Molecular Markers for Tracking the Origin and Spread of Sulfadoxine and Pyrimethamine Resistant Mutations

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Abstract

Molecular markers are valuable tools for monitoring the emergence of drug resistance. Single nucleotide polymorphisms (SNPs) in the antimalarial drug target genes (eg: Pfcrt for chloroquine, *dhfr* for pyrimethamine) have been correlated with clinical resistance. Microsatellite markers, which are random repeats of nucleotides spread across different parts of the chromosomes, can be used as tools to track the origin and spread of drug resistant mutations. We have used several microsatellite markers to track the drug resistant *dhfr* and *dhps* genotypes in parasite isolates from different endemic areas of Africa and South America. In this presentation, how these molecular tools helped us to determine the lineage relationship between different *dhfr* genotypes in Agrica and South America will be described.

Introduction

The emergence of drug resistant *Plasmodium falciparum* is a serious public health problem in many countries where malaria is endemic (Gregson and Plowe, 2005; Talisuna, Bloland and D'Alessandro, 2004). Resistance to chloroquine (CQ), the least expensive and widely available antimalarial drug, has spread throughout the world except in Central America and the Caribbean. As a result, sulfadoxine-pyrimethamine (SP) became the next widely used drug treatment for uncomplicated *P. falciparum* malaria.
Unfortunately, resistance to SP has become established in many parts of the world, as well. Currently, artemisinin based combination therapy (ACT) is advocated as the best treatment option to circumvent the rapid emergence of resistance to malaria.

Understanding the mechanisms involved in drug resistance is critical to developing methods for the prevention of emergent drug resistance, the development of molecular surveillance tools, and the formulation of appropriate policy to respond to drug resistance. It has become evident that there is a genetic basis for drug resistance to CQ, SP and other drugs (Gregson and Plowe, 2005; Talisuna, Bloland and D'Alessandro, 2004). Resistance to CQ is strongly associated with mutations in the *pfcrt* gene which encodes a transmembrane protein in the digestive vacuole (Sidhu, Verdier-Pinard and Fidock, 2002). SP acts as an inhibitor of the *P. falciparum* folic acid pathway, and point mutations in the genes encoding dihydrofolate reductase (*dhfr)* and dihydropteroate synthetase (*dhps*) have been implicated in pyrimethamine and sufadoxine resistance, respectively (Hayton and Su , 2004).

Microsatellite loci, random repeats of DNA bases (example: ATATATATATATATATATATAT), are distributed across the genome and are considered to be neutral with respect to the fitness of an organism; i.e. the microsatellite loci are assumed not to be under selection pressure because of the microsatellite sequence itself. However, these loci can be influenced by natural selection when they are physically close to a sequence under selection. When a beneficial mutation spreads through a population, the regions of the genome surrounding the mutation can become "linked" to the selected site due to their proximity and will subsequently increase in frequency

along with the beneficial mutation within the population (Smith and Haigh, 1974). This phenomenon, known as the hitchhiking effect, results in decreased genetic variation around the selected site in the population (Smith and Haigh, 1974). Microsatellite loci that are physically close to genes that confer drug resistance have become valuable tools in studying the origin and spread of resistant alleles. Using such an experimental approach it has been determined that CQ resistance originated in at least four different sites: Asia, Papua New Guinea, and two in South America (Wootton et al, 2002). In this review, we describe the latest findings of how SP-resistant alleles have emerged and spread in different parts of the world.

