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Full Length Research Paper

Mutations within folate metabolising genes of *Plasmodium falciparum* in Cameroon

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Sulfadoxine-Pyrimethamine (S-P) still used in some parts of the country was suggested as the second line drug to amodiaquine following widespread failure of chloroquine in Cameroon in 2002. We investigated the efficacy of S-P and determined the baseline mutations on marker genes for folate metabolism (*dhfr* and *dhps*) in the forest and Guinea-Savanna ecozones of Cameroon, as a way of tracking resistance in patients aged between 0.5 and 10 years in Limbe (n=138), Nkambe (n=103), Fontem (n=100) and Dschang (n=93). Filter paper blood sample were collected prior to treatment and on clinical failure days to determine the prevalence of molecular markers of resistance and to assess the mutation rates on the folate metabolising genes by restriction fragment length polymorphism assays or dot-blot assays with 32-P labeled mutation-specific probes. Sequencing using the dideoxy-chain termination method by PCR was conducted to confirm doubtful cases. Late parasitological failure (LPF) was higher in Limbe (30.6%) compared to Nkambe 10.3% (p=0.001). The prevalence of the 437-Gly mutation though lower in Nkambe, 57.6%, than in Limbe, 60% were statistically not different (p=0.2). All genotypes with the 108N mutation also carried the 51-Ile and 59-Arg mutations. All sensitive alleles (S108) also carried the amino acids, 51-Asn and 59-Cys. S-P is no longer efficacious in Limbe and Nkambe, Cameroon for treating uncomplicated malaria in children below 10 years. Instead 437G rather than the 108 N of *Plasmodium falciparum* may be determinant as the marker for tracking the spread of S-P resistance in Cameroon.

Key words: Mutations, plasmodium, sulfadoxine-pyrimethamine, resistance, *dhfr*, *dhps*

INTRODUCTION

Malaria continues to be a major public health problem in tropical Africa, accounting for more than 90% of the 300 – 500 million cases occurring annually (WHO, 2006). The control of malaria relies mainly on chemotherapy, leading to overuse of the commonly available antimalarial drugs with an ensuing spread of resistance. Chloroquine is still the cheapest and most widely used antimalarial drug

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despites its prohibition by the government of Cameroon (Mbacham et al., 2005). The disease had been fairly managed with chloroquine until resistance to chloroquine became widespread in Cameroon (Brasseur et al., 1986, 1988, 1992a, 1992b). Introduced in the late 1970s, studies on Sulfadoxine-Pyrimethamine (S-P) resistance reported a 42% pyrimethamine resistance among clinical isolates in Yaounde using the *in vitro* semi-microtest (Ringwald et al., 1996). Among 1,407 children enrolled in the studies, 460, 444, and 503 were treated respectively with chloroquine, amodiaquine or sulfadoxine-pyrimethamine. Chloroquine treatment resulted in high failure rates (proportion of early and late failures, 48.6%). Amodiaquine was effective at all study sites (proportion of failures, 7.3%). Sulfadoxine-pyrimethamine therapy was less effective than amodiaquine ($P < 0.05$), with failures observed in 9.9% of patients (Basco et al., 2006).

Basco et al. (2002) further used a 28 day *in vivo* clinical trial protocol based on the WHO, 1996 guidelines involving AQ, S-P and AQ+S-P, to show that AQ cleared parasites better than S-P and that AQ+S-P could be used to reduce the spread of CQ resistant parasites. Tahar and Basco (2007) demonstrated the fixed nature of triple mutants on *dhfr* in Cameroon in an *in vitro* test that regrouped 194 isolates from samples collected between 1994 and 2005. Only 7 isolates had the wild-type *dhfr* while 187 were mutants.

Molecular markers were analysed from 1,430 blood samples either of symptomatic children or asymptomatic carriers collected from 14 sites throughout the country for the analysis of dihydrofolate reductase (*dhfr*) gene. Of the 1,368 samples (95.7%) that were successfully amplified, 1,180 were analyzed for the prevalence of the wild-type, single Asn-108 mutation, double Arg-59/Asn-108 mutations, double Ile-51/Asn-108 mutations, triple Ile-51/Arg-59/Asn-108 mutations, and mixed alleles were 20.8, 2.8, 5.7, 0.8, 62.2 and 7.6%, respectively. The proportions of triple *dhfr* mutations were greater than 60% at all study sites, with the exception of the eastern province (42% triple mutants in Bertoua in 1999) and the northern provinces (11-35% triple mutants in Ngaoundere, Garoua, and Maroua). In these two provinces, the proportion of mutant parasites increased significantly over a 2-4 years period. Furthermore, there was a higher proportion of wild-type parasites in the Northern provinces, compared with the rest of the country.

