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Detection of molecular markers for chloroquine and pyrimethamine/sulfadoxine resistance in imported cases of *Plasmodium falciparum* malaria in Poland

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Abstract

The identified mutations in the *pfcrt*, *dhfr* and *dhps* genes of *Plasmodium falciparum* show a very high correlation with resistance to chloroquine, pyrimethamine and sulfadoxine, the drugs that are still used as malaria chemoprophylaxis or treatment. We undertook a molecular screening of 82 Polish *P. falciparum* isolates, mainly imported from different countries of sub-Saharan Africa to assess their molecular drug-resistance profiles. Only 4 isolates showed no mutations in the three analyzed gene fragments. In the remaining isolates from one to six mutations in one or more examined genes were found. Different mutations in the *pfcrt*, *dhfr* and *dhps* genes were found in ca. 76%, 80% and 70% of *P. falciparum* isolates, respectively. About forty our patients used chloroquine or pyrimethamine + sulfadoxine as malaria chemoprophylaxis and/or antimalarial treatment, but without success. In all but 5 of the *P. falciparum* isolates obtained from these persons, mutations associated to resistance of the parasite to chloroquine and the antifolate drugs were found.

Key words

Plasmodium falciparum, drug resistance, molecular markers, chloroquine, pyrimethamine, sulfadoxine

Introduction

At present, malaria is one of the most important infectious diseases in addition to AIDS and tuberculosis. The incidence of malaria is estimated at between 300 and 500 million cases annually and the annual mortality is estimated at about 3 million, mainly children and pregnant women (WHO 1999). Malaria is also recognized as an increasing problem in tourists and in other travelers returning from the tropics.

During the last 10 years, there were 16 to 37 malaria cases diagnosed yearly in Poland (Dzbeński *et al*. 2004). Most probably, not all imported cases were reported and some cases may have been treated before the infected person returned to Poland. Available data suggest that despite the low incidence rate, the mortality rate in persons with diagnosed malaria in Poland is 7 to 16 times higher than in the other European countries (Dzbeński *et al*. 2004). After the entry of Poland to the European Union in 2004, the number of persons traveling to malaria endemic areas is increasing. Thus, an increase of imported malaria cases in Poland is expected.

Drug resistance has become a major public health hazard in malaria. The widespread distribution of resistant *Plasmodium falciparum* strains is a major concern both for chemoprophylaxis and treatment. The distribution of resistance of *P. falciparum* strains is accelerated by migration resulting in introduction of resistant malaria strains into new areas. The prevalence of multiple drug resistance throughout the endemic malarious areas has complicated the control of this disease. Assessment of the frequency of resistance and of its dynamics may help define the standards of management and prophylaxis of *P. falciparum* malaria. Therefore, it is important to monitor the occurrence of drug-resistant *P. falciparum* both in endemic areas as well as in malaria cases imported into temperate regions, including Europe.

In the last decades several molecular markers for *P. falciparum* drug resistance have been identified, including among

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others the *pfcrt* point mutations associated with chloroquine resistance and *dhfr* and *dhps* point mutations associated with sulfadoxine/pyrimethamine and proguanil resistance (Wongsrichanalai *et al*. 2002).

The aim of our investigations was to assess the molecular drug-resistance profiles of *P. falciparum* isolates imported to Poland from different countries of the world, mainly from Africa, by using molecular techniques, namely PCR followed by DNA sequencing. The resulting genotypes (mutations) were compared with the outcomes of prophylaxis and treatment.

Materials and methods

Blood specimens

Plasmodium falciparum isolates (n = 82) imported to Poland from 1996 to 2005 from endemic malarious areas by tourists, business or service travelers (including missionaries) were studied. The most common were travelers from sub-Saharan African followed by Central America and Papua New Guinea. Among Polish subjects, 34% of the *P. falciparum* infected patients returning from Africa used chloroquine as the main chemoprophylactic drug. Several patients used other drugs; however, the largest group of patients did not use any prophylaxis at all.

The patients were diagnosed and treated in two Polish hospitals: Department of Tropical and Parasitic Diseases, Medical University of Gdańsk and the Department of Zoonotic and Tropical Diseases, Medical University of Warsaw. In all patient specimens malaria parasites were detected by microscopic examinations (thin and thick blood films) and confirmed by PCR (Myjak *et al*. 2002, Johnston *et al*. 2006). For molecular examinations whole blood anticoagulated with EDTA was used. Written informed consent was obtained from all patients.

