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Defining the origin of *Plasmodium falciparum* resistant *dhfr* isolates in Senegal

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Abstract

We previously reported a high baseline prevalence of mutations in the *dhfr* and *dhps* genes of *Plasmodium falciparum* throughout Senegal. The highest prevalence of the triple *dhfr* pyrimethamine associated mutations were found in isolates obtained in the western part of the country near the capital city of Dakar. In this study, we sought out to determine the relatedness of *dhfr* wild type and mutated strains by analyzing three microsatellite regions upstream of the *dhfr* locus. Twenty-six of the 31 wild type strains had a unique microsatellite pattern. In contrast of the 17 isolates containing the triple mutation in *dhfr*, 11 had an identical microsatellite pattern. Diverse geographical isolates in Senegal containing the triple *dhfr* mutation have arisen from a limited number of ancestral strains. In addition, we demonstrate that these isolates have shared ancestry with the previously reported triple mutation haplotype found in Tanzania, South Africa, southeast Asia. This common ancestry may have implications for the malaria control strategy for reducing the spread of sulfadoxine–pyrimethamine resistance in Senegal and elsewhere in Africa.

Keywords

Plasmodium falciparum; Drug resistance; Senegal; *Dhfr*; Sulfadoxine–pyrimethamine

1. Introduction

Drug therapy remains the mainstay of management in *Plasmodium* sp. infections; however, only a limited number of compounds are available and drug resistance is common. Because of high prevalence of chloroquine resistance in Senegal, the national malaria treatment policy was recently changed to sulfadoxine–pyrimethamine-amodiaquine (SP-AQ) from chloroquine. A high prevalence of isolates containing the *dhfr* pyrimethamine drug resistance haplotype (N51I, C59R, S108N) was described prior to the introduction of SP-AQ as first line therapy in Senegal (Ndiaye et al., 2005). Furthermore, there is a marked difference in the prevalence of resistance across the country. The highest prevalence of *dhfr* genotypic resistance occurs in

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the western coastal region near the capital city of Dakar, whereas, a lower prevalence of resistance is found in the eastern, interior region of the country. Upstream flanking microsatellites of *dhfr* in wild type and genotypic resistant *dhfr* isolates was analyzed and compared between these two regions to understand the origin and spread of pyrimethamine resistance. Microsatellite size polymorphisms in the upstream flanking region of *dhfr* are highly variable in wild type isolates as well as in those isolates with one or two mutations in *dhfr*. In contrast, isolates containing the triple mutation in *dhfr* are less polymorphic, suggesting they arose from a small number of ancestral strains. In addition, the microsatellite sizes of the strains containing the triple mutation is similar between the two geographic regions and those previously reported from southeast Asia.

2. Material and methods

2.1. Patient population

To define the relationship of parasite isolates at the *dhfr* locus we analyzed samples from two geographically separated regions in Senegal (Fig. 1). Samples were collected from patients with a blood smear positive for *Plasmodium falciparum*, mild malaria, 5 years or greater and no history of recent treatment with antimalarials or chronic illness. Forty-one blood samples were analyzed from western Senegal in Pikine and Thies and the eastern villages near Tambacounda and Santhioumaleme which had been collected as part of a previous study in 2003 (Ndiaye et al., 2005). To increase the sample size from eastern Senegal, an additional 18 samples from were collected and analyzed from villages near Velingara in 2004. Patient blood samples were collected after informed consent was obtained. The Human Subjects Committee of Harvard School of Public Health in Boston and the Ethics Committee of Cheikh Anta Diop University in Dakar approved the protocol used in this study.

2.2. Study sites

In the western part of Senegal, the study region included Pikine, which is 15 km from the coastal city of Dakar and has an entomological inoculation rate (EIR) of 1 as well as Thies (EIR = 1–20), which is 70 km from Dakar (Trape et al., 1992). The second study region is greater than 400 km from Dakar in the eastern portion of the country and has, in contrast to the west, a high transmission rate with EIR > 100 in the small villages surrounding Santhioumaleme, Tambacounda and Velingara (Faye et al., 1995).

2.3. Molecular analysis

DNA was extracted from finger prick samples collected on filter paper using DNA Minikit (Quiagen) following the manufacturer's instructions and used for *dhfr* sequencing and microsatellite analysis. *Dhfr* haplotypes were already defined for all villages except Velingara. To determine the prevalence of *dhfr* mutations from the Velingara 2004 samples, nested PCR was followed by cloning and sequencing using primers and protocol as previously described (Ndiaye et al., 2005). To determine the number of clones in each sample, genotyping was performed for the merozoite surface protein (MSP) 1 and MSP 2 alleles by use of polymerase chain reaction (PCR) with nested primers as previously described (Snounou et al., 1999).