S-P has the advantages of excellent compliance, good tolerance and relatively few side effects. However, its potential drawback is the rapid emergence of resistant parasites when massively employed. In 2006 all monotherapies were ordered withdrawn from the Cameroon market with the change to take effect in January 2007 and only S-P was allowed to be used as the drug of choice in Intermittent Preventive Therapy in pregnancy (IPTp) (PNLP, 2006) and is still the case presently. In light of these changes the present study was completed as part of an ongoing investigation by the Laboratory of Public Health Biotechnology, University of Yaounde I and the National Malaria Control Program with the specific objectives to retrospectively establish baseline prevalences of resistance markers on *dhfr* and *dhps* genes for the *in vivo* S-P efficacy in two ecological settings; littoral forest and guinea savannah of Cameroon.

MATERIALS AND METHODS

Study sites

This study was permitted by ethical clearance and an administrative approval of the National Ethics Committee and the Ministry of health in 2002. Two hospital centres in the town of Limbe were chosen for the study -the Bota District Hospital, which is a semi-Urban Integrated Health Centre (UIHC). Limbe is a town in the South-West Province, situated on the Atlantic coast. It is a forest zone with an equatorial climate. The second site for the study was the Nkambe/Ndu area which is in the mountain ranges of the northwest of Cameroon. Patients were attended to at either of the three hospital centres of the Baptist Mission or the Nkambe District Hospitals within 5 km walking distances from each other. Samples were collected from two other towns for the study of the prevalence of mutations. These were Dschang which is a semi-urban centre in the Guinea savannah belt where its people are basically traders or subsistent farmer on coffee cash crop. The fourth town is Fontem found in the mountainous guinea savannah-belt of Cameroon and bordered by an upland forest. Dschang is separated from Fontem by 35 Km road distance. The St. Mary's Hospital in Fontem and the Dschang District Hospital were the sites of samples collection. The study was conducted during the months of March to June (Fontem and Dschang) in 2002 and in the months of March - October (Limbe and Nkambe) in 2003.

Patient recruitment and sample collection

Children between 6 months to 10 years, who were presented with symptoms of uncomplicated malaria infection, a positive thick blood film with a parasite density of greater 2000 parasites/µl of blood, an axillary temperature greater than 37.5°C or a history of fever within the past 24 h were enrolled in the study after informed consent was obtained from the parent or guardian. The children who were excluded were those who presented with signs and symptoms of severe malaria as defined by WH0 1996, history of allergy to the sulfonamides and refusal of parental or guardian consent. The children were examined by a clinician and are referred to the laboratory where capillary blood samples were collected on filter paper by finger pricking and thick blood smears prepared and stained with Giemsa for a microscopic examination. Parasite density was obtained by counting the number of asexual parasites against 200 white blood cells based on a mean count of 8000 white blood cells/µl of blood. The samples from Fontem (n=100) and Dschang (n=93) were collected from the out patient department of malaria positive patients without any further clinical follow up.

Drug administration

The children were administered a single oral dose of S-P (25mg per Kg body weight of sulfadoxine + 1.25 mg per Kg body weight of pyrimethamine). The same dose was repeated to children who vomited within thirty minutes of drug administration. Children who showed early or late clinical failure or a late parasitological failure on day 14 were treated with oral Quinine-HCl (10 mg per Kg body weight per dose per day (30 mg/kg) as three divided doses for six days). Paracetamol was also administered for fever clearance on

the day of enrolment. All medications were purchased as a quality controlled generics from the Baptist Health Board Central Pharmacy, Cameroon.

Follow up procedures

The 1996 WHO-fourteen-day protocol (WHO, 1996) was used to assess clinical outcome and follow up. By this protocol, patients' parasitological and clinical responses were evaluated on days 3, 7 and 14. Those who did not return to the hospital on any of the follow up days were visited at home. At the recurrence of parasites in the thick smear and/or fever, capillary blood was collected again on filter paper and stored as post-treatment samples. The therapeutic responses were graded using the WHO classification

DNA extraction and the PCR

These procedures were performed at the Biotechnology Centre, University of Yaounde I. *P. falciparum* DNA was extracted from blood samples obtained on day 0 and on failure days post-therapy with S-P, from filter paper by the chelex method (Abdel-Muhsin et al., 2002). Three parasite loci, msp-1 and msp-2 and glurp with repeat sequences were amplified by nested PCR from the DNA of each isolate as described by Ranford-Cartwright et al. (1997). The *dhps* gene was also amplified by outer and nested rounds of PCR. All primers were obtained from MR4, Manasass, Virginia, USA. The PCR products were detected by electrophoresis on a 2% agarose gel and visualised by staining with ethidium bromide and observed by UV fluorescence. A 100-base pair molecular weight marker was electrophoresed alongside the samples.