Genetic basis of SP resistance

At least five mutations in *dhfr* (codons 50, 51, 59, 108, and 164) and a similar number of mutations (codons 436, 437, 540, 581, and 613) in *dhps* confer resistance to pyrimethamine and sulphadoxine, respectively. The *dhfr* point mutation S108N is sufficient to confer a low level of resistance to pyrimethamine; however, additional mutations at codons 50, 51, 59, and 164 act synergistically to increase the levels of resistance (Cortese and Plowe, 1998; Plowe, Kublin and Doumbo, 1998). At least 3 different triple mutant alleles and one quadruple mutant allele have been associated with high level of resistance to pyrimethamine in different parts of the world (Table 1). The triple mutant allele 51I, 59R, 108N is found in Asia and Africa. In South America this allele has not been found and two other alleles (50R, 51I, 108N and 51I, 108N, 164L) have been reported. Among these two South American *dhfr* triple mutant alleles, only the 51I, 108N, 164L allele has been found in the Amazon region of Peru and the other *dhfr* triple mutant allele (50R, 51I, 108N) has been reported in Venezuela, Guyana, Suriname, Bolivia and Brazil. The quadruple mutant allele 51I, 59R, 108N, 164L is considered to confer the highest level of resistance to pyrimethamine, and this allele was originally found in Southeast Asia and subsequently reported in India as well (Gregson and Plowe, 2005). Detection of the presence of the mutation in codon 164 in Africa was elusive until recently when a study from our laboratory among others found evidence for the presence of this highly resistant allele in Kenya (Hastings et al, 2002; Farnert et al, 2002; Staedke et al, 2004; Alker et al, 2005; McCollum et al, 2006).

Table 1: Common *dhfr* alleles found in different geographical regions

dhfr alleles	Location	Reference
N51I/C59R/S108N	Asia,Africa	Reviewed in Gregson and Plowe, 2005
N51I/C59R/S108N/I164L	Asia, Africa	Gregson and Plowe, 2005; McCollum et al., 2006
N51I/S108N/I164L	South America	Cortese et al., 2002
C50R/N51I/ S108N	South America	Cortese et al., 2002

Common *dhps* alleles found in different geographical regions

dhps alleles	Location	Reference
A437G/K540E	Africa	Gregson and Plowe, 2005
A437G/K540E/ A581G	Asia, South America	Cortese et al., 2002; McCollum et al., 2006

Similarly, different *dhps* alleles associated with high levels of resistance have been found in different geographical regions. In Southeast Asia, triple mutant *dhps* genotype 437G, 540E, 581G has been predominantly reported. In Africa, the double mutant allele 437G, 540E is commonly found. Although other minor variants involving mutations in codons 436, 581 or 613 have been reported, they are relatively rare in Africa. In South America, the triple mutant allele 437G, 540E, 581G has been found to be the major allele associated with high level resistance (Gregson and Plowe, 2005).

Microsatellite studies: understanding the evolution of resistant alleles

An important question has been whether highly resistant alleles (triple and quadruple mutants) have come from a single common ancestor or from multiple origins. Researchers have predicted that the occurrence of simple point mutations, as seen in the case of *dhfr* and *dhps* genes, will not be a rare event, and experimental data was needed to test this hypothesis (Hastings, 2001). Microsatellite loci have been successfully used to address this hypothesis. If a given allele has a single origin then microsatellite loci surrounding all isolates of this resistant allele will be the same because these microsatellite loci are linked to the gene under selection and are consequently maintained with the resistant allele (Fig. 1). On the other hand, if there are multiple origins for the resistant allele then the microsatellite profile or haplotype will be different across samples, as explained in Fig. 1. Therefore, one can experimentally test the origin of a resistant allele by characterizing microsatellite alleles that are physically close to the allele under selection.

Using this approach, Nair et al. (2003) utilized multiple loci to show a single origin for highly resistant *dhfr* alleles among multiple sites in Southeast Asia, focusing on the Thai-Myanmar border (Nair et al, 2003). Around the same time, Roper et al. (2003) utilized three microsatellites near both *dhfr* and *dhps* (all loci were less than 10kb away from the genes) to show multiple haplotypes for the wildtype *dhfr* and *dhps* alleles, a few haplotypes for single or double mutant alleles, and one haplotype for the triple mutant *dhfr* allele (51I, 59R, 108N) in samples from both South Africa and Tanzania (Roper et al, 2003). Combining their data, these researchers suggested a single origin for highly resistant *dhfr* alleles, originating in Southeast Asia and then migrating to the African continent (Roper et al, 2004; Anderson and Roper, 2005). Work by our group in western Kenya has shown multiple haplotypes for the triple mutant *dhfr* allele, thus demonstrating that some triple mutant *dhfr* haplotypes have also emerged locally in Africa. In addition, we showed that quadruple (51I, 59R, 108N and 164L) *dhfr* alleles have evolved locally in Africa. Our findings emphasized the importance of local ecology and evolutionary history when considering the origin of resistant alleles in different geographical settings. (McCollum et al, 2006; Roper et al, 2004) (Fig. 2). Recent work in Southeast Asia using microsatellite markers has further showed an additional Southeast Asian origin for resistant double mutant *dfhr* alleles which has gone through a selective sweep in a Pacific island (Fig. 2) (Mita, 2007).

