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Monitoring for multidrug-resistant *Plasmodium falciparum* isolates and analysis of pyrimethamine resistance evolution in Uige province, Angola

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Summary

OBJECTIVES To assess the extent of drug resistance in Uige through molecular genetic analysis and to test whether the *dhfr* triple mutant alleles present in Angola are of southeast Asian origin.

METHODS Seventy-one samples of blood from children admitted to the Pediatric Emergency Unit of Uige Provincial Hospital in 2004 were screened for resistance mutations at *pfcrt, pfmdr1, pfdhfr, pfdhps* and *pfATPase6*.

RESULTS Mutations in *pfcrt* (codon76), *pfmdr1* (codon86), *pfdhfr* (codons 51, 59, 108) and *pfdhps* (codons 436, 437) were common. Among the 66 isolates for which we were able to determine complete genetic information 13.7% carried all seven of these mutations. Flanking microsatellite analysis revealed the triple mutant *pfdhfr* was derived from the southeast Asian lineage, while the N51I+S108N double mutant *pfdhfr* alleles are a local origin. *pfATPase6* mutations were rare and S769N was not found.

CONCLUSION The parasite population of Uige Angola has high frequency mutations in *pfcrt*, *dhfr* and *dhps* associated with resistance to chloroquine and sulphadoxine pyrimethamine, reflecting past reliance on these two drugs which were the mainstay of treatment until recently. Our findings show that drug resistance in Uige has occurred through a combination of local drug pressure and the regional and international dispersal of resistance mutant alleles.

keywords *Plasmodium falciparum,* Angola, drug resistance molecular markers, microsatellites, pyrimethamine resistance evolution

Introduction

Malaria is the primary cause of morbidity and mortality in Angola, accounting for 35% of the overall mortality in children under five and 25% of maternal deaths (WHO/UNICEF 2005). Malaria is endemic in Angola, with *Plasmodium falciparum* being the predominant pathogen (WHO 2005a). The epidemiological characteristics vary from hyper-endemic (mainly in the northern and north-eastern areas of the country) to mesoendemic stable and mesoendemic unstable, with 90% of the population being exposed to an endemic risk of malaria. Drug resistance is a major obstacle in controlling *falciparum* malaria. Resistance to both chloroquine (CQ) and sulfadoxine-pyrimethamine (SP), used as the standard antimalarials in Angola until 2006, was reported among resident foreigners some 20 years ago (Kyronseppa *et al.* 1984; Olsen *et al.* 1984; Lindberg *et al.* 1985) and, in recent years, documented in central Angola with high-level of failure rate (Guthmann *et al.* 2005).

Currently, arthemether–lumefantrine (AM-LUM; Coartem®) is the first-line treatment and amodiaquine-artesunate (ASAQ) the recommended alternative therapy for *falciparum* malaria (WHO 2005b). Finally, Sulfadoxine/Pyrimethamine Intermittent Preventive Treatment (IPT) is recommended in pregnant women.

Plasmodium falciparum resistance to sulfadoxine and pyrimethamine has been ascribed to polymorphisms of dihydropteroate synthase (*pfdhps*) and dihydrofolate reductase (*pfdhfr*) genes, respectively, while single nucleotide polymorphisms in the multidrug resistance (*pfmdr1*) and chloroquine resistance transporter (*pfcrt*) genes have

been associated with altered susceptibility to CQ and amodiaquine (reviewed in Hayton & Su 2008). *Pfmdr1* polymorphism was shown to be also involved in the development of tolerance/resistance to lumefantrine (Sisowath *et al.* 2005; Dokomajilar *et al.* 2006). More recently, pfATPase6 protein coded by the only SERCA-type Ca^{2+}-ATPase gene in the *P. falciparum* genome, has been suggested as a possible target for artemisinins and the observed substitution S769N in the *pfATPase6* has been associated with lowered *in vitro* sensitivity to artemether in *P. falciparum* isolates from French Guyana (Jambou *et al.* 2005) but not from elsewhere (White 2008). Prevalence of known molecular drug resistance markers are a useful proxy for surveillance of *in vivo* or *clinical* resistance in a population.