PCR and RFLP of anti-folate genes

The entire *dhfr* gene was first amplified resulting in a 708 bp product that was subjected to a nested amplification by the polymerase chain reaction as described (Abdel-Muhsin et al., 2002). The nested PCR products were precipitated with 95% ice-cold ethanol in the presence of 0.3 M Sodium Acetate, 5 ul containing 450-550 ng of the reconcentrated PCR product were incubated with 1 unit of *Alu I* and/or *Bsr I* as described by the manufacturers (New England Biolabs, North Berverly, MA, USA). The 437 and 540 mutations on the *dhps* gene were similarly determined respectively by the use of enzymes, Ava II and Fok I essentially as described by Zindrou et al. (1996), Plowe et al. (1998). The reaction mixtures were electrophoresed as above.

Dot blot assay

Amplified samples were thawed from -20°C and 10 ul of each were analyzed using a gene screen membrane, and dot blotter as described by Abdel-Muhsin et al. (2002). The DNA on the membrane was fixed by exposure to UV, air dried and then used immediately for hybridization. Probes were used at 8 pmol of primer ends and labelled with [α-32 P] ATP by use of Ready-to-go Polynucleotide Kinase (PNK) (Amersham Pharmacia Biotech, Amersham, UK). The reactions were stopped by the addition of 5 ul of a 250 mM EDTA solution and unincorporated isotopes removed by brief centrifugation in a screw cap microspin G-25 column. The filtrate with labeled probe was stored at -20°C until used within 1 week. Blots were placed in rotisserie hybridization bottles with 25 ml of hybridisation buffer for 30 min at the respective temperatures and 20 ul of the labeled probe from above were added and allowed to hybridize for 13 h (overnight) with slow rotation. Membranes were washed as necessary with 2XSSC or 1xSSC/0.1% SDS Blots were allowed to air dry briefly and sealed in clingfilm and autoradiographed for 24-48 h to X-ray film(Kodak). For re-probing, blots were stripped by adding an excess of 0.1 M NaOH and incubating it for 15 min at room temperature. These stripped blots were re-washed with 5X SSC and placed in clingfilm and stored at -20°C until used again in hybridization with another probe.

Sequencing of PCR products

Samples which could not be resolved by Dot blot or RFLP were sequenced. The genotypes of the *dhfr*-amplified fragment in samples C1, C4, F2, F12, L43 and L49 were sequenced by the dideoxy-termination method using the "Thermosequenase Radiolabeled Terminator Cycle Sequencing Kit" as described by the manufacturers (Amersham, Amersham, UK). Sequences were aligned and compared to sequences of known strains - K1, Dd2 and HB3 of *P. falciparum*.

RESULTS

Study population characteristics

Therapy seeking habits were investigated as part of the general information obtained from participants. Most of the patients reporting to the hospital presented at the out-patient department after 3 days of having a fever with higher than 65% of the population self-administering one of the quinolines (quinine, chloroquine or amodiaquine). These populations had most of their anti-malarias from the hospital or health clinic. Although in Limbe 26.8% would purchase their drugs from patent medicine stores or the pharmacy.

All patients were not febrile at the time of enrollment. In Limbe, 56% (n=77) and 46.5% in Nkambe (n=48) were febrile. The rest 44% (Limbe) and 53.5% (Nkambe) had a history of fever within the past 24 h. Though the temperature of the participants ranged between 36.5 to 40.5°C the mean temperature for both sites were similar (38.0°C (Limbe) and 38.1°C (Nkambe). Mean parasitaemia was 15239p/ul for Limbe (with perennial transmission) and 8679 p/ul for Nkambe (seasonal transmission). Clinical symptoms of the participants besides fever included principally aches, chills/shivers, diarrhoea and vomiting (Table 1).

Parasite polymorphisms and allelic diversity in Nkambe and Limbe

The parasite population sampled from patients showed diversity based on length polymorphism of three antigen loci. There were 7 alleles identified for msp-1 with sizes that ranged from 460 to 600bp, 12 alleles for msp-2 and 11 alleles for glurp with sizes, which ranged from 420bp to 910bp and 660bp to 1260bp respectively. Recrudescing parasites determined by the msp 2 and glurp polymorphisms on day of failure compared to day of recruitment were used to correct potentially erroneous interpretations of failure to S-P. These results were

Table 1. Patient characteristics at enrolment – D0.

