Malaria Journal

BioMed Central

Research

Open Access

Molecular surveillance of the antifolate-resistant mutation I164L in imported african isolates of *Plasmodium falciparum* in Europe: sentinel data from TropNetEurop

Ole Wichmann*†[1], Tomas Jelinek[1], Gabriele Peyerl-Hoffmann[3], Nikolai Mühlberger[1], Martin P Grobusch[2,4], Joaquim Gascon[8], Alberto Matteelli[10], Christoph Hatz[11], Hermann Laferl[12], Marco Schulze[5], Gerd Burchard[1,6], Saraiva da Cunha[14], Jiøi Beran[15], Paul McWhinney[16], Herwig Kollaritsch[13], Peter Kern[7], Juan Cuadros[9], Michael Alifrangis[17], Ida Gjørup[17] and the European Network on Surveillance of Imported Infectious Diseases (TropNetEurop)

Address: [1]Institute of Tropical Medicine. Charité, Humboldt University, Berlin, Germany, [2]Department of Medicine (Infectious Diseases), Charité, Humboldt University, Berlin, Germany, [3]Department of Infectious Diseases and Tropical Medicine, University of Munich, Germany, [4]Institut für Tropenmedizin, Eberhard-Karls-Universität Tübingen, Germany, [5]Städtische Kliniken "St. Georg", 2. Klinik für Innere Medizin, Leipzig, Germany, [6]Bernhard-Nocht-Institute for Tropical Medicine, University of Hamburg, Germany, [7]Sektion Infektiologie und Klinische Immunologie, Universität Ulm, Ulm, Germany, [8]Secció Medicina Tropical, Hospital Clinic, IDIBAPS, Barcelona, Spain, [9]Department of Clinical Microbiology and Parasitology, Hospital Príncipe de Asturias, Madrid, Spain, [10]Clinica di Malattie Infettive e Tropicali, Universitá di Bresci, Italy, [11]Swiss Tropical Institute, Basel, Switzerland, [12]Kaiser-Franz-Josef-Spital der Stadt Wien, 4. Medizinische Abteilung mit Infektions-und Tropenmedizin, Germany, [13]Abteilung für spezifische Prophylaxe und Tropenmedizin am Institut für Pathophysiologie, University of Vienna and Kaiser-Franz-Josef-Spital der Stadt Wien, Vienna, Austria, [14]Consulta de Medicina do Viajante, Departamento de Doenças Infecciosas, Hospital Universitário, Coimbra, Portugal, [15]Department of Infectious Diseases, University Hospital, Hradec Kralove, Czech Republic, [16]Bradford Royal Infirmary, Infection and Tropical Medicine, Bradford, United Kingdom and [17]Department of Infectious Diseases, University Hospital and Centre of Medical Parasitology, Panum Institute, University of Copenhagen, Denmark

Email: Ole Wichmann* - ole.wichmann@charite.de; Tomas Jelinek - jelinek@bbges.de; Gabriele Peyerl-Hoffmann - peyerl@lrz.uni-muenchen.de; Nikolai Mühlberger - muehlberger@bbges.de; Martin P Grobusch - martin.grobusch@uni-tuebingen.de; Joaquim Gascon - jgascon@clinic.ub.es; Alberto Matteelli - amatteelli@bsnet.it; Christoph Hatz - hatz@keep.touch.ch; Hermann Laferl - herman.laferl@kfj.magwien.gv.at; Marco Schulze - mhschulze@yahoo.com; Gerd Burchard - gerd.burchard@bni-hamburg.de; Saraiva da Cunha - saraiva@huc.min-saude.pt; Jiøi Beran - jiri.beran@vakcinace.cz; Paul McWhinney - pmcw@doctors.org.uk; Herwig Kollaritsch - herwig.kollaritsch@univie.ac.at; Peter Kern - peter.kern@medizin.uni-ulm.de; Juan Cuadros - jcuadros@efd.net; Michael Alifrangis - Malif@biobase.dk; Ida Gjørup - Ida.Gjorup@dadlnet.dk; the European Network on Surveillance of Imported Infectious Diseases (TropNetEurop) -

* Corresponding author †Equal contributors

Published: 25 June 2003

Malaria Journal 2003, **2**:17

Received: 06 May 2003
Accepted: 25 June 2003

This article is available from: http://www.malariajournal.com/content/2/1/17

Abstract

Background: Malaria parasites that carry the DHFR-mutation I164L are not only highly resistant to sulfadoxine-pyrimethamine but also to the new antimalarial drug chlorproguanil-dapsone. The spread of this mutation in Africa would result in a public health disaster since there is a lack of effective alternatives that are both affordable and safe. Up to now, this mutation has only been described in Asian and Latin-American countries. The objective of this study was to assess the prevalence of this mutation in African isolates of *Plasmodium falciparum* that have been imported into Europe through travellers.

