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Indigenous evolution of *Plasmodium falciparum* pyrimethamine resistance multiple times in Africa

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Objectives: **Resistance to pyrimethamine in *Plasmodium falciparum* is conferred by mutations in the gene encoding dihydrofolate reductase (*DHFR*). It is known that *DHFR* double mutants have evolved independently in multiple geographic areas, whereas the triple mutant prevalent in Africa appears to have originated in south-east Asia. In this study, we investigated whether other triple mutants may have evolved independently in Africa.**

Methods: **We determined the *DHFR* genotypes and haplotypes of five microsatellite loci flanking the *DHFR* locus between 4.49 kb upstream and 1.48 kb downstream of 159 isolates collected from three African countries (Republic of Congo, Ghana and Kenya).**

Results: **The CIRNI type of *DHFR* triple mutant (with mutations underlined at amino acid positions 51, 59 and 108) was predominant in the Republic of Congo (82%) and Ghana (81%) and was the second most prevalent in Kenya (27%), where the CICNI type of *DHFR* double mutant was dominant. Three distinct microsatellite haplotypes were identified in the *DHFR* triple mutant. One haplotype was identical to that originating in south-east Asia. The other two haplotypes occurred in Ghana and Kenya, which were unique, previously undescribed and identical to those of the two *DHFR* double mutants found in the same locations.**

Conclusions: **This study presents strong evidence for the unique, multiple independent evolution of pyrimethamine resistance in Africa. Indigenous evolution of the triple mutant from the double mutant appears to have occurred in a step-wise manner in Kenya and Ghana or in nearby countries in east and west Africa.**

Keywords: malaria, *dhfr*, microsatellite

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Introduction

The spread of *Plasmodium falciparum* resistance to commonly used antimalarial drugs is a major public health problem in malaria-endemic regions. The antifolate drug pyrimethamine/sulfadoxine inhibits two enzymes in the parasite's folate synthesis pathway. Amino acid changes at positions 50, 51, 59, 108 and 164 in one of these enzymes, dihydrofolate reductase (DHFR), are strongly associated with pyrimethamine resistance.[1,2] At present, isolates harbouring four mutations (CIRNL, with mutated amino acids underlined) show the highest degree of resistance to pyrimethamine. There are several *DHFR* genotypes in malaria-endemic regions,[3] but the number of times the resistant genotype has emerged in independent parasite lineages is thought to be considerably limited.[4–7] In south-east Asia, an analysis of microsatellite markers flanking *DHFR* indicated that all resistant parasites bearing two or more mutations share a single lineage.[4] In contrast, in Africa, multiple indigenous lineages have been reported for *DHFR* double mutants.[5,8] Nevertheless, it is currently accepted that these indigenous double mutants have not yet produced a triple mutant. Rather, nearly all lineages showing the *DHFR* triple mutant found in Africa have been ascribed to the migration of triple-mutant parasites from south-east Asia.[6–8] However, given the occurrence of multiple lineages of *DHFR* double mutants in Africa, it is likely that triple mutants may have evolved independently within the continent. In this study, we determined the *DHFR* genotype and flanking microsatellite haplotypes of *P. falciparum* isolates from the Republic of Congo, Ghana and Kenya. We discovered two previously undescribed lineages of the *DHFR* triple mutant, from Ghana and Kenya, showing the independent evolution of *DHFR* triple mutants within Africa.

Materials and methods

Study site and patients

Blood samples were collected from symptomatic individuals from Pointe-Noire, Brazzaville and Gamboma in the Republic of Congo in 2006 and from asymptomatic individuals at three villages near Winneba in Ghana in 2004 and at two villages in Kisii District in Kenya in 1998. In all regions, surveys were necessarily conducted prior to the official introduction of pyrimethamine/sulfadoxine treatment for malaria and so were not feasible with current populations. These studies were approved by the administrative authority of the Ministry for Research and Ministry for Health in the Republic of Congo, the Ministry of Health/Ghana Health Service and the Ministries of Health and Education in Kenya. In all surveys, each patient was informed about the study prior to sampling, consent was obtained (in the case of children, parents/guardians gave informed consent) and medical follow-up was provided if needed.

DHFR genotyping and microsatellite haplotyping

Parasite DNA was purified using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) for samples from Ghana and Kenya and the EZ1 BioRobot™ (QIAGEN) for samples from the Republic of Congo. The first 184 codons of *DHFR*, encompassing all known polymorphic loci were amplified by nested PCR. Amplified products were directly sequenced with a BigDye terminator v1.1 cycle sequencing kit in an ABI 377 DNA Sequencer (Applied Biosystems, CA, USA) as described previously.[9] In order to determine the evolutionary history of pyrimethamine-resistant *DHFR* genotypes, we identified individual haplotypes based on the association of five microsatellite markers closely linked to the gene (0.1, 3.87 and 4.49 kb upstream, and 0.52 and 1.48 kb downstream of *DHFR*).[4] Briefly, variation in the number of TA repeats in microsatellite loci was measured by semi-nested PCR using fluorescent end-labelled primers, followed by electrophoresis on an ABI 377 sequencer and analysed with the Genescan software (Applied Biosystems).

