320 bp). Another PCR product (436 bp) was digested by *HindIII* to identify the 436-phenylalanine mutation (410 bp). A PCR product of 161 bp was digested by *BstUII* to detect the 581-alanine wildtype (30 bp and 130 bp) and by *BsII* to discover 581-glycine mutations (30 bp and 130 bp). In the same way, the PCR product was digested by *MwoI* to identify 613-alanine (137 bp) and by *BsaWI* to detect the 613-serine/threonine muta-

**Figure 2**

Proportion of point mutations related to resistance to group I antifolates in different regions of sub-Saharan Africa (percentage showing proportion of mutation-positive samples from every geographical region)

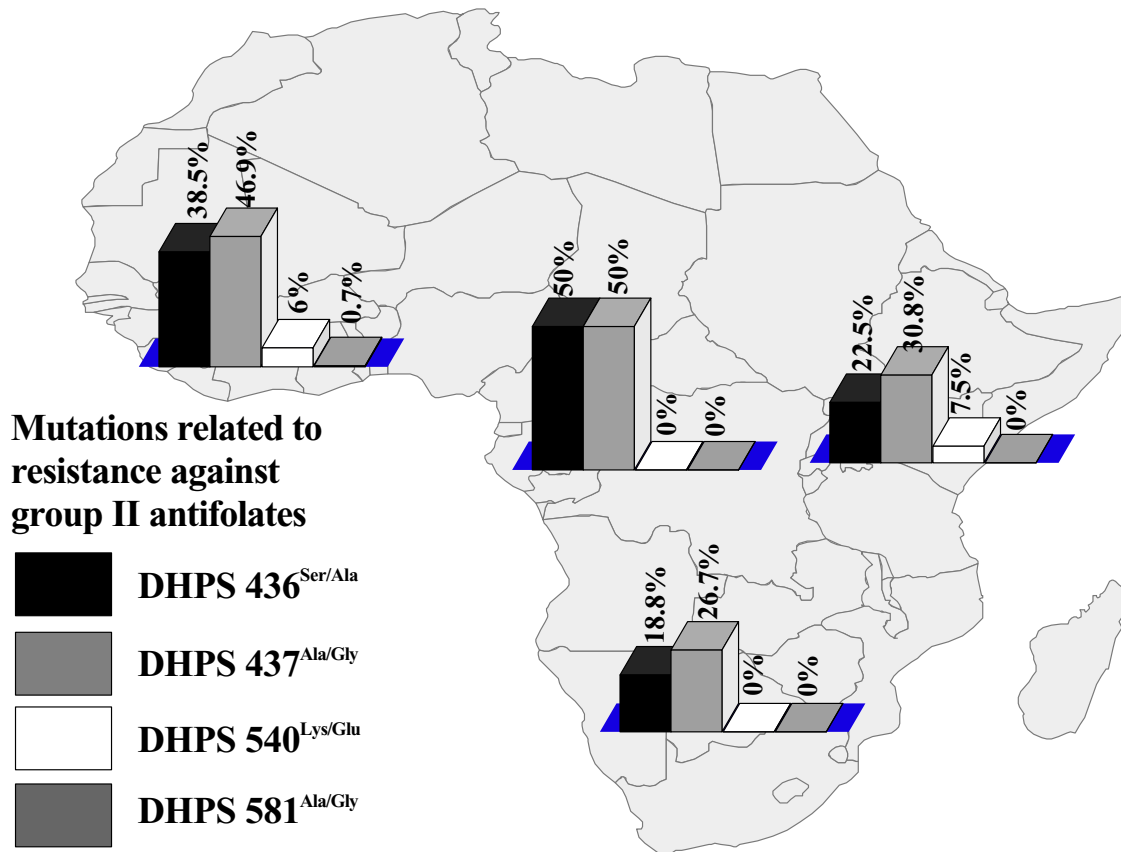
tion (130 bp). It was possible to differentiate 613-serine from 613-threonine by digestion with *AgeI*, which cuts in case of an 613-threonine mutation (128 bp).

All digestion products were separated by electrophoresis in an 1% SeaKem™ plus 1% NuSieve™ gel (FMC BioProducts, Rockland, ME, USA). As representative controls we used the established *Plasmodium falciparum* laboratory clones K1 and FCR3 as well as three own isolates that were gained during earlier studies in Uganda.

