ASSOCIATION OF *P. FALCIPARUM* DHFR GENOTYPES WITH HOST AGE AND SEX IN SOUTH-WEST NIGERIA

AMODU, O.K.,* OLANIYAN, S.A. and **OMOTADE, O.O.**
Institute of Child Health, University of Ibadan, Ibadan, Nigeria

*Corresponding author: *amkemi@hotmail.com*

Abstract

Parasite drug resistance remains a great hindrance to effective control of malaria. Despite the widespread resistance, Sulphadoxine-Pyrimethamine (SP) is still used as one of the Artemisin-based combination therapies (ACTs). SP resistance has been associated with mutations in the dihydrofolate reductase (DHFR) gene of *Plasmodium falciparum.* We hypothesized that other factors may contribute to the prevalence of DHFR resistant genotypes in areas of limited use of SP in Ibadan, south-west Nigeria. Blood samples were collected from 100 children presenting with microscopically confirmed *P. falciparum.* Parasite DNA extracted from dried blood spots by Chelex method was analysed with a primary Polymerase Chain Reaction (PCR) and nested PCR for specific DHFR codons; 108, 51 and 59. Overall, 83% had resistant DHFR 108 genotypes. Mutations in the Ile^{51} and Arg^{59} were present in 69% and 76% respectively. The proportion of Ser^{108} increased significantly with age while the proportion of resistant genotypes Asn^{108} reduced with age. The prevalence of DHFR alleles differed between genders. Among the females, the prevalence of the Ser^{108} and Cys^{59} increased with age while the prevalence of the Asn^{108} and mixed infections Ser^{108}/Asn^{108} decreased with age. There was an increased risk of the Asn^{108} resistant genotypes being present in the females in a 2:1 ratio [OR=2.3, 95% CI=1.2 – 4.5] when compared to the males [OR = 1.0, 95% CI= 0.8 – 2.1]. This study shows that other factors in addition to drug selection, specifically age and sex, may determine the distribution of DHFR resistant genotypes in areas of limited SP usage.

Keywords: malaria, drug resistance, sulphadoxine-pyrimethamine, dihydrofolate reductase, artemisinin-based combination therapy.

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Introduction

Malaria remains one of the leading causes of morbidity and mortality in many tropical regions of the world, especially in Africa. Malaria causes about 600,000 deaths worldwide every year, of which 91% are in Africa. Nigeria, together with the Democratic Republic of Congo and India accounted for 40% of estimated malaria cases and over 40% of deaths globally (WHO, 2012).

Malaria control focuses on the development of new tools and improved integration of existing interventions such as insecticide-treated mosquito nets (ITNs), indoor residual spraying with insecticides, intermittent preventive treatment for pregnant women (IPTp), and artemisinin-based combination therapy (ACT). ACT is currently the recommended anti-malarial treatment and has been adopted in many countries in Africa as the first line of treatment for both uncomplicated and severe malaria. Chloroquine (CQ) monotherapy was highly effective in treating malaria for several years, but the development of CQ resistance to *P. falciparum* became a major challenge for control programs (Clyde, 1987; Bjorkman and Phillips-Howard, 1990). The onset of wide-ranging CQ resistance led to its replacement with anti-folate drugs, particularly sulphadoxine-pyrimethamine (SP), as a first-line therapy in many parts of the world. However, resistance to anti-folate drugs emerged rapidly. ACT has since been introduced

to either work in combination with anti-folates or replace them as first-line therapies in many countries (Bjorkman and Phillips-Howard, 1990; Wongsrichanalai *et al* 2002).