Molecular methods

DNA was extracted from 100 µl of whole EDTA-stabilized blood as described by Myjak *et al*. (2002). To determine the mutations and genotype of the relevant part of the *pfcrt, dhfr* and *dhps* genes, a fragment of these genes were amplified through PCR and sequenced.

PCR primers

The following PCR primers were used to amplify the: 211 base pair diagnostic region of the *pfcrt* gene: TCRP2A (Fidock *et al*. 2000) and TCRP2 (Severini *et al*. 2006); 718 base pair diagnostic region of the *dhfr* gene: AMP1 and AMP2 (Plowe *et al*. 1995), AMP3: 5'-CAAGATTGATACATAAAG-3' and AMP4: 5'-TAAAATAAACAAAATCATC-3' (Pieniazek N.J. personal communication); 748 base pair diagnostic region of the *dhps* gene: (Pieniazek N.J. personal communication) DHPSF: 5'-TTATGATTCTTTTTCAGATG-3', DHPSR: 5'-CCAATTGTGTGATTTGTCAC-3', DHPS-IF2: 5'-TTATAAAATTATTAAAAAAAAAAAAC-3' and DHPS-IR4: 5'-GAATTTGTTTTTTTTTTTTAATAA-3'.

PCR reaction setup

Master-mixes used for PCR reaction are as follows: 5 µl 10 × PCR buffer, 5 µl (4 for *dhfr*) $MgCl_2$ 25 mM, 4 µl dNTP 2.5 mM mixture, 1 µl (10 pmol) of each primer, 0.25 µl polymerase (AmpliTaq Gold polymerase, Applied Biosystems, USA, 1 µl = 5 IU), 1 µl genomic DNA or product from I PCR, 32.75 µl (33.75 for *dhfr*) distilled water.

PCR reaction conditions were as follows: initial activation of polymerase at 95°C for 15 min, 45 cycles: denaturation at 94°C (92°C for *dhfr*) for 30 s, annealing at 60°C for 30 s (49°C and 45 s for *dhfr*), and extension at 72°C for 1.5 min (65°C and 60 s for *dhfr*), followed by extension at 72°C for 10 min and finished with a hold step at 4°C. DNA amplification was performed in the 7600 Gold thermocycler (Applied Biosystems, USA).

Purification of PCR and sequencing products

PCR products were purified directly from the reaction by using the Clean-up kit or from gel slices using the Gel-out kit. Sequencing reaction products were purified by using the Ex-Terminator kit. All kits were from A&A Biotechnology, Gdynia, Poland.

Purified sequencing reaction products were thermally denatured and analyzed by using the ABI PRISM 310 DNA sequencer (Applied Biosystems, USA), according to the manufacturer's protocol. Sequences were analyzed with the use of ABI PRISM DNA Sequencing Analysis v.3.7 for the Windows (Applied Biosystems) and GeneStudio Pro software (GeneStudio, Inc.).

Results

Out of the 82 *P. falciparum* isolates examined, only 4.9% (three from Africa and one from Central America) were found not to have any mutations in each of the three examined gene fragments. The most frequent mutation in the *pfcrt* gene was found to be in codon 76; in the *dhfr* gene – in codon 108 and in the *dhps* gene – in codon 437 (Table I). In none of the examined *P. falciparum* isolates mutations (66, 342 and 511 nucleotide in the *dhfr* gene) associated only to the resistance to proguanil were found.

Basing on the lack or the presence of the mutation and their number in a respective isolate, the 3, 7 and 9 genotypes in *pfcrt*, *dhfr* and *dhps* genes, were distinguished, respectively (Table II).

Comparison of genotypes occurring in P. falciparum *isolates obtained from Polish travelers to East Africa and West Africa*

Among the 80 persons returning from Africa, 25 traveled to East Africa, 40 to West Africa and for following 15 we have no data. It was found that the percentage of wild (sensitive) *P. falciparum* isolates, as far as the *pfcrt* and *dhfr* genes were concerned, was higher in those traveling to West Africa (26.3% and 23.1%, respectively) than in travelers to East Africa

Table I. Mutations detected in fragments of genes: *pfcrt*, *dhfr* and *dhps* of *Plasmodium falciparum* isolates (most important mutations associated to the drug-resistance are in bold type)

