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Molecular Markers of *Plasmodium falciparum* Drug Resistance in Parasitemic Pregnant Women in the Middle Forest Belt of Ghana

Joseph Osarfo,[1]* Harry Tagbor,[2] Pascal Magnussen,[3,4] and Michael Alifrangis[3,5]

[1]*Ghana Health Service, Effiduase District Hospital, Effiduase, Ashanti Region, Ghana;* [2]*School of Medicine, University of Health and Allied Sciences, Ho, Ghana;* [3]*Centre for Medical Parasitology, Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark;* [4]*Department of Veterinary and Aquatic Sciences, Section for Parasitology and Aquatic Diseases, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark;* [5]*Copenhagen University Hospital (Rigshospitalet), Copenhagen, Denmark*

Abstract. Data on prevalence of antimalarial molecular resistance markers in pregnant women in Ghana is scarce. Prevalence of single nucleotide polymorphisms/haplotypes in the *Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps* genes was assessed in a cross-sectional study involving 200 pregnant women. Almost 90% of infections were wild type at the *Pfcrt* gene whereas the *Pfmdr1* NFD mutant haplotype occurred in 43% of samples. Prevalence of *Pfdhfr*/*Pfdhps* quadruple mutation was 92.6% whereas *Pfdfr*/*Pfdhps* quintuple mutation with K540E was not observed. The study provides important updates of antimalarial resistance markers in Ghanaian pregnant women and suggests increased tolerance to one of the first-line treatment options in Ghana: artemether–lumefantrine. The data support the view that sulfadoxine–pyrimethamine is still efficacious for intermittent preventive treatment in Ghana, but the impact of increased doses on selection of mutations needs to be assessed. Continuing the surveillance of resistance markers is important to inform changes in antimalarial drug policy in pregnancy.

Pregnancy-associated malaria (PAM) leads to maternal and newborn morbidity and mortality.[1] Control measures include intermittent preventive treatment with sulfadoxine–pyrimethamine (IPTp-SP); regular and correct use of long-lasting insecticide-treated nets; and prompt diagnosis and treatment with efficacious antimalarials, including artemisinin-based combination therapies (ACTs). However, parasite resistance to antimalarial drugs and mosquito resistance to insecticides mitigate these measures.[2]

Particularly, *Plasmodium falciparum* resistance to SP has resulted in the declining protective efficacy of IPTp-SP mainly in eastern Africa.[3] Stepwise mutations in mainly codons 108, 51, and 59 of the *Pfdhfr* gene and codons 436, 437, 540, 581, and 613 in the *Pfdhps* gene lead to resistance in vitro and in vivo.[4,5] The *Pfmdr1* single nucleotide polymorphisms (SNPs) are linked to altered sensitivity to various antimalarials including monotherapies lumefantrine, amodiaquine (AQ), quinine, and artemisinins and the ACTs as well.[6,7] Altered parasite sensitivity to AQ is also linked to similar mechanisms for chloroquine (CQ) resistance, which in turn is associated with mutations at codons 72–76 of the *Pfcrt* gene, particularly at codon 76 (K76T).[8] Wild-type *Pfcrt* variants have been reportedly selected by artemether–lumefantrine (AL).[7]

In Ghana, PAM accounts for 17.6% of outpatient department attendance, 13.7% of admissions among pregnant women, and 3.4% of maternal deaths.[9] Quinine is used to treat uncomplicated malaria in the first trimester whereas since 2005 and 2008, artesunate–amodiaquine and AL, respectively, are used in the second and third trimesters. Sulfadoxine–pyrimethamine has, since 2005, been used for IPTp alone.

Surveillance of molecular markers allows early detection of changing antimalarial drug susceptibility and may inform changes in drug policy. However, data on current prevalence of antimalarial resistance markers in Ghanaian pregnant women, compared with the general population, are scarce. Prevalences of 69% for *Pfcrt* K76T, 66% for *Pfmdr1* N86Y, 80% for *Pfdhfr* N108S, and 36–73% for *Pfdhfr* triple mutations were reported in studies conducted from 1998 to 2006.[10–12] With increasing coverage and doses of IPTp-SP and wider ACT utilization, it is appropriate to evaluate how these changes have influenced the prevalence of antimalarial resistance markers in this population.

We assessed the prevalence of SNPs/haplotypes in the *Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps* genes in a cross-sectional study of asymptomatic pregnant women of all gravidity enrolled in a drug trial from antenatal clinics at St. Michael's and Bekwai Government hospitals in the Bosomtwe and Bekwai districts, respectively, in Ghana.[13] The geometric mean parasite density was 224/μL (95% confidence interval; 193, 289).[13] Some women were likely exposed to IPTp-SP in previous pregnancies whereas ACT use cannot be ruled out given that second and third trimester women were enrolled.

Filter paper spots from 200 women, picked by simple random selection, were collected at recruitment from July 2011 to October 2012. The study protocol was approved by the Committee for Human Research and Publication Ethics, Kwame Nkrumah University of Science and Technology, Ghana (CHRPE 190/10 and CHRPE/AP/236/12). Written informed consent was obtained from each participant.