Sulphadoxine and Pyrimethamine resistance have been associated with single point mutations in dihydropteroate synthase (*dhps*) (Brooks *et al* 1994; Triglia *et al* 1998) and the dihydrofolate reductase (*dhfr*) (Peterson *et al* 1988; Basco *et al* 1995) genes respectively. A change from wild-type Ser108 to Asn108 (S108N) in dhfr is sufficient to cause low level pyrimethamine resistance both *in vitro* and *in vivo* (Cowman *et al* 1988; Peterson *et al* 1988; Reeder *et al* 1996). The progressive addition of mutations altering Cys50 to Arg (C50R), Asn51 to Ile (N51I), Cys59 to Arg (C59R), and Ile164 to Leu (I164L) in dhfr can yield higher levels of SP resistance *in vitro* and *in vivo* (Basco *et al* 1995; Reeder *et al* 1996; Contreras *et al* 2002; Khalil *et al* 2003). Genotypes consisting of multiple mutations in the dhfr gene have evolved in different parts of the world and are most often associated with higher levels of resistance than the single mutant genotypes. The triple dhfr mutant genotype consisting of N51I, C59R, and S108N has demonstrated strong association with *in vivo* SP treatment failure (Kublin *et al* 2002; Happi *et al* 2005). While these DHFR genotypes are selected for by continuous use of Pyrimethamine-containing drugs (Curtis *et al* 1996; Plowe *et al* 1996, Doumbo *et al* 2000), other factors may contribute to the prevalence of these resistant genotypes, in areas of limited use of these drugs.

Drug resistance continues to present a major threat to remaining effective anti-malarial therapies, such as ACT, which is currently the main line of treatment for malaria (WHO, 2010). Therefore, the success of control efforts relies heavily upon surveillance methods to adequately monitor and promptly respond to emerging resistance. Sulphadoxine-Pyrimethamine (SP) was used as a second-line treatment for uncomplicated malaria for a long time in Nigeria. National treatment policy changed in 2000, with effect from 2002 due to widespread resistance of Chloroquine (the original first-line treatment) and SP. However SP is still used as one of the Artemisin based combination therapies (ACTs), though sparsely in combination with artesunate (AS) (WHO, 2010). It is therefore important that the efficacy of the SP component should be closely monitored to ensure the effectiveness of current ACTs including SP.

In this study, we describe the prevalence of these DHFR genotypes in Sasa community, Akinyele Local Government Area of Ibadan, southwest Nigeria.

Materials and methods

The study was carried out in Sasa Community, about 10 km north of Ibadan in south-west Nigeria. Sasa Community, with a population of 7,992 is located in Ward 8 (Ojo) which is one of the wards in Akinyele Local Government, Ibadan (National Population Census, 2006). Children between the ages of 0 months and five years were recruited from the Primary Health Care Centre serving the rural community. Ethical approval was obtained from the UI/UCH Joint Ethical Committee. Thick and thin blood smears were prepared from blood samples collected by finger-prick from the children to determine parasite densities. Malaria parasites were examined on 5% Giemsa-stained thick and thin blood smears stained for 20 minutes. The parasites were counted against 200 white blood cells and the parasite densities were calculated based on an assumed total white blood cell count of 8,000/µL. Five ml of blood were collected from 100 children presenting with microscopically confirmed *P. falciparum.* 100µl of blood was spotted on filter paper.

Parasite DNA was extracted from filter paper samples by the Chelex method (Polski *et al* 1998). *P. falciparum* infections were confirmed with a species-specific Polymerase Chain Reaction (PCR). Samples were analysed with a primary PCR to amplify a fragment of the DHFR gene. Five µl of DNA from each sample was added to 45µl of the reaction mix containing 1x buffer -10mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton X-100, 1.5mM MgCl2, 1µM of the paired outer reaction primer AMP 1 and AMP 2, 2.5 units of Taq polymerase and 200µM of each deoxynucleotide dNTP. The PCR conditions were primary denaturing at 95°C for 3 minutes followed by 45 cycles of 3 seconds at 92°C denaturing, annealing at 45°C for 45 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 3 minutes. A nested PCR was also carried out for the specific codons, 108, 51 and 59. The primer sequences were as follows: Codon 108; complementary primer, SPI – ATGATGGAACAAGTCTGCGAC), primer DIA 108 GAATGCTTTCCCAGC (Ser^{108}) and DIA 12 GGAATGCTTTCCCAGT (Asn^{108}), codon 51; MUM-D complementary primer TTTATCCTATTGCTTAAAGGTTA), FR-51W B1 GAGTATTACCATGGAAATGCA (Asn^{51}) and FR-51M B1 GGAGTATTACCATG GAAATGTCT (Ile^{51}) and codon 59; the complementary primer 59-Com ATGATGGAACAAGTCTGCGAC, FR-59W ATGTTGTAAC TGCAC**A** (Cys^{59}) and FR-59M ATGTTGTAACTGCAC**G** (Arg^{59}).

