Submicroscopic *Plasmodium falciparum* Infections Before and After Sulfadoxine-Pyrimethamine and Artesunate Association Treatment in Dienga, Southeastern Gabon

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Background: It has been shown that *Plasmodium falciparum* submicroscopic infections (SMI) can contribute to malaria-associated anemia as well as to cerebral malaria. Polymerase chain reaction (PCR) assays are usually used as an alternative to microscopy in detecting subpatently infected individuals.

Objectives: The main objective of this study was to investigate the occurrence of SMI before and after a suppressive antimalarial treatment in the population of the village of Dienga in Gabon.

Methods: Nested PCR was used to detect SMI and to determine genotypes.

Results: The prevalence rates of SMI were 13.67% (38/278) at day 0 and 8.99% (25/278) at day 14 after sulfadoxine-pyrimethamine-artesunate treatment. Genotype analysis of two polymorphic regions of the merozoite surface protein (MSP)-I block 2, MSP-2 and a dimorphic region of the erythrocyte binding antigen (EBA-175) revealed that as many as 88% (22/25) of SMI detected after treatment were completely new alleles, indicating either previously sequestered parasites or newly acquired infections.

Conclusion: These results demonstrate the usefulness of sulfadoxine-pyrimethamine-artesunate association treatment in the population of Dienga and confirmed early parasite genotype change after a suppressive antimalarial treatment in endemic areas.

Keywords: Dienga, Gabon; Endemic area; PCR; Prevalence; Submicroscopic *Plasmodium falciparum*; Sulfadoxine-pyrimethamine-artesunate

The diagnosis of *Plasmodium falciparum* infections based on microscopic examination of stained blood smears remains to date the most practical and reliable means for detecting parasites in blood samples.¹ However, the sensitivity of this technique decreases with malaria parasite density in blood.² When parasitemia is low, during mixed infections, after drug treatment or during the chronic phase of the infection, diagnosis by microscopy requires long periods of observation and experienced microscopists.³ Therefore, polymerase chain reaction (PCR) assays have been used as an alternative to microscopy because of their high sensitivity and specificity.⁴ Interestingly, since the work of Jarra and Snounou⁵ in 1998, it has been shown that malaria parasite DNA is cleared very quickly from the blood circulation and that a positive PCR amplification is mainly

associated with the presence of viable parasites and represents submicroscopic infections (SMI).

While it has been shown in endemic areas that *P. falciparum* SMI contribute to malaria-associated anemia and inflammation,⁶ the clinical and epidemiological relevance of such infections remains unclear. However, more recently it has been documented that cerebral malaria is frequently associated with SMI in semi-immune individuals.⁷

The evidence for subpatent parasitemia detectable by PCR, as well as a rapid turnover of *P. falciparum* isolates in infected individuals have been previously reported,^{8,9} Nevertheless, the influence of antimalarial treatment, such as sulfadoxine-pyrimethamine-artesunate association on the occurrence of SMI, has not been investigated.

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A prospective longitudinal study was undertaken in Dienga to assess whether hepatitis viruses B or C infection may influence the delay of emergence of asexual malarial parasites in the blood after suppressive treatment. This study has provided an opportunity to determine the prevalence rates of SMI before and after sulfadoxine-pyrimethamine-artesunate combination treatment (Ouwe-Missi-Oukem-Boyer et al, manuscript in preparation).

Materials and Methods

Study Site and Subjects

This study was carried out in Dienga (Ogooué Lolo Province), a village in southeastern Gabon where *P. falciparum* malaria is highly endemic with a perennial mode of transmission and some seasonal fluctuation.^{10,11} Preliminary data from studies done in this area reveal that the transmission of *P. falciparum* is predominantly due to *Anopheles gambiae*, with an entomological infection rate of 100 infective bites per human per year.¹¹

Informed consent was obtained from 278 individuals or from their parents. Participants ranged in age from 14 to 75 years. The study was approved by the ethics committee of the International Centre for Medical Research of Franceville (CIRMF) Gabon, the Governor of the Province and the village authorities, and performed in accordance with the guidelines for human experimentation in clinical research of the Ministry of Public Health and Population of Gabon (Libreville).

