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The Polymorphic Linker Domain of *pfmdr1* Is Associated with Resistance-Conferring Mutations in *Plasmodium falciparum* Populations from East and West Africa

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Sequence variation in the asparagine/aspartate-rich domain of *pfmdr1* in 215 isolates of *Plasmodium falciparum* from three African countries was compared with published data. The role of this domain in modulating antimalarial sensitivity has not been established. The *pfmdr1* 86Y allele was significantly associated with different configurations of the Asn/Asp-rich domain in West and East Africa. In Kenya, a specific form of the Asn/Asp-rich domain was significantly linked to the 86Y, 184Y, and 1246Y haplotype of *pfmdr1*.

The malaria parasite *Plasmodium falciparum* is becoming less sensitive to the artemisinin class of drugs in Asia (1, 2), and variable responses in East Africa have been reported (3). Artemisinin is a component of all current combination therapies, and while their clinical efficacy remains high, understanding of parasite genetic factors that contribute to variation in artemisinin sensitivity is essential. Variants of the *pfmdr1* gene of *P. falciparum* (PF3D7_0523000) have been associated with increased probability of parasite survival after artemisinin combination therapy (4, 5). The locus encodes the ABC transporter, PgH1, comprising nine *trans*-membrane domains interrupted by an asparagine-rich (Asn-R) "hinge" domain known to exhibit length polymorphism (6–9).

Asn-R domains have been shown, in homologous ABC transporters of other taxa, to mediate ubiquitination and protein turnover (10, 11). Conversely, Asn repeats in the proteasome lid regulatory subunit 6 protein of *P. falciparum*, Rpn6, appear to be nonessential during intraerythrocytic growth *in vitro* (12), although similar data are not available for any other parasite ABC transporter. In this report, we present an analysis of the extent of sequence diversity in the *pfmdr1* Asn-R domain in samples from four countries where malaria is endemic and explore associations with resistance-associated polymorphisms at codons 86, 184, and 1246 of *pfmdr1*.

Clinical samples were obtained from Mbita, Kenya, in 2009 (a random sample of 80 pretreatment isolates from a total of 300 enrollees) (13), Gedaref, Sudan, in 2006 (all 47 pretreatment DNA samples) (4), and Bobo-Dioulasso, Burkina Faso, between Oct 2009 and Jan 2010 (random sample of ~25% of available DNA extracts) (I. Zongo, P. Milligan, Y. D. Compaore, A. F. Some, B. M. Greenwood, J. Tarning, P. J. Rosenthal, C. J. Sutherland, F. Nosten, and J.-B. Ouedraogo, unpublished data). Published sequence data on 26 isolates collected in The Gambia in 1996 were also included in the analyses (7). Permission for parasite genotyping studies was obtained from the relevant Ethics Committees. The Asn-R domain was amplified using primers and cycling conditions described elsewhere (7). Ten reference laboratory strains were included for comparison. Genotypes with resistance-associated polymorphisms at codons 86, 184, and 1246 were available from previous analyses. The sequences were determined with an ABI Prism 3730 DNA analyser (Applied Biosystems, United Kingdom) and analyzed using SeqMan (DNAStar Lasergene 7; Madison, WI) and BioEdit version 7.0.9. Amino acid haplotype diversity (Hs) and Wright's F statistic (F_{ST}) were determined using DnaSP version 5.10 (13). Associations between *pfmdr1* codons 86, 184, and 1246 and Asn-R domain polymorphisms were assessed for statistical significance using the χ^2 distribution, or Fisher's exact test where appropriate, using STATA (College Station, TX, USA).

Sequences of the Asn-R domain of *pfmdr1* were determined successfully in 215 samples from the three African studies and compared to data from a Gambian report (7). A high level of diversity in the Asn-R domain was evident (Table 1). When Gambian data were included, a significant association was observed between the *pfmdr1* 86Y allele and a linker region configuration of 8-2-8/9/10 ($P = 0.008$ by the χ^2 test). However, after stratification by population, this association persisted only in Gambian ($P = 0.006$ by Fisher's exact test) and Burkina Faso (odds ratio [OR] of 38.5; 95% confidence interval [95% CI] of 7.01 to 253; $P < 0.001$) data, consistent with previous findings in West Africa (6). No Sudanese isolate bore this particular linker configuration. In Kenya, *pfmdr1* 86Y was instead significantly associated with the 7-2-10 profile (OR, 8.75; 95% CI, 1.72 to 14.6; $P = 0.001$). Regarding *pfmdr1* 184F, the only significant association was observed in Kenya with the form 7-0-4 ($P = 0.0006$). A third polymorphism in

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TABLE 1 Polymorphisms in the linker region sequence of 215 *P. falciparum* isolates and the proportion in each class linked to the codon 86Y, codon 184F, and codon 1246Y alleles[a]