									Without mutations (wild)	Total sample number
Mutations in the codon of the *pfcrt* gene	Mutations	72	73	74	75	**76**				
	number	0	0	60	60	62[a]			20 + 2[a]	82
	per cent	0	0	73.2	73.2	75.6			24.4 + 2.4	
	mutations	**51**	**59**	**108**						
Mutations in the codon of the *dhfr* gene	number	49	50	64[b]					16 + 1[b]	82
	per cent	59.7	61.0	78.0					19.5 + 1.2	
	mutations	431	**436** G	**436** T	**437**	**540**	**581**	613		
Mutations in the codon of the *dhps* gene	number	4	16[c]	1c	35[c]	7	2	1	18 + 2[c]	61[d]
	per cent	6.6	26.2	1.6	57.4	11.5	3.3	1.6	29.5 + 3.3	

[a]Two mixed isolates (mutant and wild), [b]1 mixed isolate (mutant and wild), [c]2 mixed isolates (mutant and wild), [d]in 21 samples PCR product was not obtained.

Table II. Results of the search for mutations in three genes of *Plasmodium falciparum* isolates obtained from Poles returning from the tropics

Pyrimethamine			**Chloroquine**		**Sulfadoxine**									
	Genotype	n	genotype	n	mutation at codon position (R-1 – R-8: genotype symbol)								no mutation (wild)	PCR product not obtained
					437	437, 540	437, 540, 581	437, 613	431, 436G, 437	436G	436G, 437	436G, 436T		
Symbol	mutation in position		symbol (mutation)		R1	R2	R3	R4	R5	R6	R7	R8	S	
R1	51-59-108	34	S (no mutation)	7									4	3
			E1 (74, 75, 76)	27	7	3	2	1	4	4				6
R2	59-108	14	S (no mutation)	5							1	1*	2	1
			E1 (74, 75, 76)	8	2					2			4	
			S and W1b (0 and 76)	1		1								
R3	51-108	12	S (no mutation)	2										2
			E1 (74, 75, 76)	10	5 + 1*						2		2	
R4	108	2	E1 (74, 75, 76)	2	1								1	
R5	51-59	1	E1 (74, 75, 76)	1									1	
R6	51	1	E1 (74, 75, 76)	1									1	
R1 and R2	51-59-108 and 59-108	1	E1 (74, 75, 76)	1		1								
S and R4	0 and 108	1	S and W1b (0 and 76)	1	1									
S	0	16	S (no mutation)	6	2								3	1
			E1 (74, 75, 76)	10						1	1*			8
Total		82		82	19	5	2	1	4	7	4	1	18	21

*Mixed infection with wild (sensitive) isolate; 0 – no mutation (wild isolate).

Table III. The comparison of the efficiency of chemoprophylaxis and/or treatment with chloroquine and Daraprim or Fansidar depending on the mutations of *P. falciparum* isolates in genes *pfcrt*, *dhfr* and *dhps* (only mutations in the gene related to the resistance to used drugs are marked)

		Chloroquine			Pyrimethamine + Sulfadoxine			Mutations (genotype)											
		chemo-prophy-lactic	treat-ment	drug resis-tance	chemo-prophy-lactic D	treat-ment F (D)	drug resis-tance	*pfcrt* (chloroquine)		*dhfr* (pyrimethamine)			*dhps* (sulfadoxine)						
Place of infection	n							wild (E1)	74, 59, 108 (R1)	51, 75, 76 (R2)	59, 108 (R3)	51, 108 (R1)	437 (R2)	437, 450 (R4)	437, 613 (R5)	431, 436G, 437 (R6)	436G (R7)	436G, 437	no
Central America	1	1						1											
Papua NG	1	1	1	1					1										
Africa	19	19						3	16										
	6	3	6	4				1	5										
	7	3	4	4	1	6	6		7	4		3	2		1	1	1		2
	6				6	1	1			3	3			1		1		1	3
Total – chloroquine	34	27	11	9				5	29										
Total – F/D	13				7	7	7			7	3	3	2	1	1	2	1	1	5

D – Daraprim, F – Fansidar, no – PCR product not obtained.

(20.0% and 8.0%, respectively); however, for the *dhps* gene the opposite (22.2% and 47%, respectively) was true. Nevertheless, these differences for these findings are not statistically significant (χ^2 = <2.966; p>0.05).