Microsatellite (MS) repeats can be used to assess the evolutionary history of drug resistance genotypes (Ferdig & Su 2000; Cortese *et al.* 2002). In particular, MSs polymorphism flanking resistance genes has been used to examine the evolutionary origins of mutant alleles at *pfcrt* (Fidock *et al.* 2000; Wootton *et al.* 2002) and *pfdhfr* (Nair *et al.* 2003; Roper *et al.* 2003, 2004). In the case of *pfcrt* and the *dhfr* a highly resistant mutant allele has originated in Southeast Asia and subsequently spread to Africa. In the case of *pfdhfr* there are a variety of mutant alleles common in Africa, including single mutant (S108N) double mutant (S108N, C59R or S108N, N51I) and triple mutant (S108N, C59R and N51I). Analysis of flanking microsatellites shows these have multiple independent origins, yet the triple mutant allele (S108N, C59R and N51I) alone has a flanking microsatellite haplotype in common with resistance alleles in southeast Asia (Roper *et al.* 2004). To date it is confirmed that *pfdhfr* triple mutants sampled in Tanzania and South Africa (Roper *et al.* 2003), Senegal (Ndiaye *et al.* 2006), Kenya (McCollum *et al.* 2006; Certain *et al.* 2008), Congo, Gabon, Ghana, Mali and Uganda (Maïga *et al.* 2007) and Uganda (Lynch *et al.* 2008) all derived from the southeast Asian lineage.

In order to have further information to the limited knowledge of antimalarial resistance in Angola, we estimated the prevalence of drug resistance associated mutations in five genes and we analysed the microsatellites around *pfdhfr* gene in blood samples obtained from *P. falciparum*-infected individuals from the Northern Angolan province of Uige.

Methods

Samples

Between 27 July and 12 August 2004, 71 samples of blood from children admitted to the Pediatric Emergency Unit of Uige Provincial Hospital for suspected malaria, were collected after obtaining informed consent from their parents or guardians. Prior to treatment, for each *P. falciparum* trophozoite-positive (mean parasitaemia 113 210/μl, range 160–464 640/μl) febrile children (mean age 10 months, range 4–108 months), samples of capillary blood were spotted onto a 2.5-cm glass-fibre membrane disc supported on a sector of Whatman filter-paper disc. DNA was extracted from a fragment of the glass-fibre membrane blood-spot by using QIAamp DNA blood Kit following the manufacturer's instructions (Qiagen, CA, USA). Following extraction, DNA was concentrated using Microcon-100 (Millipore) up to 50 μl of final volume.

Molecular analysis

In order to evaluate the presence of multiple infections of two or more clones of *P. falciparum*, we have amplified polymorphic regions of *pfmsp1* and *pfmsp2* genes, according to the method of Wooden *et al.* (1993). Samples with polyclonal infections were not excluded from the analysis and this was taken into consideration when results analysis was done. The prevalence of the genetic polymorphisms associated with antimalarial resistance was evaluated as described below. PCR-restriction fragment length polymorphism (RFLP) based protocol was employed to analyse *pfmdr1* gene fragment spanning codons 86, as previously described in Duraisingh *et al.* (2000). *Pfmdr1* PCR products were restricted at 50 °C with 0.5 U of *Apo1* (New England Biolabs, Schwalbach, Germany) restriction enzyme for the recognition of *pfmdr1* 86N, adhering to the manufacturer's protocol. The resulting digests were resolved in a 3% (3:1) Nusieve GTG agarose gel for RFLP electrophoresis for 2 h at 100 V. Analysis of *pfcrt* alleles was done by using a PCR-based method and direct sequencing, as previously described (Palmieri *et al.* 2004). Nested-PCR was employed to amplify a fragment of *pfdhfr* gene spanning codons 50–108. The first-round PCR was performed with specific primers AMP1forward and AMP4reverse described by Palmieri *et al.* (2004). In the second round, first amplification product was added to fresh PCR mixture with primers AMP3forward (5′-CCATGGAAATGTAATTCCCTAGAT-3′) and AMP4reverse (5′-TCCCAAGTAAAACTATTAGATCTTCAA-3′) obtaining a fragment of expected size of 325 base pair. The PCR cycling parameters were 30 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min. The *pfdhps* domain (719 bp) was amplified by semi-nested PCR. The oligonucleotides used were: 1st fw DHPS forward (5′-TTATGATTCTTTTTCAGATGG-3′); 1st rev DHPS reverse (5′-CCAATTGTGTGATTTGTCCAC-3′); semi-nest fw DHPS-N forward (5′-TTGTTGAA-

CCTAAACGTGCTGT-3′). The PCR cycling parameters were 30 cycles of 94 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min 30 s. *pfdhfr* and *pfdhps* PCR products were purified by Microcon-PCR devices (Millipore), according to the manufacturer's instructions, and sent to MWG Biotech company (Germany) for sequencing. Polymorphic sites were identified in sequence alignments compiled and analysed at the ISS laboratory by OMIGA 2.0 computer program. Detection of single nucleotide polymorphisms for the *pfATPase6* locus was performed by PCR and sequencing method, as described in Menegon *et al.* (2008).