Blood Sample Collection

On day 0, peripheral venous blood samples were obtained from each individual and collected in EDTA sterile tubes for malaria diagnosis. A thick blood smear was made and three drops of blood were also deposited on Serobuvar calibrated pre-punched paper disks (LDA²², Zoopole, Ploufragan, France).¹² After 4 hours at room temperature, dried blood spots were put in envelopes, transported to CIRMF and stored in an air-conditioned room until use. Thick peripheral blood films were stained with Giemsa and examined by two microscopists following standard, quality-controlled procedures. Parasite load was expressed as the number of asexual forms of *P. falciparum*/μl of blood, assuming an average leukocyte count of 8000/μl. After sampling, all the 278 individuals were treated using a single dose of sulfadoxine-pyrimethamine (25 mg/kg/day and 1.25 mg/kg/day, respectively) given on day 0 and artesunate (4 mg/kg/day) given on days 0-2 (Ouwe-Missi-Oukem-Boyer et al, manuscript in preparation).

Blood was collected from each individual on day 14 by finger prick. The blood was absorbed onto Serobuvar discs. Thick blood smears were also made and staining for *P. falciparum* proceeded as described above.

DNA Extraction

DNA templates were extracted using dried blood spots as previously described.¹³ Each dried blood spot was placed in 1 ml of phosphate buffered saline (PBS) containing 0.5% saponin and incubated overnight at 4°C. The resulting brown solution was replaced with 1 ml PBS and incubated for an additional 15-30 minutes at 4°C. After this incubation, 200 μl of 5% Chelex 100 (Bio-Rad Laboratories, CA) was placed in clean tubes and heated at 100°C in a water bath. The disks were removed from the PBS and placed in the preheated 5% Chelex 100, vortexed at high speed for 30 seconds and placed in a water bath at 100°C for 10 minutes with gentle agitation. Samples were then centrifuged at 10,000 x g for 2 minutes, and the supernatant removed and centrifuged as before. The supernatant was then collected into a clean tube and used for PCR immediately or stored at 20°C until use.

DNA Amplification for *P. falciparum* Diagnosis

Five microliters of the Chelex supernatants were amplified using a Perkin Elmer thermal cycler in a 50 μl reaction containing 1x PCR buffer as supplied by the manufacturer (200 mM Tris-HCl, pH 8.7, 100 mM KCl, 100 mM (NH₄)₂ SO₄, 20 mM MgSO₄, 1% Triton-X 100, 1 mg/ml of bovine serum albumin), 200 μM each of dATP, dCTP, dGTP and dTTP, 1 μM of each primer and 1 unit of Taq DNA polymerase (Invitrogen, Cergy Pontoise, France). PCR and nested PCR were carried out as previously documented⁴ to amplify the *P. falciparum* small subunit ribosomal RNA gene. After amplification, 10 μl of each nested PCR product was mixed with 1 μl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% w/v sucrose in water) and analyzed by electrophoresis on a 1.5% agarose gel. The gel was stained with ethidium bromide and the DNA was visualized and photographed under ultraviolet light.

DNA Amplification for *P. falciparum* Genotyping

Before genotype determination, five separate, randomly chosen, nested PCR products were sequenced. The alignment of these sequences showed that they were 100% identical to the original sequence of the *P. falciparum* small subunit ribosomal RNA gene (data not shown).

Genotypes of the parasite population were analyzed on paired primary and post treatment samples. Block 2 of merozoite surface protein (MSP)-1, block 3 of MSP-2, and erythrocyte binding antigen (EBA)-175 were amplified by nested PCR as previously reported.¹⁴⁻¹⁶ Briefly, after amplification, 10 μl of each nested PCR product were analyzed by electrophoresis on a 1.5% agarose gel. PCR-positive samples at day 0 and day 14 were processed concomitantly and the bands compared. Identical bands were classified as treatment failure, while nonidentical bands were considered new infections.

Results

At the start of the study (day 0), 278 individuals ranging in age from 14 to 75 years were examined. After the standard microscopic examination, 20 (7.2%) specimens were positive

Table 1. Comparison of *P. falciparum* submicroscopic infections (SMI) before and after sulfadoxine-pyrimethamine and artesunate association treatment.

Day	Total samples	Blood smear positive	Blood smear negative	PCR positive (SMI)	PCR negative
0	278	20* (7.2%)	258 (92.8%)	38 (13.67%)	220 (79.13%)
14	278	0 (0%)	278 (100%)	25 (8.99%)	230 (82.73%)

*, not tested by polymerase chain reaction (PCR).

for *P. falciparum*. The remaining 258 individuals with negative blood smears were assessed by nested PCR; 38 of these samples tested positive for *P. falciparum*. Overall, the prevalence of *P. falciparum* infection (detected by standard microscopy and PCR) before treatment was 20.8% (58/278, table 1). Among those infections, SMI were the most prevalent (13.67%).