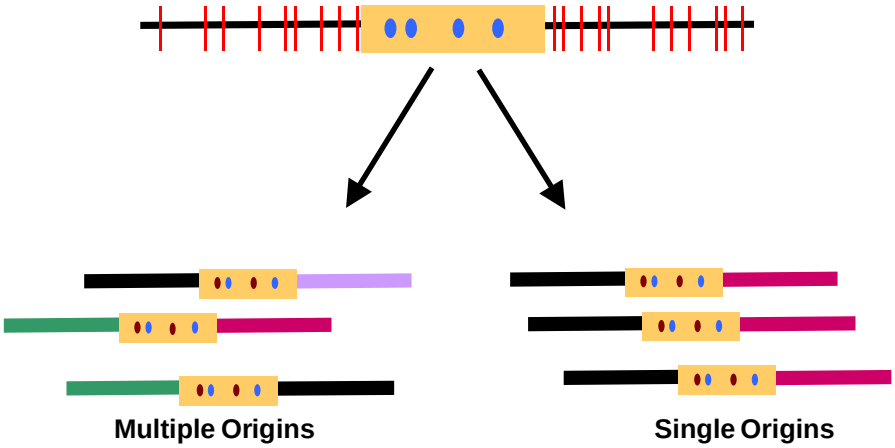
We have continued to try to understand the origins of pyrimethamine and sulfadoxine resistance by conducting a similar study in Venezuela since the ancestral origins of South American parasite isolates are not well understood. We found a single haplotype for the *dhfr* 50R, 51I, 108N and 51I, 108N alleles; and this haplotype was different at the majority of the loci than the haplotypes present in western Kenya (Fig. 2). Thus, this work has shown yet another origin for highly resistant *dhfr* alleles in South America. In addition, we have shown a common ancestral origin for the *dhps*

Table 2: Summary of published microsatellite haplotypes for *dhfr* multiple-mutant alleles[a].

	dhfr microsatellite haplotype												
Genotype	-7.55[b]	-5.3	-4.49	-4.4	-3.87	-1.22	-0.3	0.2	0.52	4.05	5.87	location found	Ref.
51I/108N	145	199	195	178	206	221	95	157	97		113	Venezuela	20
	131	191	202	174	188	224	85	158	93	190		Kenya	13
59/108			220		202				100		111	Papua New Guinea	19
			198		188				93			Solomon Islands; Vanuatu	19
50R/51I/108N	145[c]	199	195	178	206	221	95	157	97		113	Venezuela	20
51I/59R/108N	131	202	198	174	192	213	105	173	103	188		Kenya; S Africa; Tanzania; Thailand; Myanmar; Laos; Cambodia; Vietnam	13, 17
			198		192				93			Thailand; Cambodia	19
	131	202	198	174	188	213	85	158	93	189		Kenya	13
	131	191	202	188	186 / 198	213	85	172	91	196		Kenya	13
	131	191	202	176	188	224	85	158	93	190		Kenya	13
	131	191	202	172	188 / 192	213	85	158	93 / 103	189		Kenya	13
51I/108N/164L	131	191	202	183	188	224	85	167	97	183		Kenya	13
	131	191 / 202	202	174	184	200 / 213	85	158 / 166	110	187		Kenya	13
51I/59R/108N/164L	135	191 / 212	202	180	184	200 / 219	85	166	97	187		Kenya	13
	131	202	198	174	192	213	105	173	103	188		Thailand; Myanmar; Laos; Cambodia; Vietnam	17
			198		188				93			Thailand; Cambodia	19