Pfdhfr and *pfdhps* polymorphism analysis has been carried out also at LSHTM laboratory using a different methodological approach previously described in Pearce *et al.* (2003). A nested PCR was used to amplify a 594-bp fragment of *pfdhfr* and a 711-bp fragment of *pfdhps* each containing the sequence where mutations are found. The amplified PCR products were screened for *pfdhfr* and *pfdhps* sequence variants at codons 51, 59 and 108 of *pfdhfr* and codons 436, 437, 540, 581 and 613 of *pfdhps* by *Sequence Specific Oligonucleotide Probing* (SSOP), as described by Pearce *et al.* (2003). The probed blots were visualized using ECF substrate and detection using a phosphoimager (GE Healthcare, Buckinghamshire, UK). Output was recorded through viewing of digitally captured images of chemifluorescent signal. The stringency and specificity of the hybridization process was confirmed by inspection of a series of four controls of known single genotype variant sequence. All blots with non-specifically bound probes were stripped and re-probed. A single nucleotide polymorphism (SNP) was considered to be present in the PCR product when the intensity of signal was higher than that of the background. The presence, absence, and relative abundance of hybridization signal was recorded for every probe at each locus. A sample was considered to have a single haplotype when only one sequence variant was found at each locus.

Microsatellite analysis

To examine the evolutionary origins of *pfdhfr* resistance haplotypes, we studied polymorphic microsatellite repeats within 8 kb flanking regions of the gene. We analysed microsatellite sequences located 0.1, 4.4 and 5.3 kb upstream from the *pfdhfr* gene which is on chromosome 4. We amplified each locus by PCR then assessed the size of the fragments (reflecting the number of repeats) by electrophoresis on ABI 3730 and using the software Genemapper (Applied Biosystems, Warrington, Cheshire, UK). A semi-nested PCR design was used and primer sequences and PCR reaction conditions are described in an earlier paper (Roper *et al.* 2003).

Ethics

Scientific and ethical permissions were given by the Ministry of Health of Angola and the ethics committee of the London School of Hygiene and Tropical Medicine.

Results

Genotyping

Seventy-one blood samples, collected at the Uige Provincial Hospital from children with uncomplicated malaria were selected for this study. Of the 71 original samples, 66 were successfully characterized for all drug resistance molecular markers considered for the present study. The genotyping of the isolates performed by amplification of the *pfmsp1* and *pfmsp2* loci allowed the identification of several alleles of both genes, that indicating the presence of polyclonal infections in the 34.8% (n = 23/66) of samples.

By PCR-RFLP or PCR amplicons sequencing, it has been possible to characterize the following codons: 72, 74, 75 and 76-*pfcrt*; 86-*pfmdr1*; 50, 51, 59 and 108-*pfdhfr*; 436, 437, 540, 581 and 613-*pfdhps*; 243, 263, 431, 623 and 769-*pfATPase6* (Figure 1). The analysis of *pfcrt* showed that 93.9% (n = 62/66) of the isolates harboured the *pfcrt* 74I-75E-76T mutations and only four isolates carried the wild type codon (MNK haplotype). The p*fcrt*72S allele was not found in any of the samples. The asparagine-to-tyrosine mutation at codon 86 of the *pfmdr1* gene was found in 45 isolates (68.2%), although 21 of these mutant samples exhibited mixed wild type/mutant alleles.

As regards SP resistance markers, the *dhfr* 51I and 108N mutations were detected in 95.4% (n = 63/66) and 96.9% (n = 64/66) of the tested isolates, respectively, while the 59R mutation was identified in 36.4% (n = 24/66) of samples. Likewise, nearly all samples showed the *pfdhps* 437G (92.4%; n = 61/66) while 51.5% (n = 34/66) showed *dhps* 436A residue. The known *pfdhfr* mutation at codon 50 and *pfdhps* mutations at codons 540, 581 and 613 were not detected. Typing of *pfdhfr* and *pfdhps* alleles was confirmed using the SSOP technique. The isolates that exhibited both wild-type and mutant type alleles of the drug resistance markers were considered as mutants.