Methods: TropNetEurop is a network for the surveillance of travel-associated diseases and seems to cover approximately 12% of all malaria cases imported into Europe. Within this network we screened 277 imported African isolates of *P. falciparum* with the help of PCR- and enzyme-digestion-methods for the antifolate-resistant mutation I164L.

Results: The I164L mutation was not detected in any of the isolates tested.

Discussion: Continuous molecular surveillance of mutations in *P. falciparum*, as it is practised within TropNetEurop, is an essential tool for the understanding and early detection of the spread of antimalarial drug resistance in Africa.

Background

Drug resistant malaria is a major problem in malaria control. At the present, there are few antimalarial drugs available in endemic areas which are both cheap and save. Since resistance to chloroquine has spread across sub-Saharan Africa, several countries have switched their first-line drug to the antifolate sulfadoxine-pyrimethamine [1].

Soon after sulfadoxine-pyrimethamine was introduced to malaria-control programs in the late 1960s, resistance to this drug was noted [2]. The efficacy of sulfadoxine-pyrimethamine primarily depends on the sensitivity of the parasite to pyrimethamine [3]. The dihydrofolate reductase domain of *Plasmodium falciparum* (pfDHFR) is the target of this drug. DHFR catalyzes the reduction of dihydrofolate to regenerate tetrahydrofolate which is required for one-carbon transfer reactions and deoxythymidylate synthesis of the parasites.

The discovery of changes in codons of the pfDHFR-gene strongly indicated that single amino acid changes lead to observed resistance. Mutations at amino acid position 51, 59, 108 and 164 have been shown to be linked with resistance of *P. falciparum* to antifolate antimalarials. A scheme for evolution of resistance could be derived as a result of stepwise mutations starting with the S108N mutation, which was shown to be the optimal mutation in leading to both the decreased binding affinity for inhibitors and the retention of enzyme activity [4,5]. Absolute resistance is conferred by the addition of I164L mutation in the quadruple mutant form (N108/I51/R59/L164). In this fourth mutation the enzyme is about 1,000-fold less sensitive to pyrimethamine [6].

The I164L mutation also plays an important role in the development of resistance to the more potent antifolate combination chlorproguanil-dapsone: while the triple mutant allele (N108/I51/R59) has no great impact on the sensitivity of this drug, parasites that carry the quadruple mutant allele are resistant [7,8]. This highly potent quadruple mutation was described first in South East Asia, later also in South America [9–11]. There is a concern that continued sulfadoxine-pyrimethamine pressure, as well as the widespread use of trimethoprim-sulfamethoxazole for prophylaxis against opportunistic infections in patients with AIDS in Africa, is going to select these highly resistant alleles [12]. This would rapidly make sulfadoxine-pyrimethamine, as well as chlorproguanil-dapsone, ineffective in this region. For this reason continued surveillance of this mutation is needed to evaluate the prevalence, the distribution and the speed with which these populations might be selected.

TropNetEurop is a European surveillance system that covers approximately 12% of all imported malaria cases in Europe [13]. Travellers can be used as a highly sensitive surveillance tool to detect development of drug-resistance in endemic areas, as it is suggested that most of them carry a monoclonal Plasmodium strain. Initial data on molecular surveillance, not including the I164L mutation, have already been published elsewhere [14].

Since there are only limited data available on the prevalence of the I164L mutation throughout Africa, the purpose of this survey was to screen a sample of *P. falciparum* imported from Africa for this particular antifolate mutation.

Material and methods

Sampling

The European Network on Imported Infectious Disease Surveillance (TropNetEurop) has been established in 1999 and, in 2001, covered approximately 12% of all imported malaria cases in Western and Central Europe [13]. At the present 45 clinical sites in Europe are members of the network with more than 57,000 patients seen post-travel annually.

Within the infrastructure of TropNetEurop several member sites started in 2001 to collect blood samples from patients diagnosed with a *P. falciparum* infection. 10 µl of whole blood from each patient were dotted on Whatman 3 MM® chromatography paper and air-dried at room temperature before initiation of treatment. The filter paper disks were sent to Munich for further preparation.