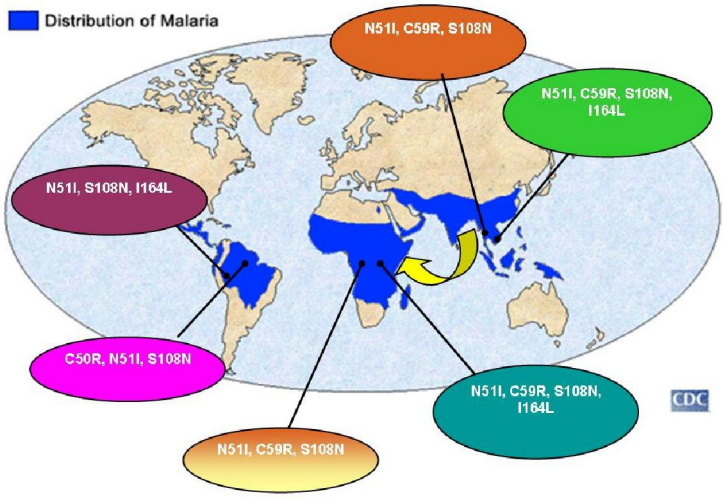
Blue alleles represent the haplotype that is commonly found in most locations. [a]We are showing the major haplotypes reported in these different regions; we are not showing minor variants. [b]Location of the microsatellite loci with respect to *dhfr* in kilobases, where negative positions are 5' to *dhfr* and non-negative positions are 3' to *dhfr*. [c]Alleles are given as PCR product sizes

Fig 1: Use of microsatellite markers to elucidate the evolutionary history of alleles under selection.



Here is a gene (yellow) with simple point mutations (blue to brown) that help the parasite in the presence of a drug. Scattered along the chromosome around the gene are microsatellite loci (red vertical lines). If the resistant allele has multiple origins, experimentally we would see multiple microsatellite profiles or haplotypes surrounding the resistant allele (left). Conversely, if the resistant allele has a single origin we would see a single microsatellite haplotype surrounding the resistant allele (right). In Table 2 we have shown a summary of microsatellite marker profiles for *dhfr* alleles based on recently published studies.

Fig 2: This figure illustrates major *dhfr* alleles found in different geographical regions. The color of the circles represents microsatellite haplotypes (lineages).



It has been hypothesized that the triple mutant *dhfr* allele N51I, C59R, S108N originated in Southeast Asia and spread to Africa (reference 17). However, it is important to note that some of the parasites with the same allele have been demonstrated to have evolved in Africa, as well (reference 13). The two alleles observed in South America have been proposed to have first occurred in the Amazon region of Brazil or Bolivia and spread to other countries in the region.

437G, 540E, 581G and 437G, 581G alleles present in Venezuela (McCollum et al, 2007).

A summary of *dhfr* haplotypes currently known has been provided in Table 2. It is important to note that we do not know the haplotypes of SP resistant alleles in India. Given that one of the *dhfr* triple mutant alleles (51I, 59R, 108N) has spread from Southeast Asia to Africa, it will be curious to know if this transition occurred through India. Further studies are needed to understand the origin of SP resistant alleles in different parts of the world.

Conclusion

Molecular markers have proven to be valuable tools for the early detection and emergence of drug resistance and also in understanding the origin and spread of resistant genotypes. It has become clear that highly resistant alleles have occurred in a few places and spread through the populations. These findings have implications for malaria control policies. For example, in some countries drug policies are changed only at the local level without sufficient understanding of how resistant alleles may be spreading through that region. If drug resistance is spreading through selective sweep, as shown here, then it is important to consider the whole region, irrespective of local or national borders, to implement a new drug policy. Thus, it is important that molecular tools become integrated into the overall malaria control program including assessment of drug efficacy through more conventional methods such as in vitro and in vivo testing for early detection and response to the emergence of drug resistance.

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