At this study site, a total of 17 different haplotypes were identified by analysis of the allele combinations in four genes analysed, all listed in Table 1. The most frequent haplotypes were the six-fold mutated haplotypes (36.3%) and particularly the haplotype *crt*T_{76}-*mdr*Y_{86}-*dhfr*$I_{51}N_{108}$-*dhps*$A_{436}G_{437}$ (21.2%). Moreover, we found five-fold mutated haplotypes in 33.3% of the isolates and seven-fold mutated isolates in 13.7%.

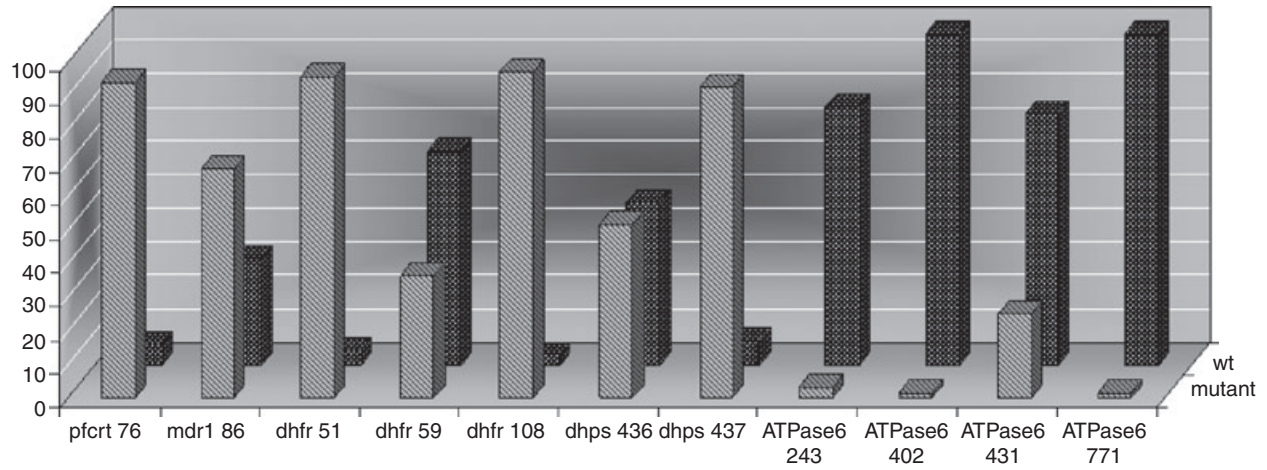

Figure 1 Prevalence of *Plasmodium falciparum* mutations associated with antimalarial drug resistance in isolates from Uige province. *pfcrt* 76 mutant allele is representative of *pfcrt* 741-75E-76T haplotype; *pfcrt* 72, *pfdhfr* 50, *pfdhps* 540-581-613 and *pfATPase6* 263-623-769 alleles were omitted from the graph because all the Angolan samples carried the wild type alleles.

Table 1 Haplotypes identified in Angolan isolates by the analysis of four molecular markers of CQ and SP resistance

No. of point mutations	*pfcrt* 76	*pfmdr1* 86	*pfdhfr* 51	*pfdhfr* 59	*pfdhfr* 108	*pfdhps* 436	*pfdhps* 437	No. of isolates TOT: 66
0	K	N	N	C	S	S	A	1
1	T	N	N	C	S	S	A	1
3	T	N	I	C	N	S	A	1
4	K	Y	I	C	N	S	G	1
4	T	Y	I	C	N	S	A	1
4	T	N	I	C	N	S	G	6
5	K	Y	I	R	N	S	G	1
5	K	Y	I	C	N	A	G	1
5	T	N	I	C	N	A	G	5
5	T	Y	I	C	N	S	G	11
5	T	Y	I	R	N	S	A	1
5	T	Y	N	R	N	S	G	1
5	T	N	I	R	N	S	G	2
6	T	N	I	R	N	A	G	5
6	T	Y	I	C	N	A	G	14
6	T	Y	I	R	N	S	G	5
7	T	Y	I	R	N	A	G	9
Reference								
Wild type	K	N	N	C	S	S	A	
	aaa	aat	aat	tgt	agc	tct	gct	
Mutant	T	Y	I	R	N	A	G	
	aca	tat	att	cgt	aac	gct	ggt	

We also performed an analysis of *pfATPase6* polymorphisms and we found a low level of SNPs frequency: wild type alleles at codons 263, 623 and 769 were found in all isolates; mutations coding for substitutions at residue 243 (H → Y), previously reported in Cojean *et al.* (2006), and at the residue 431(E → K) were observed in subsets of 2 and 16 isolates, respectively. Finally two isolates carried single novel SNPs coding for substitutions at residue 402 (L → V) and residue 771 (K → E).