The microsatellite haplotype observed in south-east Asia, harbouring an association of 200-194-176-106-203 bp at microsatellite loci 4.49, 3.87 and 0.1 kb upstream and 0.52 and 1.48 kb downstream of *DHFR* was designated the SEA haplotype.[10] Microsatellite haplotypes unique to the Republic of Congo, Ghana and Kenya are described in the Results section. Haplotypes showing minor variations at one or two loci were included in the respective major haplotypes.

Isolates showing mixed *DHFR* genotypes were excluded from the analysis. Samples with more than one allele detected at any microsatellite locus were also considered as mixed allele infections and excluded. By these criteria, 131 (45%) of 290 samples were excluded from the analysis.

Results

DHFR genotypes

We identified five distinct *DHFR* genotypes in 159 isolates: one triple mutant (CIRNI), two forms of double mutant (CICNI and CNRNI), one single mutant (CNCNI) and the wild-type (CNCSI) (Figure 1). The triple mutant was found in all three African countries with high prevalence in the Republic of Congo and Ghana (81–82%), whereas the two double mutants were distributed differently, the CICNI type in the Republic of Congo and Kenya and the CNRNI type in Ghana.

Microsatellite haplotypes

DHFR double-mutant isolates carried microsatellite haplotypes that were unique to each country (Figure 2a). In Kenya, the majority of isolates were of a microsatellite haplotype consisting

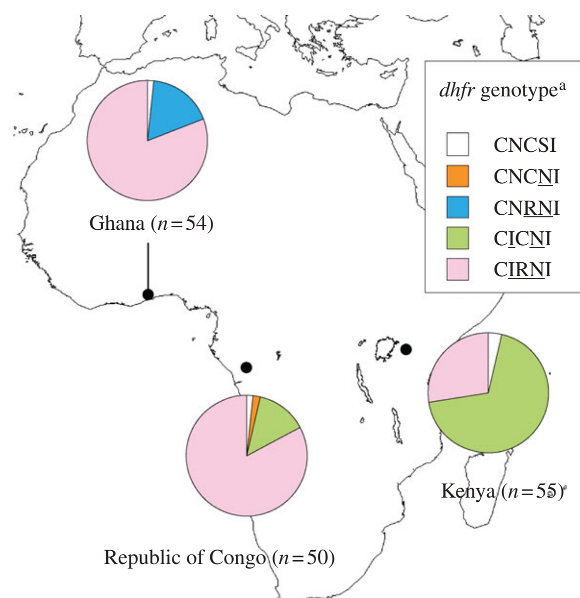

Figure 1. Frequency of *DHFR* genotypes in *P. falciparum* isolates from Kenya, the Republic of Congo and Ghana. [a]Genotype is expressed as an association of amino acids at positions 50, 51, 59, 108 and 164. Underlined amino acids indicate mutated residues.

(a)

Country	Size of microsatellite markers (bp) at indicated position					n	Haplotype
	−4.49 kb	−3.87 kb	−0.1 kb	0.52 kb	1.48 kb		
Kenya (CI<u>C</u>NI)	204	190	156	96	203	33	KEN haplotype
	196	190	156	96	203	2	
	208	190	156	96	203	1	
	204	190	156	96	211	1	
	202	186	156	96	203	1	
Republic of Congo (CI<u>C</u>NI)	198	194	170	98	197	5	CON haplotype
	206	194	170	98	197	1	
	198	190	170	96	197	1	
Ghana (CN<u>R</u>NI)	198	204	168	104	205	8	GHA haplotype
	202	186	168	104	205	1	

(b)

Country	Size of microsatellite markers (bp) at indicated position					n	Haplotype
	−4.49 kb	−3.87 kb	−0.1 kb	0.52 kb	1.48 kb		
Kenya	200	194	176	106	203	4	SEA haplotype
	204	194	176	106	203	1	
	204	190	176	106	203	1	
	200	190	156	96	203	2	KEN haplotype
	204	190	156	96	203	7	
Republic of Congo	200	194	176	106	203	36	SEA haplotype
	202	194	176	106	203	1	
	200	194	176	106	197	1	
	204	194	176	96	203	1	KEN haplotype
	200	194	156	96	203	2	
Ghana	200	194	176	106	203	29	SEA haplotype
	198	194	176	106	203	1	
	200	194	176	106	205	1	
	200	206	168	104	205	1	GHA haplotype
	202	186	168	104	205	2	
	198	204	168	104	205	9	
	204	190	156	96	203	1	KEN haplotype

Figure 2. Microsatellite haplotypes in *P. falciparum* isolates having *DHFR* double (a) and triple (b) mutations in Kenya, the Republic of Congo and Ghana. KEN haplotype, CON haplotype, GHA haplotype and SEA haplotype are unique associations at five microsatellite loci of alleles. Haplotypes showing minor variations at one or two loci (unboxed) were included in the respective major haplotypes. These alleles are likely to be generated by a TA repeat mutation and/or recombination events between unlike haplotypes. (b) A variant belonging to the SEA haplotype from the Republic of Congo had an allele at 1.48 kb that was seen in the DHFR double mutant in the same country.

of an association of 204-190-156-96-203 bp (KEN haplotype), and the rest had minor variations from the predominant haplotype. Similarly, all isolates from the Republic of Congo were of one predominant haplotype (198-194-170-98-197 bp; CON haplotype), as were those from Ghana (198-204-168-104-205 bp; GHA haplotype). No SEA haplotype was observed.