Table 1: Frequencies of geographical regions in which the observed *Plasmodium falciparum* infection has been acquired

	Frequency (%)	Mutation (%)	Wild type (%)
West-Africa	170 (62.7)	0 (0%)	166 (100%)
East-Africa	46 (16.6)	0 (0%)	46 (100%)
Central-Africa	36 (13)	0 (0%)	36 (100%)
South-Africa	17 (6.1)	0 (0%)	17 (100%)
Madagascar	2 (0.7)	0 (0%)	2 (100%)
Total	**271 (100)**	**0 (0%)**	**271 (100%)**
PCR-negative	6		

For this survey we screened all blood samples that were obtained between January 2001 and July 2002 from travellers returning from Africa with a microscopically confirmed *P. falciparum* infection.

Parasite DNA and Polymerase chain reaction

Parasite DNA was prepared from the dried blood spots on the filter paper by the Chelex method as described by Kain and Lanar [15]. Nested mutation-specific PCR was done as previously described for analysis of the DHFR 164 mutation site [16,17].

Restriction fragment length polymorphisms

A volume of 4 μl of PCR product was incubated for three hours at a temperature of 37°C with the mutation specific restriction enzyme DraI according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA). To discriminate between the two variants of codon 164, the 522 bp PCR product was digested with DraI to detect the leucine mutation (143 bp). The digestion products were separated by electrophoresis in a 1% SeaKem™ plus 1% NuSieve™ gel (FMC BioProducts, Rockland, ME, USA) containing ethidium bromide. DNA from established laboratory strains of P. falciparum served as controls of PCR and enzyme digests. Gels were recorded by photography.

Results

Over the 18-month time period we collected 277 blood samples from patients with a microscopically confirmed *P. falciparum* infection acquired in Africa. The DNA from all blood samples was amplified by nested PCR systems as described above. 271 samples showed the expected bands at 522 bp for the DHFR gene, while six samples turned out to be negative for *P. falciparum* DNA.

Among the 271 observed samples of *P. falciparum*, 170 (62.7%) were imported from West Africa, 46 (16.6%) from East Africa, 36 (13%) from Central Africa, 17 (6.1%) from South Africa and two from Madagascar (Tab 1).

After digestion none of the 271 isolates revealed the leucine-164 mutation. All 271 isolates of *P. falciparum* presented as the wild type with the amino acid isoleucine in position 164.

Discussion

The burden of falciparum malaria is carried mainly by tropical Africa. Since resistance to Chloroquine is now widespread in Africa, the antifolate combination sulfadoxine-pyrimethamine (SP) is often the only affordable alternative.

Unfortunately, SP is particularly prone to the rapid emergence of resistance [18]. There is evidence, that *in vitro* pyrimethamine chemosensitivity will be predictive of *in vivo* SP efficacy, and that parasitological resistance in Africa is primarily due to the triple mutant in DHFR: S108N, N51I and C59R [19–21].

At the moment, the only drug available in Africa to deal with SP failure is often further treatment with SP. Researchers are, therefore, seeking new, safe and inexpensive, second line drugs or combination regimens. Among these regimes chlorproguanil-dapsone is considered a potential replacement for SP in Africa because of its effectiveness against infections associated with triple mutants in DHFR, its short half-life and its low price [21–23]. However, the efficacy of combinations involving old drugs may be short-lived, since resistance-conferring mutations already exist. In the case of chlorproguanil-dapsone, the change in DHFR enzyme structure resulting from the I164L mutation is known to be associated with resistance to this drug [19,20].

Experience with SP in South East Asia and South America has shown that continued use of SP will select for the quadruple mutant DHFR, including I164L. So far, mutations of this potency have not been observed in Africa in any study using standard PCR analyses [10,17,21,23–27]. The I164L mutation was also not found in any of the isolates we analyzed within this survey.

Recently, Hastings *et al.* [28] developed a yeast-based system that is sensitive enough to identify point mutations in

the DHFR gene even if they are rare. With the help of this technique alleles that encoded the I164L mutations were isolated from 3 of 6 patient samples with SP treatment failure from Tanzania. However, mutations of all kinds occur during DNA replication, but not all establish themselves and are functional.

Overall, efficient surveillance systems to detect and monitor resistance to SP are required, since malaria infections will become fully resistant to SP and chlorproguanil-dapsone with the selection of the DHFR mutation I164L in Africa. For this reason, several national and regional networks of sentinel sites have been set up in recent years and collaborate in order to monitor antimalarial drug resistance. The East African Network for Monitoring Antimalarial Treatment (EANMAT), for example, started in 1998 to monitor drug resistance with standard *in vivo* tests [29]. TropNetEurop, as the largest network for infectious diseases acquired by travellers worldwide, can function as an additional sensitive tool in the surveillance of antimalarial drug resistance.