Microsatellite markers

Microsatellites loci were analysed among a subset of 49 isolates, of which 19 (38.8%) were triple mutant, 29

(a)

Microsatellite loci			*Pfdhfr codons*		
5.3 kb	4.4 kb	0.1 kb	51	59	108
203	177	108	I	R	N
.	176 > 178	.	■	■	■
.	.	.	■	■	■
.	.	.	■	■	■
.	.	.	■	■	■
193	.	.	■	■	■
.	.	.	■	■	■
.	.	.	■	■	■
203 > 193	.	.	■	■	■
203 > (193 + 217)	.	.	■	■	■
.	.	.	■	■	■
.	.	.	■	■	■
.	.	.	■	■	■
.	.	.	■	■	■
.	.	.	■	■	■
.	.	.	■	■	■
.	.	111	■	■	■
193 > 203	.	102 > 108	■	■	■
.	.	108 > 88	■	■	■
193	.	102 > 108	■	▨	■

(b)

5.3 kb	4.4 kb	0.1 kb	51	59	108
193	177	102	I	R	N
.	.	.	■		■
223	152	.	■		■
.	.	.	■		■
191	.	.	■		■
.	.	.	■		■
209	180	.	■		■
.	.	.	■		■
.	.	.	■		■
.	na	.	■		■
.	na	.	■		■
.	.	.	■		■
.	.	.	■		■
.	.	.	■		■
.	na	.	■		■
.	na	.	■		■
na	.	.	■		■
.	.	.	■	▨	■
.	.	.	■	▨	■
na	.	.	■	▨	■
191	.	.	■	▨	■
.	.	.	■	▨	■

(c)

5.3 kb	4.4 kb	0.1 kb	51	59	108
193	177	100	I	R	N
.	.	.	■		■
191	.	.	■		■
.	.	.	■		■
.	.	.	■		■
.	.	.	■		■
.	.	.	■		■
.	180	.	■		■
205	174	87	■		■

5.3 kb	4.4 kb	0.1 kb	51	59	108
191	na	96	N	C	S

(59.2%) were double mutant (all S108N and N51I) and just one isolate (2%) was wild type. We compared microsatellite sequences by measuring the size of PCR amplified products on a capillary sequencer, so the alleles are individually identified as fragment sizes. In Figure 2 alleles at loci 0.1, 4.4 and 5.3 kb upstream from the major point mutation haplotypes of *pfdhfr* are shown. The 108, 177 and 203 bp alleles at the 0.1, 4.4 and 5.3 kb loci, respectively were seen in association with the triple mutant allele (Figure 2a). This haplotype matches the canonical Asian-derived flanking haplotype previously described (Roper *et al.* 2004). In some isolates these alleles were found in mixture with another allele of different size, and we attribute this to the co-occurrence of multiple genotypes in the same infection. There were three isolates where a single allele different from the canonical type was found. In each case the alleles of other two loci matched and so we infer this was due to recombination events. The double mutant *pfdhfr* was found in association with two microsatellite haplotypes: 102, 177, 193 bp and 100, 177, 193 bp. The microsatellite allele sizes in isolates of the two types are shown in Figure 2b,c, respectively. Only a single wild type isolate was analysed (Figure 2d) but this was clearly associated with a different haplotype with a unique 96 bp allele found at the most closely linked locus.

Discussion

Uige province has high perennial levels of transmission in the forest and savannah regions and experiences one of the highest incidences of severe malaria and malaria-related mortality if compared with the other Angolan provinces (Dr Pisani and Dr Matondo, personal communication). At the time of this study, the summer of year 2004, there were no data available on the efficacy of the different antimalarials in the Northern province of Uige and, still, data on this issue are scanty even from the country as a whole.