We identified three microsatellite haplotypes associated with the *DHFR* triple mutant (Figure 2b). In Kenya, both the KEN haplotype and SEA haplotype were observed. Chimeric haplotypes between the SEA and KEN haplotypes were observed, probably generated by recombination between the two haplotypes. In the Republic of Congo, the SEA haplotype was the most prevalent (93%) and the KEN haplotype was also found at low prevalence (7%). All three samples of the KEN haplotype were chimeric haplotypes, having alleles of the SEA haplotype at two loci. In Ghana, the SEA haplotype was most dominant (70%), followed by the GHA haplotype (27%) and the KEN haplotype (3%). Overall, among the three *DHFR* triple-mutant lineages, the SEA haplotype was the most prevalent (75%), and the prevalence of the KEN (13%) and GHA (12%) haplotypes was low but significant.

Discussion

The present study clearly shows that there are three lineages of the CI<u>RN</u>I pyrimethamine-resistant *DHFR* triple mutant in the Republic of Congo, Ghana and Kenya. One of the three lineages

is identical to the SEA lineage and the other two lineages, the KEN lineage and GHA lineage, are unique to Africa. The SEA lineage was the most prevalent of the *DHFR* triple mutants, but the prevalence of the two African lineages was significant. Importantly, microsatellite haplotypes of the two African lineages were also found from parasites with *DHFR* double mutants, while no double mutants shared the SEA microsatellite haplotype. These findings present strong evidence for the indigenous evolution of the *DHFR* triple mutant from the double mutant in Africa.

Previous reports have not identified indigenously generated African triple *DHFR* mutants. These include two studies analysing 24 triple mutants from South Africa,[6] and 204 from 11 sub-Saharan African countries.[7] In these countries, all triple mutants except one showed an identical or very similar microsatellite haplotype to the SEA haplotype. This suggests that the *DHFR* triple mutant currently predominant in Africa was imported from SEA, rather than indigenously generated. In the present study, the SEA lineage was the most prevalent in all countries considered, but two other indigenous lineages were also observed.

The co-prevalence of the SEA haplotype and the KEN/GHA haplotypes in Africa suggests that the indigenous *DHFR* triple mutant may have evolved earlier than or contemporaneously with the migration of the SEA type from south-east Asia—an initial triple mutant, either indigenously evolved or imported, would quickly spread out through the continent, as observed in south-east Asia, where only a single lineage of *DHFR* triple or quadruple mutant is presently prevalent.[4,10] This scenario is based on the assumption that pyrimethamine pressure was continuously and extensively present. However, in Africa, the history of pyrimethamine administration differs greatly country-by-country. Indeed, we observed the *DHFR* wild-type, a single mutant and double mutants in three African countries, a situation in sharp contrast to that in south-east Asia where all *P. falciparum* populations show a single lineage of *DHFR* triple/quadruple mutant.[4,10] Dispersal of drug resistance obviously depends on many factors, including movements of infected people, the intensity of malaria transmission, the level of host population immunity and the history and extent of drug usage. Thus, the strength of any selective sweep by pyrimethamine differs depending on area, making it difficult to infer which occurred earlier; the migration of the *DHFR* triple mutant from south-east Asia into Africa or the indigenous evolution of the triple mutant in Africa.

This study also presents strong evidence for the indigenous evolution of *DHFR* double mutants in Africa. All three countries studied have a unique, country-specific lineage of a *DHFR* double mutant, suggesting that the generation of *DHFR* double mutants is not a rare event in Africa, a situation that differs from south-east Asia and Melanesia, where the generation of double mutants has been very rare.[10] As the prevalence of *P. falciparum* is much greater in many parts of Africa than in most of south-east Asia and Melanesia, the pool of potential drug-resistant mutant parasites available for selection by drug pressure is much larger. We believe that this situation may explain the generation and selection of multiple independently originating drug-resistant mutants in Africa.

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Transparency declarations

None to declare.

T. M. designed the study, conducted the survey, analysed the data and wrote the report. K. T. participated in the design, analysed the data and reviewed the report. N. T. carried out molecular genetic analysis. R. C. participated in the survey in the Republic of Congo and reviewed the report. M. N. organized and participated in the survey in the Republic of Congo. M. D. organized and participated in the survey in Ghana. W. S. A. organized and participated in the survey in Kenya. A. K. organized and participated in the survey in Kenya and Ghana. T. K. participated in the study design, organized the survey in Kenya and Ghana and reviewed the report.

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