Parameter	Limbe (n=138)	Nkambe (n=103)	P-value
Mean temperature (^oC)	38.1±1.4 (36.5 – 40.5)	38.0±1.1 (36.5-39.0)	0.60
Proportion of fevers with parasite	56.0%	46.5%	0.04
Geometric Mean Parasitaemia (p/ul)[b]	15239 (2400-104800)	8679 (2040-98180)	<0.05[a]
Aches/Irritability	22.5%	20.5%	0.40
Chills/shivers	12.0%	10.5%	0.10
Diarrhoea	18.0%	13.0%	0.07
History of vomiting in past 24H	22.5%	15.7%	0.05

[a] Kruskall-Wallis test set at p-value <0.05.
[b] p/ul = parasites per microliter. P-values were determined by performing Chi-squared analysis.

Table 2. Prevalence of wild type & mutant folate metabolising genes

Gene	Limbe (%,n=138)	Nkambe (%, =103)	Dschang (%,n=93)	Fontem (%, n=100)
dhfr NCS*	56.0	54.0	96	86
dhfr IRN	44.0	46.0	04	14
dhps 437G	60.0	57.6	22.2	39.7
dhps 540E	01.4	00.0	00.0	00.0

NCS represents the wild type of asparagine-cystein-serine amino acids at position 51, 59 and 108 of the *DHFR*.
IRN is the triple mutant of *DHFR*.
437G is the glutamate amino acid change at codon 437.
540E is the aspartate amino acid change at codon 540 both of the *DHPS* for which the wild types are respectively A437 and K540. Other *dhfr* (16 and 164) and *dhps* (436, 581, and 613) codons that may undergo mutation and be associated with drug resistance were not analyzed in this study.

similar for the two sites and demonstrated that 87.5 and 95% of the parasites on the day of failure were recrudescent respectively in Limbe and Nkambe.

Frequency of anti-folate gene mutations

138 patients in Limbe and 103 in Nkambe, who completed treatment, respectively in Limbe and Nkambe; ETF were 10.4 and 12.6%, LCF were 15.7 and 30.1%, LPF were 30.6 and 10.7%. The ACPR were 43.3% (n=58) in Limbe and 46.6% (n=48) in Nkambe.

A scoring by the dot blot assay showed 4% of samples from Fontem, 14% from Dschang, and 42% from Limbe carried the 108N mutation. Sample F12 from Fontem could not give results with either *Alu I* or *Bsr I* during the restriction fragment polymorphim. Sequencing results obtained demonstrated the presence of a serine at position 108 in samples F12 whereas all the other samples had an asparagine at that same position. In addition, sample F12 had an asparagine and a cysteine respectively at positions 51 and 59 whereas the five others had an isoleucine and an arginine at the same respective positions. The RFLP, dot blot and sequencing results put together reveal that parasites from Dschang and Fontem in 2002 remained mostly of the wild type with the mutational rates for 108N being respectively 4 and 14% (Table 2). The mutational rates of 108 N was 46% in Nkambe and was no different from that in Limbe (44%) (Tables 2 and 3, p = 0.4). From these results it can be observed that all samples carrying the 108N are tripple mutants.

Determination of mutation rates of *dhps* gene

Among the amplified and digested isolates, the Gly437 mutation was present in 60% of the D0 samples from Limbe with a 56.7% of failures to S-P whereas the 437-Gly mutation was 57.6%, in Nkambe. The search for 540E mutation only yielded a 1.4% prevalence (n=2), in Limbe and was not seen anywhere else. These 540 E mutations were confirmed by dot blots in the two participants in Limbe. They had the other mutant types of *dhfr* 51I, 59 R and 108N and the *dhps* 437G. These 2 patients were neither more sick nor showed any other atypical clinical characteristics.

DISCUSSION

The present study was conducted with the objectives to evaluate the cure rates for S-P in 2 ecozones and to investigate the prevalence of the mutation rates of folate

Table 3. Clinical and molecular characteristics of study population.

Parameter	Index	Limbe (%)	Ndu/Nkambe (%)	p-value
Clinical response	ETF	10.40	12.25	0.1
	LCF	15.50	30.75	0.01
	LPF	30.60	10.30	0.001
	ACPR	43.50	46.60	0.5
Molecular markers - D0	108N	44.00	46.00	0.4
	437G	60.00	57.60	0.2

ETF; early treatment failure, LCF; late Clinical failure, LPF; late parasitological failure, ACPR; adequate clinical and parasitological response.
DHFR 108N is associated with pyrimethamine resistance and *DHPS* 437G is associated with sulfadoxine resistance.