At present, high-throughput genotyping methods, such as the analysis of single nucleotide polymorphisms and microsatellites, provide reliable tools for monitoring drug resistance and assessing the evolutionary history of the resistant genotypes (Hayton & Su 2008). In the current study, we used both the above methodological approaches to study the prevalence of the mutations linked to the drug resistance in the target genes (*pfcrt*, *pfmdr1*, *pfdhfr/pfdhps* and *pfATPase6*) and to track the evolution of pyrimethamine resistance (*MSs* flanking *dhfr* gene) in this endemic African area. Table 1 shows that, overall, the mutational

Figure 2 *pfdhfr* alleles at codons 51, 59, 108 and flanking microsatellites at loci 5.3 kb. 4.4 kb and 0.1 kb upstream from *pfdhfr* gene identified in *P. falciparum* isolates from UIGE province.

analysis of genetic polymorphisms in four drug-resistance genes considered in the present study identified 17 different haplotypes. In summary, 24 isolates (36.3%) carried six point mutations segregated in three haplotypes; five point mutations (and seven haplotypes) were found in 22 isolates (33.3%). Nine isolates (13.7%) carried a seven-fold mutated haplotype. Haplotypes characterized by three mutations, one mutation and wild type genotype were observed only in rare single samples. The overall molecular markers high frequencies observed in the present study and the detection at high frequency of some haplotypes linked to antimalarial treatment failure (for example the 91% frequency of the haplotype carrying *dhfr*$I_{51}N_{108}$–*dhps*G_{437} triple mutant allele, critical to SP resistance) are consistent with the drug policy switch to ACT as the first-line treatment established in Angola in 2006 (and if available at that time, they could have supported the results of the Efficacy Therapeutic Tests, the gold standard tests to make the local health agency consider actual changes in the malaria treatment drug policy). In this scenario, we can easily anticipate the difficulty in treating this kind of multi resistant *P. falciparum* in the absence of an effective artemisinin derivative combination therapy, perhaps heralded by the worrisome occurrence of *P. falciparum* artemisinin resistance development in Asia (Noedl *et al.* 2008).

Analysis of microsatellite loci flanking drug resistance genes can give important clues about the geographical origins of mutant lineages. Analysis of microsatellite loci around resistant *pfdhfr* in Thailand, Myanmar, Laos, Cambodia and Vietnam compared all allelic combinations of point mutations at *pfdhfr* and found they all derived from one common ancestor (Nair *et al.* 2003). Microsatellite analysis of resistant *pfdhfr* in Africa has shown that the triple mutant (N51I C59R S108N) in Africa shares a common ancestry with resistant *pfdhfr* from South East Asia (Roper *et al.* 2004). The Asian derived triple mutant allele has been recorded at sites across the African continent (Roper *et al.* 2003; McCollum *et al.* 2006; Ndiaye *et al.* 2006; Maïga *et al.* 2007; Certain *et al.* 2008; Lynch *et al.* 2008) and its presence in Angola is confirmed in this study. The double mutant N51I+S108N *pfdhfr* allele is associated with two microsatellite flanking haplotypes 102 + 177 + 193 bp and 100 + 177 + 193 bp at the 0.1, 4.4 and 5.3 kb microsatellite loci (illustrated in Figure 2b,c), confirming that these double mutant *pfdhfr* alleles evolved separately from the triple mutant lineage. The microsatellite haplotypes associated with N51I+S108N double mutant alleles in Uige are different from that associated with the N51I+S108N in Tanzania and South Africa (Roper *et al.* 2003), Kenya (McCollum *et al.* 2006; Certain *et al.* 2008; Mita *et al.* 2009) or Uganda (Lynch *et al.* 2008) which suggests that these double mutant alleles of *pfdhfr* are of local origin.

The microsatellite analysis results show that, as already observed in other African endemic countries, the *pfdhfr* triple mutant Asian haplotype has been introduced in Angola. Also, however, the presence of unique double mutant alleles in the local *Plasmodium* population, indicates that an increasing number and variety of S/P resistant parasites circulate in this region.

In conclusion, the results obtained in the present study highlight the importance of molecular markers screening to monitor the evolution of *P. falciparum* drug resistance in African endemic regions. The investigation on drug resistance mutations prevalence as well as the analysis of the *MSs* flanking the target genes provide crucial information that could support data from the Efficacy Therapeutic Tests, allowing local health agencies to set up an effective country-wide antimalarial drug policy.

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