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SHORT REPORT: MOLECULAR MARKERS ASSOCIATED WITH *PLASMODIUM FALCIPARUM* RESISTANCE TO SULFADOXINE-PYRIMETHAMINE IN THE DEMOCRATIC REPUBLIC OF CONGO

SANDRA COHUET, MARYLINE BONNET, MICHEL VAN HERP, CHANTAL VAN OVERMEIR, UMBERTO D'ALESSANDRO, AND JEAN-PAUL GUTHMANN*

Epicentre, Paris, France; Médecins Sans Frontières, Brussels, Belgium; Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium

Abstract. Sulfadoxine-pyrimethamine (SP) is the first line antimalarial treatment in the Democratic Republic of Congo. Using polymerase chain reaction, we assessed the prevalence of mutations in the *dihydrofolate reductase* (*dhfr)* (codons 108, 51, 59) and *dihydropteroate synthase* (*dhps*) (codons 437, 540) genes of *Plasmodium falciparum,* which have been associated with resistance to pyrimethamine and sulfadoxine, respectively. Four hundred seventy-four patients were sampled in Kilwa ($N = 138$), Kisangani ($N = 112$), Boende ($N = 106$), and Basankusu ($N = 118$). The proportion of triple mutations *dhfr* varied between sites but was always > 50%. The proportion of *dhps* double mutations was < 20%, with some sites as low as 0.9%. A quintuple mutation was present in 12.8% (16/125) samples in Kilwa; 11.9% (13/109) in Kisangani, 2.9% (3/102) in Boende, and 0.9% (1/112) in Basankusu. These results suggest high resistance to pyrimethamine alone or combined with sulfadoxine. Adding artesunate to SP does not seem a valid alternative to the current monotherapy.

Since 1997, the Democratic Republic of Congo (DRC) has been affected by continuous civil war, despite a 2003 ceasefire. With ongoing poor access to health care, the health status of the civilian population has continued to deteriorate. Since 2002, Médecins Sans Frontières (MSF) has supported health structures in areas neighboring the ceasefire line (Kisangani area in Oriental province, Boende and Basankusu areas in Equator province, and Kilwa area in Katanga; Figure 1), where access to the most vulnerable population is difficult. These are areas of perennial seasonal malaria affecting mainly small children. Malaria is one of the most significant health problems in these areas, accounting for 35% of all health center attendances in 2002 (Ministry of Health, unpublished data). The national protocol, adopted in October 2001, recommends sulfadoxine-pyrimethamine (SP) as the first-line treatment of uncomplicated malaria. However, resistance to this drug is expanding in many African countries.[1,2] Despite variable levels of SP resistance recently measured *in vivo,*[3,4] no data (*in vivo*, *in vitro*) were available for our intervention sites. In Kisangani and Basankusu, MSF used SP as the first-line treatment, whereas in Kilwa and Boende, a combination of SP and amodiaquine was used.

A good correlation has been shown between mutations in the *dihydrofolate reductase* (*dhfr*) and *dihydropteroate synthase* (*dhps*) genes of *Plasmodium falciparum* (*Pf*) and resistance to pyrimethamine and sulfadoxine, respectively.[5,6] Assessing the prevalence of *dhfr* and *dhps* mutations has been suggested as an alternative measure of SP resistance when *in vivo* studies are difficult to implement,[7] which was the case in our sites. We therefore determined the prevalence of the mutations in codons 51, 59, and 108 of the *dhfr* gene and in codons 437 and 540 of the *dhps* in each of our intervention sites. Triple mutations of the *dhfr* gene, double mutations of the *dhps* gene, and especially quintuple mutations of *dhfr* and *dhps* genes are strongly correlated *in vivo* with SP treatment failure.[8,9]

Authorization to conduct these analyses was given by the national health authorities. After obtaining written informed consent, a blood sample was collected from each patient presenting with uncomplicated malaria at Kisangani, Basankusu, Kilwa, and Boende health centers. All patients with *Pf* malaria confirmed both by a rapid test (Paracheck-Pf, Orchid, Goa, India) and a thick/thin smear had a second capillary blood smear collected on an Isocode stix (Schleicher & Schuell, Dassel, Germany) for genomic analysis. A minimum of 100 samples per health center (i.e., a total of 400 samples) was considered logistically manageable and sufficient to estimate the prevalence of the mutations. Samples were air dried, stored adequately, and transported to the Institute of Tropical Medicine (Antwerp, Belgium) where parasite DNA extraction and analysis of *dhfr* and *dhps* genes were performed. Mutation-specific nested polymerase chain reaction (PCR) and/or restriction digestions were used to analyze *dhfr* and *dhps* mutations as described elsewhere.[10,11] A detailed description of these methods is available at http://www.medschool.umaryland.edu/CVD/plowe.html.