	No. of polymorphisms in the linker region sequence of isolates from the following country:					Proportion (%) in each class linked to the following *pfmdr1* allele[c]:		
Asn-Asp-Asn[b]	Sudan	Kenya	Burkina	The Gambia	All four countries (total)	86Y	184F	1246Y[d]
7-2-09	39	10	29	4	82	52	73	4 (47)
7-2-10	6	34	12	1	53	65	22	50 (40)
7-2-07	0	8	12	0	20	0	55	0 (8)
8-2-09	0	3	13	0	16	69	88	0 (3)
7-2-08	0	1	4	6	11	18	64	0 (1)
7-0-02	0	7	2	0	9	33	44	0 (7)
7-0-04	0	8	0	0	8	50	100	50 (8)
7-0-01	1	6	0	0	7	86	14	71 (7)
7-2-11	0	1	6	0	7	14	57	100 (1)
7-2-06	0	0	1	5	6	0	83	
8-2-08	0	0	0	5	5	100	100	
8-1-09	0	0	2	1	3	0	0	
5-2-09	0	0	2	0	2	0	0	
7-0-03	1	0	1	0	2	50	50	0 (1)
7-0-00	0	0	0	1	1	0	100	
7-0-06	0	1	0	0	1	0	0	0 (1)
7-1-11	0	1	0	0	1	0	0	0 (1)
7-2-01	0	0	0	1	1	0	0	
7-2-04	0	0	1	0	1	0	100	
7-2-05	0	0	0	1	1	0	100	
7-2-12	0	0	1	0	1	100	100	
7-2-15	0	0	1	0	1	0	100	
8-0-00	0	0	0	1	1	100	100	
8-2-10	0	0	1	0	1	0	0	
Total	**47**	**80**	**88**	**26**	**241**	**236**	**234**	**125**

[a] The total number of linker region sequences determined from the different countries and the total number of samples evaluated for each *pfmdr1* allele are shown in boldface type.
[b] Numbers of Asn, Asp, and Asn residues in the linker sequence, respectively.
[c] Sequence data at each codon were not available for all isolates. Thus, the denominator for the codon analyses is less than 236 in each case. For codon 1246, only samples from Kenya and Sudan were included in the analysis (see the text).
[d] The number of samples is shown in parentheses.

pfmdr1, D1246Y, is common in East Africa (5) and was included in the analysis for Mbita, Kenya, and Gedaref, Sudan. The 1246Y allele, which is rare in West Africa, was present in 30 (37.5%) isolates from Mbita, Kenya, where it was significantly associated with the 7-2-10 configuration of the Asn-R domain (OR, 4.03; 95% CI, 1.40 to 11.8; $P = 0.0035$). Of these 30 isolates, 24 harbored the YYY haplotype at *pfmdr1* codons 86, 184, and 1246, previously associated with resistance to amodiaquine (5). Two isolates from Gedaref, Sudan (4.4%) also harbored the 1246Y allele, one of which displayed both the YYY haplotype and the 7-2-10 Asn-R domain configuration, as seen in Kenya.

Compared to previous reports, our study has demonstrated a greater diversity at the Asn-R domain of *pfmdr1* (Fig. 1), with 24 distinct Asp-R haplotypes in four populations. We observed very low levels of genetic differentiation between The Gambia and Burkina Faso, with an F_{ST} value (14) of 0.07 (Table 2). This was less than the differentiation between the two East African sites ($F_{ST} = 0.19$). Differences in diversity between Kenya and Sudan in the east and the West African populations could reflect the profound seasonality in transmission in The Gambia and Burkina Faso (15–17). In Kenya, Mbita is characterized by a high and perennial malaria transmission with entomologic inoculation rates of approximately six infectious bites per person per month (18), consistent with high genetic diversity. The low diversity in Sudan was unsurprising due to low transmission over a very short wet season in Gedaref, Sudan (19) and consequently more frequent self-fertilization within the parasite population. Our data suggest independent evolution of the *pfmdr1* locus in East and West Africa. Antimalarial use and resistance allele frequencies are substantially different between these settings, and both are strongly affected by seasonality in The Gambia and Sudan (20, 21).

The linker domain of ABC transporters has been shown to mediate ubiquitination and control of protein turnover (10, 11), two processes implicated in mechanisms of drug resistance in *Plasmodium* (22, 23). The association of *pfmdr1* 86Y with particular linker polymorphisms is not uniform, being found with 7-2-10 in Kenya and 8-2-8/9/10 in West Africa. This inconsistency may imply that the Asn-R domain is not involved in modulating antimalarial resistance but rather is passively carried, due to linkage, by selective sweeps around codons 86 and 184. However, our demonstration that the 1246Y mutation, rare in West Africa, was also strongly associated with the 7-2-10 form in Kenya provides an alternative explanation—that in fact the *pfmdr1* alleles are distinct in the two regions, with little mixing. Thus, the 7-2-10 form of the Asp-R domain may be required to maintain the YYY haplotype of *pfmdr1* in the Kenyan *P. falciparum* population. Further studies in