metabolizing genes *dhfr* and *dhps*. Although mutations to *dhfr* codon 108N and *dhps* codon 437G were relatively high in prevalence in Cameroon, the 437G mutation prevalence was higher than that of 108N suggesting that the triple mutant that carries 108N might not be determinant of failure to S-P. It is still difficult given the data to correlate mutation rates of 108N or 437 G with clinical outcomes requiring that more sites and more randomized studies be conducted. Other *dhfr* (16 and 164) and *dhps* (436, 581, and 613) codons that may undergo mutations were not analyzed in this study. The dot blots technique allowed for a clear distinction and scoring of parasites alleles on the same membrane. This technique was used to establish that the 540E mutation unlike in several other regions in the east of Africa may not be contributing much for S-P resistance in the west of Africa. Since the mutations were determined on D0 samples, we are inclined to suggest that the quintuple mutant may not be in circulation in Cameroon. Instead the mutant 437G might be suggestive of the marker for tracking resistance. Following DNA sequencing, the genotypes of indeterminate Cameroonian mutant strains appeared to be identical to one another, having in addition to the serine to asparagine change at position 108, an asparagine to isoleucine and a cysteine to arginine changes at positions 51 and 59, respectively. Their genotypes were similar to the isolate (Dd2) from Indochina, at all the positions (51, 59, and 108). Parasites with these mutations were referred to as highly resistant to pyrimethamine *in vitro*. These, however, differ from the Thailand isolate (K1) having an asparagine and not an isoleucine at position 51 (Snewin et al., 1989) and classified by Van Es et al. (1993) as resistant to pyrimethamine and cycloguanil, the active metabolite of the antifolate, proguanil. The amino acid substitution at position 108 is believed to take place within the active site of the *DHFR* moiety of the bifunctional *DHFR*-TS enzyme and may alter the affinity of the protein to the drug leading to the resistant phenotype.

Studies carried out in Uganda involving the use of *DHFR* and *DHPS* as markers could not demonstrate any correlation between mutational rates and *in vivo* out comes (Sendagire et al., 2005). The observed discordance may be due to the fact that parasite clearance is aided by the immunity of the host. Yet in neighbouring Tanzania Pf*dhfr* triple mutations coexisting with pf*dhps* double mutations were detected in 64.3% of the *P. falciparum* isolates. This quintuple mutation is confirmed as a predictive molecular marker for S-P treatment failure (Schönfeld et al., 2007).

Early diagnosis and prompt treatment is one of the key points of the global strategy for malaria control. The widespread availability of cheap and effective antimalarial drugs particularly chloroquine and S-P combination has undoubtedly limited both morbidity and mortality. Although S-P is present on the shelves of the patent medicine stores and local pharmacies, data collected on perception of disease and treatment (Tawe, 2002) pointed to less self-prescription of S-P for diverse reasons including lack of knowledge of drug efficacies or fear of the Stephen-Johnson syndrome.

This study confirms that there is great genetic diversity in Limbe and Nkambe and high resistance levels to S-P 43.5 and 46.6% respectively. Ndounga et al. (2007a, b), demonstrated a failure of 30% for sulfadoxine-pyrimethamine, in Congo with similar climatic conditions like Cameroon. Basco et al. (2006) found that the prevalence of the triple mutant on *dhfr* was greater than 60%. The authors in combining data from 1180 samples over 14 sites investigated between 1999 and 2003 found wild type isolates in 20.8% of the samples, and the triple *dhfr* mutant in 62.2%. However, in the eastern province the triple mutant was 42% (Bertoua) in 1999 and in the northern provinces between 11-35% from Maroua, Garoua and Ngaoundere. They did not find the 540E in any parasite in Cameroon or Sao Tomé et Principé, an island off the west cost of Democratic Republic Congo. They found the 437G in 66% of samples analysed (Tahar et al., 2006) and similarly the 437G, as part of the quadruple mutant was found in 52.5% of the 97 samples analysed following clinical trials with S-P in the Republic of Congo (Ndounga et al., 2007a, b). These high failure rates to S-P in Cameroon are accompanied by equally high prevalence rates for the 437G mutation on *dhps*.

The 540E mutant on *dhps* is rare and so the quintuple mutant for S-P resistance described by Plowe et al. (1998), Kublin et al. (2002), cannot be used in parts of Central Africa as the marker for S-P resistance in Cameroon.

From these findings we conclude that S-P is no longer efficacious in Limbe and Nkambe, Cameroon for treating uncomplicated malaria in children below 10 years. The 540E marker is of extremely low prevalence suggesting the absence of the quintiple mutant. Mutation 437G rather than the 108N of *P. falciparum* may be determinant as the markers for tracking the spread of S-P resistance in Cameroon.

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