The classification of samples was based on a published methodology.[9] In brief, each *dhfr* and *dhps* codon was characterized as wild-type (no mutation present), mixed (both wild and mutant genotypes clearly present in the same infection), or pure mutant (only mutant genotypes detected). Then, *dhfr* and *dhps* genotypes for each infection were categorized as follows: wild-type, no mutation detected; single, infection involving parasites with a single mutation; double, infection involving parasites with a double mutation; triple, infection involving parasites with all three mutations detected. Finally, infections involving parasites both with triple *dhfr* mutations and double *dhps* mutations were categorized as quintuple mutations.

Between September 2003 and March 2004, > 100 samples were collected in each site, and most of them could be genotyped (Table 1). The proportion of triple mutations *dhfr* varied between sites but was always > 50%. The proportion of *dhps* double mutations was much lower, < 20%, with some sites as low as 0.9%. Overall, a quintuple mutation was present in 12.8% (16/125) of the samples in Kilwa; 11.9%

* Address correspondence to Jean-Paul Guthmann, Epicentre, 8 rue Saint Sabin, 75011 Paris, France. E-mail: jguthmann@epicentre.msf.org

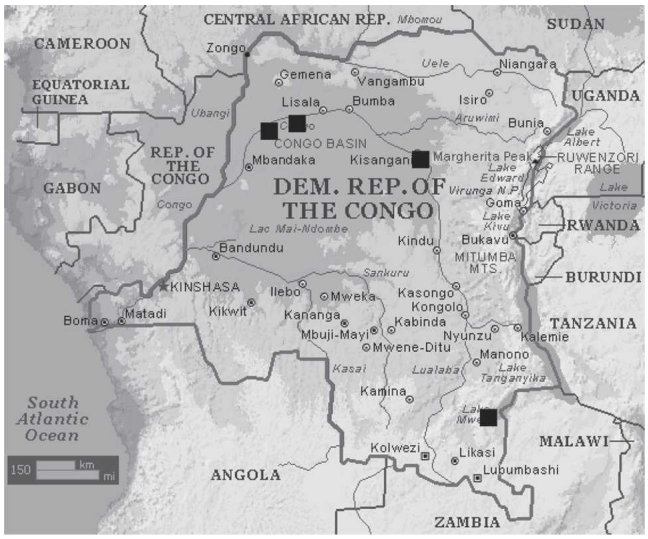

FIGURE 1. Location (■) of health structures where the survey was carried out.

(13/109) in Kisangani; 2.9% (3/102) in Boende; and 0.9% (1/112) in Basankusu.

This survey provided an estimation of the SP resistance in different areas where *in vivo* test data were not available. Our sample was relatively small in size, because of the logistic difficulties. However, the confidence intervals are relatively narrow, allowing a fair estimate of the situation in the study sites In our sites, the high frequency of *dhfr* triple mutations and the rather high frequency of *dhps* double mutations in two sites suggest that SP resistance is already well established. These findings are not surprising considering that SP resistance has been shown to increase quickly when used as monotherapy.[12–14] This situation could rapidly lead to low SP efficacy because of selection of resistant parasites by widespread use of SP. Previous studies have suggested that they may be a stepwise accumulation of mutations in response to increasing drug pressure.[15] This could be also the case in our sites. However, the data presented here cannot confirm this hypothesis. It is important to note that differences between regions may also reflect variation in the duration and magnitude of SP use,[16] although these differences were not significant. This study on molecular markers associated with SP resistance should contribute to inform health authorities on antimalarial efficacy in these sites and guide treatment policy. Indeed, in situations such as those we have described here, where *in vivo* tests cannot be carried out, the estimation of the prevalence of mutations offers an easy and rapid alternative. The prevalence of quintuple mutations varied from 12% to 1%; however, the information available (especially concerning the frequency and adequacy of antimalarial use in each site) does not provide a clear explanation for these differences between sites.