Statistical analysis

Descriptive statistics (means, standard deviations, medians, ranges) were computed for continuous variables while frequencies were computed for categorical variables. Two-group comparisons of categorical variables were done using the *chi*-square (χ^2) test.

Results

Fifty-four percent of the children were males and 46% were females, with a mean age of 40.6 months (range 1.2-180 months). The mean parasite density for the total population was 4,875/µl of blood and the mean temperature at presentation was 38.2°C. Subjects were grouped according to age-group: 0-48 months of age (the most susceptible group to malaria) and greater than 48 months. Fifty percent of the children were less than 2 years. The mean temperature differed according to age-group with the younger children, less than 2 years having significantly higher temperature of 38.2 (p = 0.028) than the older children. However, the parasite density did not differ significantly between the two groups of children (Table 1).

The prevalence among the total population of the sensitive Ser^{108} genotypes was 17%, 28% for the resistant Asn^{108} and mixed infections (Ser^{108}/ Asn^{108}); and 55% for Ser108/Asn^{108}. The prevalence of the sensitive Asn^{51} was 31%, 25% for the mutant Ile^{51} and 44% for the mixed infections Asn^{51}/Ile^{51}. The prevalence of the sensitive Cys^{59} was 24%, 38% for the mutant Arg^{59} and 38% for the mixed infections Cys^{59}/ Arg^{59}. The proportion of Ser^{108} increased significantly with age but the proportion of mutant alleles ($Asn^{108}$and Ser^{108}/Asn^{108}) decreased with age (Figure 1: χ^2 = 10.86, p = 0.003). The proportion of the Cys^{59} also increased with age and the proportion of Cys^{59}/ Arg^{59} decreased with age but this was not significant. The proportion of the N51I alleles however did not differ among the age-groups.

Table 1. Characteristics of children from Ibadan, south-west Nigeria according to age-group.

	Age groups		*p*-value
	0-48 months	>48 months	
Sex % (male)	54	46	0.379
Temperature (^{0}C)	38.2	37.4	0.028*
Parasite density (geometric mean)	4,183	7,846	0.290

All figures are median (interquartile range) unless otherwise indicated. *p* is for *chi*-square test (categorical variables) or Kruskal-Wallis non-parametric ANOVA (continuous variables).

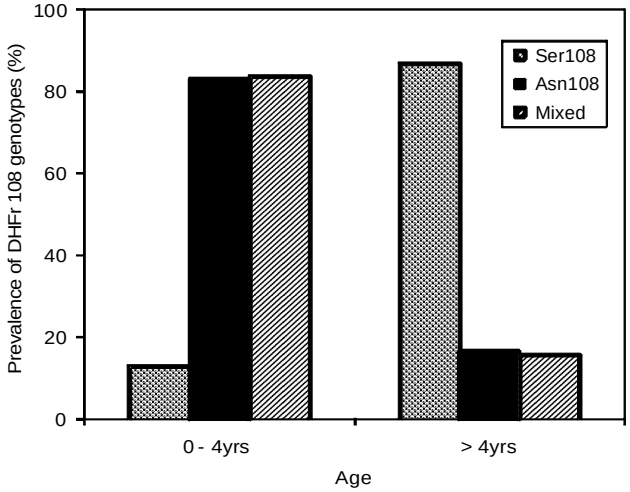

Figure 1. The prevalence of DHFR 108 genotypes among children from Ibadan, south-west Nigeria.