Parasite DNA extraction was by the Chelex-100 method.[14] Polymerase chain reaction amplification of segments of the *Pfcrt*, *Pfdhfr*, *Pfdhps*, and *Pfmdr1* genes and analyses of SNPs at these genes were as described earlier.[15,16] Laboratory parasite strains 3D7, HB3, FCR3, and DD2 and blood samples obtained from non-malaria–exposed Danish citizens served as controls. Data were entered in Microsoft Excel 2007 and analyzed using proportions for prevalence and frequency of SNPs and haplotypes. Prevalence measures included mixed infections whereas frequency excluded them.

Data analysis included 199 samples. Successful *Pfcrt* haplotyping of codons 72–76 was achieved in 83.9% of samples (167/199). Infections with the CVMNK wild-type haplotype were predominant with a prevalence of 88.6%

* Address correspondence to Joseph Osarfo, Ghana Health Service, Effiduase District Hospital, Effiduase, Ashanti Region, Ghana. E-mail: josarfo@yahoo.co.uk

TABLE 1
Prevalence and frequency of *Pfmdr1* SNPs at codons 86, 184, and 1,246

	Prevalence			Frequency*		
Pfmdr1 SNPs	Evaluable samples (*N*)	Number with specified SNP (*n*)	% (*n*/*N* × 100)	Evaluable samples (*N*)	Number with specified SNP (*n*)	% (*n*/*N* × 100)
N86	164	144	87.8	158	138	87.9
86Y	164	25	15.2	158	19	12.1
184F	149	88	59.1	137	76	55.5
Y184	146	48	46.6	137	61	44.5
D1246	155	153	98.7	155	153	98.7
1246Y	144	2	1.4	144	2	1.4

SNPs = single nucleotide polymorphisms.
* Frequency excludes mixed infections whereas prevalence includes mixed infections.

(148/167). At the *Pfmdr1* gene, wild-type SNPs N86 and D1246 were dominant with a prevalence of 87.8% and 98.7%, respectively (see Table 1). The single mutant N86-184**F**-D1246 (NFD) and the wild-type N86-Y184-D1246 haplotypes were equally frequent at 43.2%. YYD and NFY haplotypes also occurred in equal frequencies of 1.6% whereas YFD and YFY had frequencies of 10.4% and 0.8%, respectively. The triple mutant 86**Y**-184**Y**-1246**Y** haplotype was not observed.

Mutations at *Pfdhfr* codons 51, 59, and 108 were predominant with frequencies of 75.7% (103/136), 85% (119/140), and 87.8% (122/139), respectively. Neither the S108T nor the I164L mutations were observed (see Table 2).

At the *Pfdhps* gene, the combined frequency of the 437G mutation (as either 436/437AG or 436/437SG) was 96.5%. Only one sample that was a mixed infection showed the K540E mutation (see Table 2). The frequencies of A581G and A613S mutations were 2.7% and 6.2%, respectively.

TABLE 2
Prevalence and frequency of SNPs at the *Pfdfhr* and *Pfdhps* genes

		Prevalence			Frequency		
Codon	SNPs	Evaluable sample (*N*)	Number with specific SNP (*n*)	% (*n*/*N* × 100)	Evaluable sample (*N*)	Number with specific SNP (*n*)	% (*n*/*N* × 100)
Pfdhfr							
50/51	CN	142	36	25.5	136	33	24.3
	CI	142	106	75.5	136	103	75.7
59	C	146	24	16.4	140	21	15.0
	R	146	122	83.6	140	119	85.0
108	S	139	17	12.2	139	17	12.2
	N	139	122	87.8	139	122	87.8
	T	0		0			
164	I	132	132	100.0	132	132	100.0
	L	0		0			
Pfdhps							
436/437	AA	147	3	2.0	141	3	2.1
	AG		95	64.6		95	67.4
	SA		3	2.0		1	0.7
	SG		45	30.6		41	29.1
	FA		1	0.7		1	0.7
540	K	143	142	99.3	141	141	100.0
	E		1	0.7	0		
581	A	152	147	96.7	150	146	97.3
	G		5	3.3		4	2.7
613	A	148	138	93.2	146	137	93.8
	S		10	6.8		9	6.2
Pfdhfr and *Pfdhps* haplotypes*							
Pfdhfr† (*N* = 126)							
CIRNI			97	77			
CNCSI			9	7.1			
Pfhdps (*N* = 130)							
AGKAA			80	61.5			
SGKAA			39	30			
AGKAS			6	4.6			
AGKGS			1	0.8			
AAKAA			3	2.3			
SAKAA			1	0.8			
Pfdhfr/ (*N* = 81)							
CIRNI/AGKAA (quadruple mutation)			42	51.9			
CIRNI/SGKAA (quadruple mutation)			33	40.7			
CIRNI/AGKAS (quintuple mutation)			5	6.2			
CIRNI/AGKGS (sextuple mutation)			1	1.2			

SNPs = single nucleotide polymorphisms.
* Only frequencies are reported for haplotypes and *N* is evaluable samples whereas *n* is the number of haplotypes.
† The *Pfdhfr* double mutations (CN**RN**I + C**IC**N**I** + C**IR**SI) collectively constituted 15.9%.