The implementation of artemisinin-based combinations (or ACT) recommended by WHO[17] needs careful consideration of the partner drug to be associated with the artemisinin component. The choice of new protocols should be seriously discussed among relevant authorities and malaria experts in DRC. The information provided here should be added and discussed considering other data available for the country. The high SP resistance suggested by our studies indicate that combining artesunate to SP would not increase the efficacy of the first-line treatment, at least not in the long term. Artemether + lumefantrine (Coartem, Novartis Pharma, Basel, Switzerland) has been shown to be very efficacious even in unsupervised conditions,[18] but one major limitation is the cost. At the current cost of 2.4 US$/adult treatment, many African countries are unable to afford Coartem for public sector use without the support of international donors (e.g., the Global Fund). Artesunate + amodiaquine is another interesting alternative, less expensive than Coartem, and should

TABLE 1
Prevalence of mutations at codons 108, 51, and 59 in *dhfr* and of mutations at codons 437 and 540 of *dhps,* by site

	Kilwa (n = 128)			Kisangani (n = 109)			Boende (n = 102)			Basankusu (n = 112)		
*Dhfr**	*n*	%	95% CI	*n*	%	95% CI	*n*	%	95% CI	*n*	%	95% CI
Wild	5	3.9	1.4–9.3	3	2.8	0.7–8.4	3	2.9	0.6–8.3	1	1	0–5.6
Single mutation	4	3.1	1–8.3	0	0	–	2	2.0	0.2–6.9	5	4.5	1.6–10.6
Double mutation	19	14.8	9.4–22.5	43	39.4	30.3–49.3	35	34.3	25.2–44.4	33	29.4	21.4–38.9
Triple mutation	100	78.2	69.8–84.7	63	57.8	47.9–67.0	62	60.8	50.6–70.3	73	65.1	55.5–73.7
Mixte	49	38.4	29.9–47.3	35	32.1	23.7–41.8	44	43.1	33.4–53.3	64	57.1	47.4–66.3
Pure	51	39.8	34.4–48.9	28	25.7	18.0–35.1	18	17.6	10.8–26.4	9	8.0	3.9–15.1
	Kilwa (n = 135)			Kisangani (n = 109)			Boende (n = 102)			Basankusu (n = 112)		
Dhps†	*n*	%	95% CI	*n*	%	95 CI	*n*	%	95% CI	*n*	%	95% CI
Wild	102	75.6	62.3–82.4	62	56.9	47–66.2	30	29.4	20.8–39.2	38	34	25.4–43.5
Single mutation	15	11.1	6.6–17.9	26	23.8	16.4–33.1	68	66.7	56.6–75.7	73	65	55.5–73.8
Double mutation	18	13.3	8.3–20.5	21	19.3	12.6–28.2	4	3.9	1.1–9.7	1	0.9	0.0–5.6
Mixte	10	7.4	3.8–13.5	8	7.4	3.4–14.4	1	1.0	0.0–5.3	0	0	–
Pure	8	5.9	2.8–11.7	13	11.9	6.7–19.9	3	2.9	0.6–8.3	1	1	0.0–5.6
	Kilwa (n = 125)			Kisangani (n = 109)			Boende (n = 102)			Basankusu (n = 112)		
Dhfr + dhps	*n*	%	95% CI	*n*	%	95% CI	*n*	%	95% CI	*n*	%	95% CI
Quintuple mutation	16	12.8	7.7–20.2	8	7.3	3.4–14.3	3	2.9	0.6–8.3	1	0.9	0.0–5.6
Mixte	11	8.8	4.7–15.5	5	4.6	1.5–10.4	2	2	0.2–6.9	1	0.9	0.0–5.6
Pure	5	4	1.5–9.5	3	2.7	0.6–7.8	1	0.9	0.0–5.3	0	0	–

* *Dhfr,* single = 108, 51, or 59, double = 108 and 51 or 59, triple = 108-51-59.
† *Dhps:* simple = 437 or 540, double = 437 and 450.

be soon available in blister packs as a fixed combination. Information available for amodiaquine[19] indicates that this drug is safe. Other promising alternatives (such as chlorproguanil-dapsone-artesunate, piperaquine-dihydroartemisinin, or pyronaridne-artesunate) are currently under development and should soon become available, increasing considerably the options for the National Malaria Control Programs.

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Authors' addresses: Sandra Cohuet, Maryline Bonnet, and Jean-Paul Guthmann, Epicentre, 8 rue Saint Sabin, 75011 Paris, France. E-mails: sandra.cohuet@lshtm.ac.uk, maryline.bonnet@geneva.msf.org, and jguthmann@epicentre.msf.org; Michel van Herp, Médecins sans Frontières, 94 rue Dupré, 1090 Brussels, Belgium, E-mail: michel.vanherp@msf.be; Chantal van Overmeir and Umberto D'Alessandro, Prince Léopold Institute of Tropical Medicine, 155 Nationalestraat, B-2000 Antwerp, Belgium, E-mails: cvoverm@itg.be and udalessandro@itg.be.

Reprints requests: Jean-Paul Guthmann, Epicentre, 8 rue Saint Sabin, 75011 Paris, France. E-mail: jguthmann@epicentre.msf.org.

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