Acknowledgements

We wish to thank all TropNetEurop site staff, who has been invaluable in collecting material and data. Financial support from Dr. Democh Maurmeier Stiftung and the Förderprogramm für Forschung und Lehre der Medizinischen Fakultät, both at Ludwig-Maximilians-University, Munich, Germany, is gratefully acknowledged.

References

1. White NJ, Nosten F, Looareesuwan S, Watkins WM, Marsh K, Snow RW, Kokwaro G, Ouma J, Hien TT, Molyneux ME, Taylor TE, Newbold CI, Ruebush II TK, Danis M, Greenwood BM, Anderson RM and Olliaro P: **Averting a malaria disaster** *Lancet* 1999, **353:**1965-1967.
2. Bjorkman A and Phillips-Howard PA: **The epidemiology of drug-resistant malaria** *Trans R Soc Trop Med Hyg* 1990, **84:**177-180.
3. Sibley CH, Hyde JE, Sims PFG, Plowe CV, Kublin JG, Mberu EK, Cowman AF, Winstanley PA, Watkins WM and Nzila AM: **Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what is next?** *Trends Parasitol* 2001, **17:**582-588.
4. Sirawaraporn W, Yongkiettrakul S, Sirawaraporn R, Yuthavong Y and Santi DV: ***Plasmodium falciparum*: Asparagine mutant as residue 108 of dihydrofolate reductase is an optimal antifolate-resistant single mutation** *Exp Parasitol* 1997, **87:**245-252.
5. Yuthavong Y: **Basis for antifolate action and resistance in malaria** *Microbes Infect* 2002, **4:**175-182.
6. Plowe CV, Kublin JG and Doumbo OK: ***P. falciparum* dihydrofolate reductase and dihydropteroate synthase mutations: epidemiology and the role in clinical resistance to antifolates** *Drug Resist Updat* 1998, **1:**389-396.
7. Nzila-Mounda A, Mberu EK, Sibley CH, Plowe CV, Winstanley PA and Watkins WM: **Kenyan *Plasmodium falciparum* field isolates: correlation between pyrimethamine and chlorcycloguanil activity *in vitro* and point mutations in the dihydrofolate reductase gene** *Antimicrob Agents Chemother* 1998, **42:**164-169.
8. Wilairatana P, Kyle DE, Looareesuwan S, Chinwongprom K, Amradee S, White NJ and Watkins WM: **Poor efficacy of antimalarial biguanide-dapsone combinations in the treatment of acute, uncomplicated falciparum malaria in Thailand** *Ann Trop Med Parasitol* 1997, **91:**125-132.
9. Basco LK, Eldin de Pecoulas P, Wilson CM, Le Bras J and Mazabraud A: **Point mutations in the dihydrofolate reductase-thymidylate synthase gene and pyrimethamine and cycloguanil resistance in *Plasmodium falciparum*** *Mol Biochem Parasitol* 1995, **69:**135-138.
10. Plowe CV, Cortese JF, Djimde A, Nwanyanwu OC, Watkins WM, Winstanley PA, Estrada-Franco JG, Mollinedo RE, Avita JC, Cespedes JL, Carter D and Doumbo OK: **Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance** *J Infect Dis* 1997, **176:**1590-1596.
11. Masimirembwa CM, Phuong-dung N, Phuc BQ, Duc-Dao L, Sy ND, Sköld O and Swedberg G: **Molecular epidemiology of *Plasmodium falciparum* antifolate resistance in Vietnam: genotyping for resistance variants of dihydropteroate synthase and dihydrofolate reductase** *Int J Antimicrob Agents* 1999, **12:**203-211.
12. Wongsrichanalai C, Pickard AL, Wernsdorfer WH and Meshnick SR: **Epidemiology of drug-resistant malaria** *Lancet Infect Dis* 2002, **2:**209-218.
13. Jelinek T, Schulte C, Behrens R, Grobusch MP, Coulaud JP, Bisoffi Z, Martteello A, Clerinx J, Corachan M, Puente S, Gjorup I, Harms G, Kollaritsch H, Kotlowski A, Bjorkmann A, Delmont JP, Knobloch J, Nielson LN, Cuadros J, Hatz C, Beran J, Schmid ML, Schulze M, Lopez-Velez R, Fleischer K, Kapaun A, McWhinney P, Kern P, Atougia J, Fry G, da Cunha S and Boecken G: **Imported Falciparum malaria in Europe: sentinel surveillance data from the European network on surveillance of imported infectious diseases** *Clin Infect Dis* 2002, **34:**572-576.
14. Jelinek T, Peyerl-Hoffmann G, Mühlberger N, Wichmann O, Schmider N, Grobusch MP, Corachán M, Hatz C, Harms G, Laferl H, McWhinney P, Schulze M, Kollaritsch H, da Cunha S, Beran J, Kern P, Gjørup I and Cuadros J: **Molecular surveillance of drug resistance through imported isolates of *Plasmodium falciparum* in Europe: sentinel data from TropNetEurop** *Malar J* 2002, **1:**11.
15. Kain KC and Lanar DE: **Determination of genetic variation within *Plasmodium falciparum* by using enzymatically amplified DNA from filter paper disks impregnated with whole blood** *J Clin Microbiol* 1991, **29:**1171-1174.
16. Duraisingh MT, Curtis J and Warhurst DC: ***Plasmodium falciparum*: detection of polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes by PCR and restriction digestion** *Exp Parasitol* 1998, **89:**1-8.
17. Jelinek T, Rønn AM, Lemnge MM, Curtis J, Mhina J, Duraisingh MT, Bygbjerg IC and Warhurst DC: **Polymorphisms in the dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) genes of *Plasmodium falciparum* and *in vivo* resistance to sulphadoxine/pyrimethamine in isolates from Tanzania** *Trop Med Int Health* 1998, **3:**605-609.
18. Winstanley PA: **Chemotherapy for falciparum malaria: the armoury, the problems and the prospects** *sitol Today* 2000, **16:**146-153.
19. Peterson DS, Milhous WK and Wellems TE: **Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria** *Proc Natl Acad Sci USA* 1990, **87:**3018-22.
20. Watkins WM, Mberu PA, Winstanley PA and Plowe CV: **The efficacy of antifolate antimalarial combinations in Africa: a predictive model based on pharmacodynamic and pharmacokinetic analyses** *Parasitol Today* 1997, **13:**459-464.
21. Mutabingwa T, Nzila A, Mberu E, Nduati E, Winstanley P, Hills E and Watkins W: **Chlorproguanil-dapsone for treatment of drug-resistant falciparum malaria in Tanzania** *Lancet* 2001, **358:**1218-1223.
22. Winstanley PA, Watkins W, Muhia D, Szwandt S, Amukoye E and Marsh K: **Chlorproguanil/dapsone for uncomplicated *Plasmodium falciparum* malaria in young children: pharmacokinetics and therapeutic range** *Trans R Soc Trop Med Hyg* 1997, **91:**322-327.
23. Kublin JG, Dzinjalamala FK, Kamwendo DD, Malkin EM, Cortese JF, Martino LM, Mukadam RAG, Rogerson SJ, Lescano AG, Molyneux ME, Winstanley PA, Chimpeni P, Taylor TE and Plowe CV: **Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria** *J Infect Dis* 2002, **185:**380-8.
24. Nzila AM, Mberu EK, Sulo J, Dayo H, Winstanley PA, Sibley CH and Watkins WM: **Towards an understanding of the mechanism of pyrimethamnine-sulfadoxine resistance in *Plasmodium falciparum*: genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenian parasites** *Antimicrob Agents Chemother* 2000, **44:**991-6.