2.4. Microsatellite analysis

Microsatellites at 0.3, 4.4 and 5.3 kb upstream of *dhfr* gene were analyzed based on Roper et al. primer sequences and protocol (Roper et al., 2003). Additional nucleotide sequence were added to three of these primers to minimize stutter artifact: PFDHFR0.3 kb (F: 5′-CTG TCT TAT TCC AAC ATT TTC AAG A-3′) PFDHFR4.4 kb (R: 5′-CTG TCT TCG ATA TAT CTG ATG GGT GA-3′); PFDHFR5.3 kb (R: 5′-CTG TCT TCA CAT ATT ATA CAG GAC G A-3′). PCR amplification resulted in single PCR bands by agarose gel electrophoresis and the

nucleotide sequences were confirmed after cloning and sequencing. Microsatellite size was determined by capillary gel electrophoresis of the PCR product using an Applied Biosystems 3730 × l DNA Analyzer. Genemapper version 3.5 (ABI) was used to analyze the data and the highest peak is reported in this study for statistical comparisons. To determine if the triple mutant haplotype from Senegal shared ancestry with the previously reported SE Asian, South African and Tanzanian triple mutation haplotype, the samples were simultaneously analyzed with SE Asian and African standards on an identical ABI Analyzer in Dr. Roper's laboratory. The primer sequences for this analysis were taken directly from Roper et al. (2003) published protocol without modification.

2.5. Statistical analysis

To compare the statistical association of microsatellite size polymorphisms and *dhfr* drug resistance haplotypes Fisher's exact test was used for two-tailed significance at $p = 0.05$ (STATA, 9.0, Stata Corporation).

3. Results

The additional haplotype data from villages near Velingara revealed 72% of the samples to contain wild type *dhfr*. Of the 18 samples collected, 13 were wild type, 1 had a single mutation at S108N, and 4 had the triple mutation (N51I, C59R, S108N).

To evaluate ancestry of the triple *dhfr* mutation isolates, we analyzed microsatellites upstream of *dhfr* in 59 samples. Thirty samples were derived from western Senegal and 29 samples came from eastern regions of Senegal. The *dhfr* haplotypes included 31 wild type, 11 single mutation samples (S108N) or double mutation (C59R–S108N) and 17 samples containing triple mutations (N51I, C59R, S108N).

Over 50% of the samples are monoclonal within each *dhfr* haplotype by MSP1 and 2 genotyping. There are 34 samples with monoclonal infection and the number of monoclonal samples in each *dhfr* haplotype is similar. Compared to the number of monoclonal infections in the *dhfr* wild type samples, the number of monoclonal infections in the one to two mutation group or the triple mutation group was not significantly different (Fisher's exact test $p = 1$, $p = 0.8$, respectively).

Table 1a reports the microsatellite sizes for the 31 *dhfr* wild type samples from western and eastern Senegal. The microsatellite 0.3 kb upstream from start site of *dhfr* ranges in size from 93 to 118 bps, marker 4.4 kb ranges from 175 to 188 bps and marker 5.3 kb varies between 187 and 225 bps. The 4.4 kb microsatellite demonstrates the least size variability with 75% of the samples containing 183 bp. When analyzing all three microsatellite polymorphisms, there are 26 unique microsatellite patterns within the wild type isolates. The haplotype, 109/183/211 is found once in the western region and twice in the eastern region. Of the 11 samples containing one (S108N) or two *dhfr* mutations (C59R–S108N), all have unique microsatellite patterns (Table 1b). In the 17 samples that contain the triple *dhfr* mutation, there are four patterns, with the majority containing the 109/183/211 haplotype (Table 2). The occurrence of the 109/183/211 pattern is not statistically different between wild type and one to two mutations haplotypes. Conversely there is statistically significant higher prevalence of this pattern in the triple mutation isolates compared to the samples containing the wild type haplotype (Fisher's exact test, two-sided $p = 0.000$). This remains statistically significant when samples that are monoclonal by MSP1/2 only are analyzed ($p = 0.011$).

Samples P14.03 and S17.03 which contain the triple mutation haplotype and the common 109/183/211 microsatellite haplotype were run with the previously reported SE Asian and African triple mutation haplotype samples and found to have matching microsatellite sizes.