Combining *Pfdhfr* and *Pfdhps* SNPs into haplotypes showed that only 7.1% of isolates (9/126) were pure *Pfdhfr* wild type (CNCSI) whereas 77.0% (97/126) carried the triple mutation (C**IRN**I). For *Pfdhps*, 3.1% (4/130) were pure wild type (three AAKAA and one SAKAA haplotypes). Single *Pfdhps* mutated haplotypes A**G**KAA and S**G**KAA were present in 91.5% (119/130) of samples whereas only one isolate exhibited the triple mutated haplotype A**G**K**GS** (see Table 2).

The *Pfdhfr*/*Pfdhps* quadruple mutation (C**IRN**I-437G) was present in 92.6% (75/81) of isolates. The quintuple mutation (51I + 59R + 108N/437G + 613S) was found in 6.2% (5/81) whereas the sextuple mutation (51I + 59R + 108N/437G + 581G + 613S) was present in 1.2% (1/81) of isolates. The quintuple mutation with *Pfdhps* K540E (51I + 59R + 108N/437G + 540E) was not observed.

Close to 90% of *P. falciparum* infections exhibited the CVMNK wild-type haplotype at codons 72–76 of the *Pfcrt* gene. The prevalence of the CVIET mutant haplotype (*Pfcrt* K76T) is likely the lowest reported in Ghana since changing from CQ treatment of uncomplicated malaria in 2005 and contrasts sharply with a recent report in nonpregnant populations in Ghana.[17] Apart from repopulation of wild-type *P. falciparum* parasites following CQ withdrawal, another possible reason for the predominance of the wild-type parasites could be selection by AL.[18] The findings suggest a prospect for reintroducing CQ for malaria treatment in pregnancy. However, CQ should be combined with another drug to combat early reemergence of resistance and restricted to those with laboratory-confirmed parasitamia.

The frequency of the *Pfmdr1* NFD was 43.2% and is the first such report in Ghanaian pregnant women. Increasing levels of this haplotype and the N86 SNP are reported to underlie ACT tolerance.[19] A higher frequency than observed was expected following the introduction of AL use in pregnant women since 2008, but it is possible that low drug pressure in pregnant women has kept the selection of NFD low.

The frequency of the *Pfdhfr* triple mutation and *Pfdhfr*/*Pfdhps* quadruple mutation (CIRNI-437G) is comparable with previous reports in west African pregnant women.[20] The *Pfdhfr* CIRNI triple mutation appears to have reached saturation within the pregnant population in Ghana, having recorded 73% prevalence in 2006[12] and 77% in the present study. Furthermore, the K540E mutation was present as a mixed infection in only one sample (1/142), consistent with reports of its rarity in west Africa along with the A581G and A613S mutations.[20] The low prevalence of these SNPs suggests that IPTp-SP still retains good efficacy in Ghana. The study did not include an assessment of the novel *Pfdhps* mutation I431V which may be related to SP resistance. In addition, not all samples were evaluable, thus reducing the numbers included in data analysis. This may be because of long storage durations of the filter paper spots (≥ 10 months) at possibly suboptimal temperatures/humidity with some DNA degradation. The study findings are, however, consistent with previous reports.[20]

The study provides an updated picture of antimalarial resistance markers in pregnant women in Ghana. With its presence in more than a third of the samples, the *Pfmdr1* NFD haplotype may suggest an increasing tolerance to AL. The National Malaria Control Program must strengthen education on targeted treatment with ACTs to help reduce drug pressure and delay resistance development. In line with World Health Organization recommendations, Ghana increased the number of IPTp-SP doses from 3 to ≥ 3 in 2013. It is important to monitor how this will impact the selection of *Pfdhfr*/*Pfdhps* mutations, especially the I431V and its distribution, and whether this may compromise the continuing protective efficacy of IPTp.

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Authors' addresses: Joseph Osarfo, Ghana Health Service, Effiduase District Hospital, Effiduase, Ashanti Region, Ghana, E-mail: josarfo@yahoo.co.uk. Harry Tagbor, School of Medicine, University of Health and Allied Sciences, Ho, Ghana, E-mail: htagbor@uhas.edu.gh. Pascal Magnussen, Centre for Medical Parasitology, Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark, and Department of Veterinary and Aquatic Sciences, Section for Parasitology and Aquatic Diseases, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, E-mail: pma@sund.ku.dk. Michael Alifrangis, Centre for Medical Parasitology, Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark, and Copenhagen University Hospital (Rigshospitalet), Copenhagen, Denmark, E-mail: micali@sund.ku.dk.

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