### Results & Discussion

Altogether, 337 samples were screened (table 1). The majority (n = 313; 92.9%) of these were imported from sub-Saharan Africa. Out of the 24 samples (7.1%) from other endemic areas, 14 were from Indonesia. As the small numbers of isolates from Asia and the Americas were only of limited value for the determination of prevalence rates of point mutations, we omitted these areas from further

evaluation. The total distribution of mutation-related point mutations among all isolates is shown in table 2. The mutation on Pfcrt76<sub>Lys/Thr</sub> has been identified as potentially crucial for developing resistance, while Pfmdr86<sub>Asn/Tyr</sub> appears to play a supporting role [16]. The majority of samples from sub-Saharan Africa showed at least former mutation (figure 1). However, a surprisingly high proportion of samples from areas that are generally assumed to have a very high level of drug resistance showed none of the mutations [17]. In particular, 40.2% of all samples from West Africa lacked the crucial prerequisite to develop phenotypical resistance to chloroquine (figure 1). A combination of both mutations was only present in 37.9% of the tested samples (data not shown). This pattern became even clearer when testing for mutations that are associated with resistance to antifolates. Here, DHFR108<sub>Ser/Asp</sub> has been identified as crucial mutation for resistance to group I antifolates, while DHFR51<sub>Asp/Iso</sub> and DHFR59<sub>Cys/Arg</sub> have a supporting



**Figure 3**  
Proportion of point mutations related to resistance to group II antifolates in different regions of sub-Saharan Africa (percentage showing proportion of mutation-positive samples from every geographical region)

**Table 1: Molecular surveillance of malaria drug resistance: geographical distribution of all isolates (n = 337)**

Region	Number	%
Central Africa	39	11.6
East Africa	53	15.7
Southern Africa	20	5.9
West Africa	198	58.8
Madagascar & African islands in the Indian Ocean	3	0.9
Central America	1	0.3
Caribbean	2	0.6
South America	0	0
West Asia	0	0
Indian Subcontinent	5	1.5
Indonesia	14	4.2
South East Asia	2	0.6
East Asia	0	0
Oceania	0	0
Total	337	100

**Table 2: Total distribution of resistance-associated mutations and wild type among imported isolates of *P. falciparum* in Europe**

Resistance to	Gene locus	Mutation (%)	Wild type (%)	Total
Chloroquine	Pfcr1 76	204 (68.2%)	95 (31.8%)	299
	Pfmdr 86	85 (42.3%)	116 (57.7%)	201
Group I antifolates	DHFR 51	135 (44.3%)	170 (55.7%)	305
	DHFR 108	170 (58.4%)	121 (41.6%)	291
Group II antifolates	DHFR 59	156 (55.1%)	127 (44.9%)	283
	DHPS 436	79 (33.3%)	158 (66.7%)	237
	DHPS 437	92 (40.4%)	136 (59.6%)	228
	DHPS 540	12 (5.3%)	216 (94.7%)	228
	DHPS 581	6 (2.3%)	255 (97.7%)	261

Group I antifolates: e.g. pyrimethamine. Group II antifolates: sulfonamides DHFR = dihydrofolate reductase DHPS = dihydropteroate synthetase

function in enhancing the effect [1]. In similar fashion, DHPS437<sub>Ala/Gly</sub> is viewed as prerequisite for resistance to group II antifolates. Thus, *P. falciparum* has to gain mutations on both genes in order to develop resistance to combined antifolates, e.g. sulfadoxine/pyrimethamine. In the random population of isolates gained from returning European travelers, the distribution of the relevant mutations across sub-Saharan Africa was comparatively low (figures 2 and 3). The combination of triple mutations on DHFR (108<sub>Ser/Asp</sub>, 51<sub>Asp/Iso</sub> and 59<sub>Cys/Ar</sub>) was found in 39.2% of samples in West Africa, 30.8% in Central, 42.9% in East, and 33.3% in South Africa. The combination of DHPS437<sub>Ala/Gly</sub> and DHPS 540<sub>Lys/Glu</sub> which is viewed as a necessary prerequisite for resistance to sulfonamides [9], was prevalent in 3.4% of the samples imported from West Africa, and 5.7% from East Africa, while no sample from either Central Africa, or from South Africa showed the combination. Thus, antifolates may still be effective in a comparatively high percentage of isolates from these areas.

## Conclusions

This type of molecular surveillance has very little effect on treatment decisions for the individual travelers returning with *Falciparum* malaria from endemic regions. However, when used within a large clinical network, this method has an unsurpassed advantage. It has become possible to screen large numbers of isolates from malarious regions that are collected randomly from travelers and are transported back to Europe. This study shows that the continuous mapping of the patterns of resistance to crucial antimalarials can be performed. Data gained by fast and efficient molecular methods 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.

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