The mean temperature and parasitaemia did not differ among the males and females. However, the association of the prevalence of DHFR alleles differed between the sexes. Among the females, the prevalence of the Ser^{108} increased with age and the prevalence of the Asn^{108} and mixed infections Ser^{108}/Asn^{108} decreased with age χ^2 = 15.21, p>0.001 (Figure 2). The prevalence of the Cys^{59} also increased significantly with age among the females and mixed infections decreased with age χ^2 = 8.73, p = 0.01 (Figure 3). In contrast, the prevalence of these DHFR alleles did not differ with age among the males. There was an increased risk of the Asn^{108} mutant alleles being present in the females in a 2:1 ratio [OR= 2.3 ($CI_{95\%}$= 1.2-4.5)] when compared to the males [OR = 1.0 ($CI_{95\%}$= 0.8-2.1)].

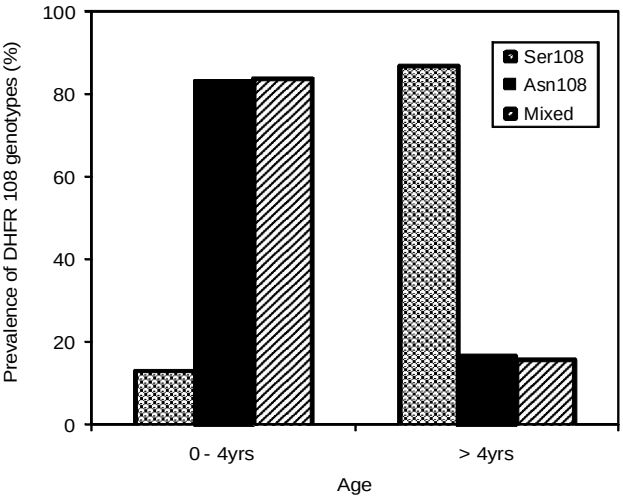

Figure 2. The prevalence of DHFR 108 genotypes among the female children in Ibadan, south-west Nigeria.

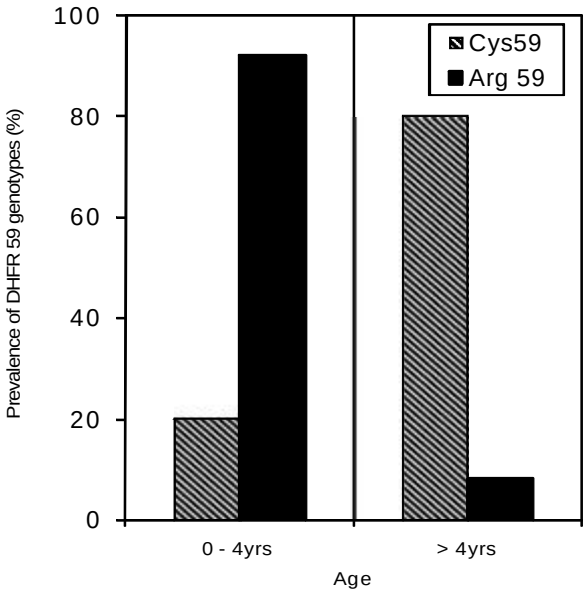

Figure 3. The prevalence of DHFR 59 genotypes in female children in Ibadan, south-west Nigeria.