4. Discussion

P. falciparum has developed resistance to many classes of antimalarials. The analysis of drug resistance development and its spread is important until non-chemotherapeutic options become available for prevention/treatment of malaria. Our previous studies demonstrated a high prevalence of isolates containing a triple *dhfr* drug resistance associated haplotype in Senegal. This triple mutation has been shown to correlate with significant antifolate drug resistance (Sibley et al., 2001). As the distance from the capital city, Dakar increases, the prevalence of the *dhfr* triple mutation decreases (Ndiaye et al., 2005). We wanted to determine the genetic relationship of these triple mutation parasites, as previous reports have found that *dhfr* drug resistant isolates have arisen from a few primary isolates, rather than occurring from independent mutational events (Nair et al., 2003; Roper et al., 2003, 2004; Pearce et al., 2005; Cortese et al., 2002).

We previously reported that the drug resistance associated *dhfr* triple mutation haplotype (N551R, C59R, S108N) in western Senegal including Pikine and Thies was 69% (Ndiaye et al., 2005). This was in contrast to a higher prevalence of wild type isolates in eastern Senegal. The additional *dhfr* haplotype data analyzed from Velingara in the eastern region of Senegal confirms the lower prevalence of *dhfr* resistance with only 28% of isolates containing the triple *dfhr* mutation. For the microsatellite analysis, we predicted that the flanking region of *dhfr* in wild type samples would be diverse, reflecting unique origins, and a lack of linkage disequilibrium. Indeed, the microsatellites at 0.3 and 5.3 kb upstream of *dhfr* show marked size polymorphism. Interestingly the microsatellite 4.4 kb upstream shows very little size variability and therefore is less informative regarding shared ancestry. Microsatellites can have unique mutation rates based on the length of the dinucleotide repeats. Microsatellites containing long dinucleotide repeats have a higher heterogeneity compared to microsatellite containing shorter repeats (Anderson et al., 2000). The 4.4 kb marker contains the shortest dinucleotide repeat and demonstrates the least heterogeneity compared to the microsatellites at 0.3 and 5.3 kb, which is consistent with this observation. The size variation in the 0.3 and 5.3 kb microsatellites in the wild type isolates suggests that this allele is ancestral. The microsatellites from isolates containing one or two *dhfr* mutations are also diverse and appear to have arisen independently. The most striking finding is the lack of diversity in the microsatellites of the isolates containing the triple *dhfr* mutation. There is dominant pattern with 65% of the isolates containing a 109/183/211 pattern and this is identical in both geographic regions. The second most common pattern found in 24% of the triple mutation samples varies only at the 0.3 kb marker (113/183/211), and is also found in both regions.

The lack of diversity in the triple mutant is consistent with the model that parasite mutations become established in the population infrequently, despite their enhanced fitness under drug pressure (Hastings, 2004). Strains with the triple mutation that are highly prevalent may contain additional mutations outside the *dhfr* locus to allow them to maintain fitness and be transmitted (Walliker et al., 2005). Further genetic analysis of strains with the *dhfr* triple mutation in high prevalence parasites versus low prevalence parasites may provide insight into mechanisms of parasite fitness and transmission.

The higher prevalence of the triple *dhfr* mutation in western Senegal suggests that the resistant strains originated in this region before spreading to eastern Senegal. The exact local origin of this drug resistant haplotype in Senegal is unknown, however we have demonstrated shared ancestry of the common microsatellite haplotype 109/183/211 with the triple mutation haplotype found in Tanzania, South Africa, southeast Asia (Roper, 2003, 2004) and most recently Kenya (McCollum et al., 2006). The slightly larger allele sizes reported here are due to differences in primer length used in our protocol, but the match was confirmed by direct comparison. We also observed the triple mutant in association with three less common but

related microsatellite backgrounds 93/183/211, 113/183/211 and 109/183/255. Similarly the Kenyan study found two related microsatellite haplotypes associated with the triple mutant also differing at the 0.3 kb locus, together with one entirely different microsatellite haplotype which was unrelated to the 'Asian' type (McCollum et al., 2006). Variation in drug pressure, transmission intensity and parasite population diversity may underlie the development of novel drug resistant strains and/or favor spread of alleles with shared ancestry. Further mapping of drug resistant strains in Africa will provide insight into mechanisms of spread and development of drug resistance.