The effect of the usage of chloroquine, Fansidar or Daraprim for malaria chemoprophylaxis and/or for the treatment of Polish travelers infected with P. falciparum *parasites depending on the genotype of the isolate*

Twenty seven patients used chemoprophylaxis (chloroquine), but, nevertheless got infected with *P. falciparum*. In 22 cases mutations K67T (the genotype E1) associated to *P. falciparum* resistance to chloroquine were detected. Moreover, 11 persons were treated with chloroquine outside Poland and in 10 of them the treatment was ineffective. Only one isolate from those cases was wild-type (sensitive to chloroquine), whereas mutations K76T were detected in all the other isolates (Table III). For example, the patient returning from Papua New Guinea, received chloroquine for prophylaxis, was treated with this drug, and the isolate showed mutations in codon 74, 75 and 76. The inefficacy of chloroquine in the case of wild (sensitive) isolates can be explained by an inadequate dosage of the drug or by its irregular use.

Seven persons unsuccessfully used Daraprim for chemoprophylaxis. *P. falciparum* isolates from these persons were found to have three (51, 59 and 108) or two mutations (59 and 108) in the *dhfr* gene related to the resistance to pyrimethamine. Seven patients were also treated with Fansidar or Daraprim, but relapses of the disease occurred in those persons after they returned to Poland. All isolates from these persons showed three or two mutations in codons 108, 59 and 51 of the *dhfr* gene as well as mutations 436G, 437, 540 and 613 in the *dhps* gene (Table III). All of these patients were subsequently treated using the standard protocol with mefloquine, artesunate or quinine, and recovered after treatment.

Discussion

Detection of point mutations in target genes for antimalarial drugs is usually done by using digestion of PCR products with different restrictive enzymes related to the given mutation (Zindrou *et al*. 1996; Jelinek *et al*. 1999, 2002), and PCR with mutation-specific primers is also used (Gyang *et al*. 1992).

The method of sequencing the PCR products used by us enables the detection of all mutations (known and new) in the fragment of the analyzed gene in one step, as well as the determination of genotypes of the examined isolates.

In our investigations, the frequency of the mutations, except for locus DHPS 346G, was always higher than in *P. falciparum* isolates obtained from European travelers from western or central Europe (Jelinek *et al*. 2002). Moreover, single mutations in loci DHPS 613, 431 and 346T, not previously found by Jelinek *et al*. (2002) were detected in the specimens analyzed by our laboratory. Also the frequency of the mutations in the *pfcrt* gene was somewhat higher in Polish travelers than in Italian travelers or immigrant (Severini *et al*. 2006).

The mutations in the *dhfr*, *dhps* and *pfcrt* genes of *P. falciparum* identified to this time showed almost 100% correlations with resistance to the particular drug (Wongsrichanalai *et al*. 2002). In some cases, however, such a high correlation was not observed (Dorsey *et al*. 2001, Mayor *et al*. 2001). Recent research on *P. falciparum* isolates obtained from Europeans returning from tropical regions, revealed an association between mutations and chloroquine and antifolates resistance. Durand *et al*. (2001) studied *P. falciparum* isolates obtained form 62 French citizens, who used chloroquine in combination with proguanil as their chemoprophylaxis. They proved that all isolates had K76T mutation in the *pfcrt* gene and 61 isolates (98%) had mutation in codon 108 of the *dhfr* gene. Chloroquine and proguanil or their derivatives in lethal doses were found in blood of the majority of these patients.

Moreover, Severini *et al*. (2006) tested *in vitro* 25 Italian imported *P. falciparum* isolates for their susceptibility to chloroquine. In 23 isolates the genotypes of these isolates were consistent with the phenotypes of isolate. However, in the two cases, *P. falciparum* isolates showed K76T and other mutations but the phenotype was susceptible to the drug. They believe that this discrepancy was probably due to the difficulty in interpretation of the results of Mark III tests in comparison to pLDH test, used for *in vitro* susceptibility to chloroquine. Thus, it can be assumed that similar comparisons may be related also to Polish *P. falciparum* isolates.

The majority of our patients using chloroquine or pyrimethamine (or pyrimethamine + sulfadoxine) as chemoprophylaxis contracted malaria and their treatment with these drugs was ineffective. It can be related with drug-resistance of *P. falciparum* isolates to the drugs used, since in most of these isolates mutations associating to the resistance of the parasite to chloroquine and the antifolate drugs were found. On the other hand, the inefficacy of these drugs in the case of wild (sensitive) isolates can be explained by an inadequate dosage regimen of the drugs or by their irregular use. Therefore, doctors and travel offices should not recommend these drugs as malaria prophylactics or treatment of malaria patients.

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