25. Eberl KJ, Jelinek T, Aida AO, Peyerl-Hoffmann G, Heuschkel C, el Valy AO and Christophel EM: **Prevalence of polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes of *Plasmodium falciparum* isolates from southern Mauritania** *Trop Med Int Health* 2001, **6:**756-760.
26. Khalil I, Alifrangis M, Ronn AM, Gabar HA, Jelinek T, Satti GM and Bygbjerg IC: **Pyrimethamine/sulfadoxine combination in the treatment of uncomplicated falciparum malaria: relation between dihydropteroate synthase/dihydrofolate reductase genotypes, sulfadoxine plasma levels, and treatment outcome** *Am J Trop Med Hyg* 2002, **67:**225-229.
27. Curits J, Maxwell CA, Msuya FHM, Mkongewa S, Alloueche A and Warhurst DC: **Mutations in *dhfr* in *Plasmodium falciparum* infections selected by chlorproguanil-dapsone treatment** *J Infect Dis* 2002, **186:**1861-1864.
28. Hastings MD, Bates SJ, Blackstone EA, Monks SM, Mutabingwa TK and Sibley CH: **Highly pyrimethamine-resistant alleles of dihydrofolate reductase in isolates of *Plasmodium falciparum* from Tanzania** *Trans R Soc Trop Med Hyg* 2002, **96:**674-676.
29. Mutabingwa TK, Watkins WM and d'Alessandro U: **Monitoring of drug-resistant malaria in Africa** *Lancet* 2002, **360:**875.