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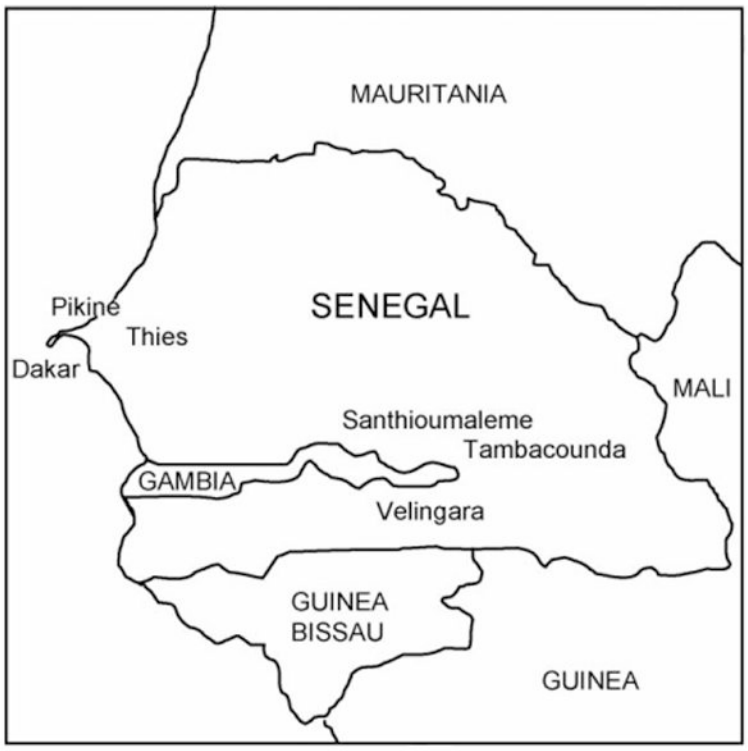

Fig. 1.
Map of Senegal and study sites. Samples derived from western Senegal were obtained in Pikine and Thies, near the capital city of Dakar. Samples from eastern Senegal were collected from villages near Tambacounda, Santhioumaleme and Velingara.

Table 1a

Microsatellite sizes of wild type *dhfr* isolates in Senegal

sample	Dhfr0.3kb	Dhfr4.4kb	Dhfr5.3kb
P33.03	93	183	211
Th6.03	103	183	200
P37.03	103	183	211
P44.03	106	177	214
Th4.03	109	183	211
P9.03	109	183	187
Th16.03	109	183	217
P45.03	109	183	221
Th9.03	113	175	211
P49.03	113	183	211
P78.03	113	183	211
P17.03	116	183	204
P20.03	118	183	211
T6.03	93	183	211
S5.03	93	183	217
T7.03	93	183	221
V32.04	93	186	217
V35.04	100	188	211
V28.04	109	183	211
V48.04	109	183	211
T1.03	109	183	214
S1.03	109	185	208
V25.04	113	183	211
V40.04	113	183	211
V51.04	113	183	217
V13.04	113	183	221
V37.04	113	183	225
V55.04	116	193	214
V20.04	116	193	221
V43.04	118	183	208
V50.04	118	188	208

Three microsatellites upstream of the start site of *dhfr* at 0.3, 4.4 and 5.3 kb are reported. Two geographic regions are studied, with non-bolded sample names from western Senegal: P = Pikine, Th = Thies; below the dark line and bolded are isolates collected from eastern Senegal: T = Tambacounda, S = Santhioumaleme, V = Velingara. Microsatellites sizes that dominate the triple mutation are in grey (109, 183, 211). Note unique patterns in most isolates.

Table 1b

Microsatellite sizes of single or double mutation *dhfr* isolates in Senegal

sample	Dhfr0.3kb	Dhfr4.4kb	Dhfr5.3kb
**P21.03	93	188	200
*Th12.03	103	183	200
*P24.03	103	183	218
*P25.03	109	179	218
*Th 10.03	109	183	211
*P22.03	118	179	218
***S18.03**	93	183	221
***V34.04**	103	188	200
****S7.03**	103	183	211
***T4.03**	109	183	218
****S13.03**	109	183	208

* Samples denoted with contain one mutation (S108N).

** Contains two mutations (C59R–S108N), note unique patterns in all isolates. For more details see the legend of Table 1a.

Table 2

Microsatellite sizes of triple (N51I, C59R, S108N) mutation *dhfr* isolates in Senegal

sample	Dhfr0.3kb	Dhfr4.4kb	Dhfr5.3kb
P19.03	93	183	211
P51.03	93	183	211
P13.03	109	183	211
P14.03	109	183	211
P15.03	109	183	211
P35.03	109	183	211
P42.03	109	183	211
P46.03	109	183	211
P53.03	109	183	211
P69 03	113	183	211
Th11.03	113	183	211
S17.03	109	183	211
V21.04	109	183	211
V52.04	109	183	211
S20.03	109	183	225
V54.04	113	183	211
V57.04	113	183	211

Three microsatellites upstream of the start site of *dhfr* at 0.3, 4.4 and 5.3 kb are reported. Two geographic regions are studied, with non-bolded names from western Senegal: P = Pikine, Th = Thies; below the dark line and bolded are isolates collected from eastern Senegal: T = Tambacounda, S = Santhioumaleme, V = Velingara. Microsatellite sizes that dominate the triple mutation are in grey (109, 183, 211). Note the loss of variation in microsatellites sizes, with one pattern, 109/183/211 dominating in both regions.