Discussion

Clinical efficacy trials that measure treatment failure of a particular drug are the gold-standard method for assessing the effectiveness of anti-malarial therapies. However, clinical efficacy trials are very expensive, making them difficult to use broadly for surveillance purposes (Price *et al* 2007). Using clinical trials as a reliable assessment of drug resistance is also masked by a number of host, drug, and parasite factors such as acquired immunity (Djimde *et al* 2001), compliance dosing (Okonkwo *et al* 2001), nutritional status (Fillol *et al* 2009) and parasite synchronicity resistance mechanisms (Gregson and Plowe, 2005; Simpson *et al* 2002). Studies that focus on specific molecular interactions among host, drug, and parasite are more informative by helping to better differentiate and identify the role of drug resistance.

Molecular studies examining genetic correlates of resistance provide the means to identify genetic markers that confer resistance. Genetic association studies have identified and validated genetic markers of resistance for older anti-malarial drugs, such as CQ and SP (Cowman *et al* 1988; Peterson *et al* 1988; Sidhu *et al* 2002). Molecular epidemiology studies of drug resistant markers are a practical means of monitoring dynamic drug resistant parasite populations in different parts of the world and could be useful in planning drug efficacy studies (Djimde *et al* 2004). The presence of the single S108N mutation in a population is the minimum requirement for pyrimethamine resistance and is the first mutation seen as PYR resistance develops (Cowman *et al* 1988; Peterson *et al* 1988; Reeder *et al* 1996). The prevalence of S108N genotype in combination with mutations at codon positions 51, 59, and 164 (Peterson *et al* 1988; Basco *et al* 1995; Nzila-Mounda *et al* 1998) confers high resistance.

Studies have shown that PYR use as prophylaxis selects rapidly for the point mutations in DHFR. Established and strong drug pressure as well as low anti-parasitic immunity probably explains the multidrug-resistance encountered in forests of south-east Asia and South America. In Africa, frequent genetic recombinations in *Plasmodium* originate from a high level of malaria transmission in addition to drug pressure (Doumbo *et al* 2000). In areas where SP is being used, development of mutation is very rapid and this has been largely attributed to selection of mutant alleles under drug pressure. Although SP is no longer a second line drug in Nigeria, there is still limited use by the Sasa Community in the Akinyele Local Government Area of Ibadan. In this rural population, about 9% had used PYR prophylaxis or SP in combination as a means of malaria control. About 83% of the population had mutant DHFR 108 genotypes. Additional mutations in the Ile^{51} and Arg^{59}, which confer high resistance, were also present in 69% and 76% respectively. No single isolate however had the triple mutations. In this study also, the prevalence of the mutant genotype (Asn^{108} and Ser^{108}/Asn^{108}) was higher than Mockenhaupt *et al* (1999) found from another rural community in south-west Nigeria.

In the study herein, the proportion of Ser^{108} increased with age, while the mixed infections (Ser^{108}/Asn^{108}) decreased with age. This was also observed with the proportion of sensitive Cys^{59} and mixed infections (Cys^{59}/Arg^{59}). In addition, the prevalence of Asn^{108} and the mutant Arg^{59} decreased with age among the female population. This demonstrates that age and sex are determinants of the presence of these genotypes; further strengthening the suggestion by Mockenhaupt *et al* (1999) that other factors in addition to drug selection may be responsible for the presence of these mutant genotypes in areas of limited pyrimethamine usage. In addition, the prevalence of Asn^{108} and the resistant Arg^{59} decreased with age among the female population. In other words, the older a child is, the more the proportion of sensitive alleles especially among the female population. This strong finding from our current study implicating gender also as a determining factor in the presence of resistant genotypes, corroborates the conclusions from Mockenhaupt *et al* (1999) that additional, unidentified factors, rather than selection by residual drug levels or drug pressure alone, might be

responsible for the emergence of pyrimethamine-resistant parasite genotypes. Findings such as these, identifying factors and mapping epidemiological spread of molecular markers of resistance to formerly efficacious anti-malarials in different populations may be useful for the improvement of molecular surveillance capabilities for the ACTs, the anti-malarials currently in use.

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