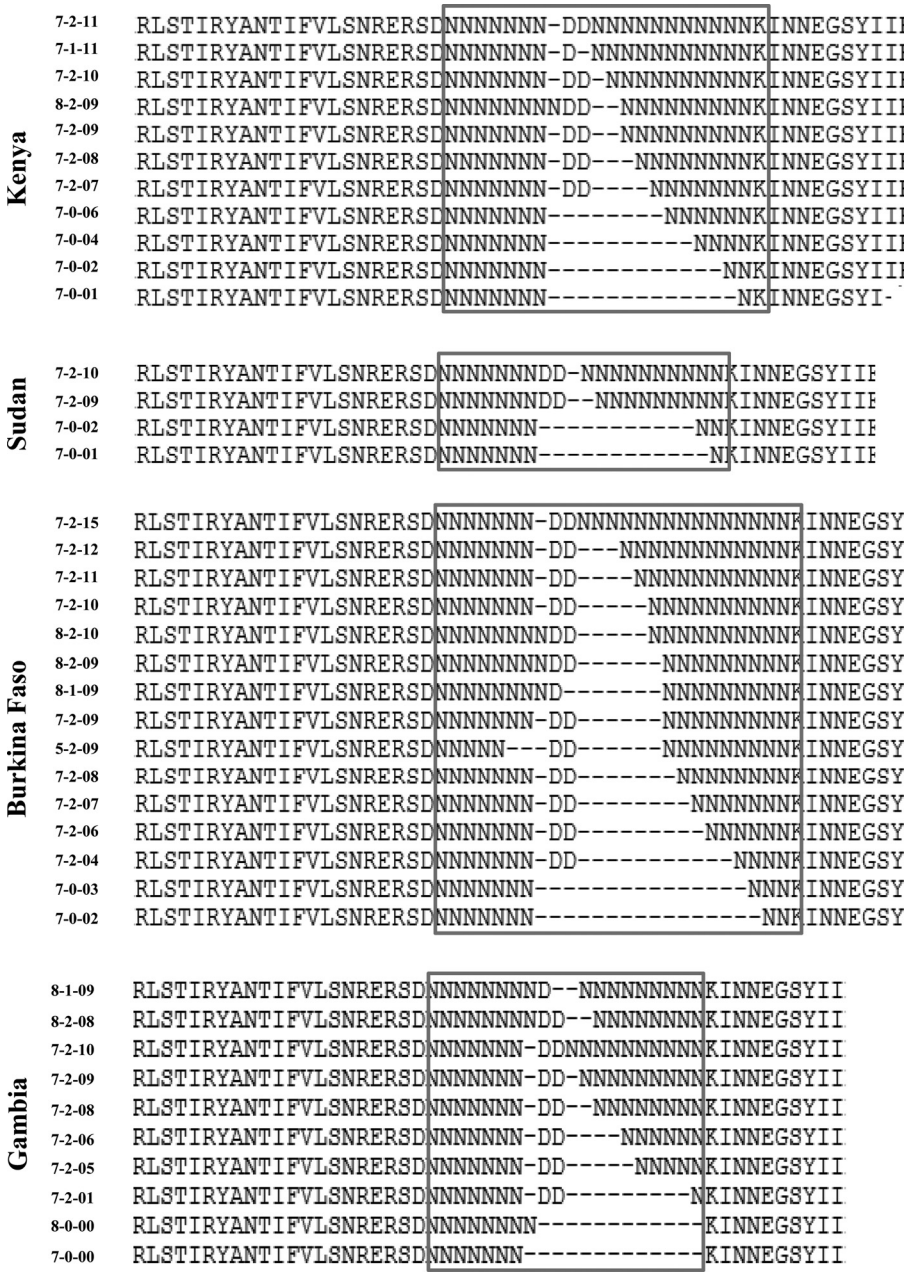

FIG 1 ClustalW alignment output of all the representative profiles of the *P. falciparum* clinical isolates from three different countries and published Gambian data. Each group represents all sequence profiles identified in the country (indicated), and the variations are highlighted in the rectangular grid. The greatest variation was observed in Burkina Faso with 15 out of the 24 distinct sequence profiles observed in all the populations, while Sudan was the least diverse with only 4 of the 24 profiles.

West Africa, where the 1246Y allele is present but very rare, could seek evidence of the spread of this haplotype, together with the 7-2-10 Asp-R domain, from East Africa.

We have demonstrated substantial diversity in the Asp-R domain of *pfmdr1* and significant within-population associations between this domain and resistance-associated single nucleotide polymorphisms in four *P. falciparum* populations. Our results suggest that the *pfmdr1* locus has evolved separately in East and West Africa, reflecting differences in transmission, seasonality, and histories of antimalarial use. Any functional role of the Asn-R

TABLE 2 Matrix of genetic differentiation showing pairwise F_{ST} values for the polymorphic linker domain in the four populations

Country[a]	F_{ST} value for comparison of pairs of populations		
	Burkina Faso	The Gambia	Kenya
Burkina Faso (88)			
The Gambia (26)	0.07089		
Kenya (83)	0.10839	0.06075	
Sudan (47)	0.05791	0.17261	0.18974

[a] The sample size for each location is given in parentheses.

domain, a potential marker for both resistance and population diversity, remains to be determined.

Nucleotide sequence accession numbers. Sequences described are available in GenBank under accession numbers KC573528 to KC573687.

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