After treatment, microscopic examination was repeated on blood samples from the 278 individuals, but all were negative for *P. falciparum*. By using a nested PCR assay, 25/278 (8.99%) blood samples tested positive for *P. falciparum* on day 14 and, therefore, represent the occurrence of SMI posttreatment (table 1). Among these 25 SMIs, nine were detected only during posttreatment, 6 were thick blood smear positive at day 0, and the remaining 10 were detected at both pre- and posttreatment (table 2). The SMIs detected only during posttreatment (9) represent new SMI.

Genotyping was done on the 6 samples that were positive by microscopic evaluation on day 0 and on the 10 SMI that were detected both pre- and posttreatment. The results showed a predominance of the K1 and FCR-3 genotypes from MSA-1 and EBA-175 genes, respectively. Interestingly, 13 of these 16 *P. falciparum* isolates, including the 6 blood smear positive samples at day 0, showed completely new alleles. The remaining 3 SMI (12%) presented identical *P. falciparum* strains during pre- and posttreatment, as characterized by identical patterns for MSA-1, MSA-2 and EBA-175 alleles (table 2).

Twenty-two of 25 (88%) SMI detected after treatment were probably new infections. This includes the 9 SMI detected only during posttreatment for which genotypes have not been determined.

Overall, 95% of *P. falciparum* isolates detected at day 0 (blood smear and PCR) were killed by sulfadoxine-pyrimethamine-artesunate association treatment. This included all blood smear positive samples and 35 of 38 SMI.

Discussion

The main objective of this study was to investigate the occurrence of *P. falciparum* SMI before and after a suppressive antimalarial treatment in the population of the Dienga village in Gabon. This study was the first to focus only on SMI during pre- and posttreatment in an endemic area of central Africa. Our data show that K1 and FCR-3

genotypes from MSA-1 and EBA-175 genes, respectively, were predominantly encountered. These findings are consistent with data reported by Kun et al.¹⁷ Our results confirmed, first, that microscopically detectable *P. falciparum* parasitemia in peripheral blood is a poor indicator of active infection and second, the persistence of SMI in endemic areas as previously demonstrated.^{8,9,18} The prevalence of SMI in Dienga was 8.99% after only 2 weeks of treatment suggesting that the entire study population may be infected at least once in 6 months.

Interestingly, these results demonstrated the usefulness of sulfadoxine-pyrimethamine and artesunate association in the treatment of uncomplicated *P. falciparum* infections, since all blood smear positive individuals were aparasitemic at day 14 posttreatment. Furthermore, all samples were negative by PCR at day 14 except those representing new infections. Although our study was not designed to investigate the efficacy of sulfadoxine-pyrimethamine plus artesunate, our results indicate a high cure rate of this combination in Dienga. However, this treatment does not prevent the early appearance of new infections. Twenty-two of 25 SMI (88%) detected in posttreatment were completely new parasite populations that had not been detected at day 0. Such changes in *P. falciparum* genotypes within individual infections could occur when PCR fails to detect either the minority parasite that is drug resistant in multiple genotype infections or previously sequestered parasites that are not present in the peripheral blood at day 0 of sampling.¹⁹⁻²¹ However, since these individuals live in a perennial transmission area with continuous exposure, it is more likely that they were infected with new parasite populations that have emerged from the liver. This later hypothesis is consistent with previous reports from other malaria endemic countries of Africa.^{9,19,22}

In this study, 3 SMI presented with the identical *P. falciparum* strain during pre- and posttreatment, as characterized by an identical pattern for MSA-1, MSA-2 and EBA-175 alleles. These 3 SMI, of an overall 58 *P. falciparum* infections (5.1%), may represent genetically similar, but new infections occurring in the posttreatment period or previously sequestered parasites. Alternatively, it is also possible that the 3 SMI treatment failures may be due to the drug resistant *P. falciparum* strains. This is not surprising as the clinical and parasitological failures in artesunate plus sulfadoxine-pyrimethamine treatment of uncomplicated malaria have been recently reported in Sudan.^{23,24}

Table 2. Comparison of genotype profiles of *P. falciparum* isolates during pre-treatment and post-treatment using sulfadoxine-pyrimethamine and artesunate association.

Isolates	Day 0 (pretreatment)			Day 14 (posttreatment)		
	Genotypes			Genotypes		
	MSA-1	MSA-2	EBA-175	MSA-1	MSA-2	EBA-175
29	K1	3D7	FCR3	K1	FC27	CAMP
132	K1	3D7	FCR3	K1 Ro33	FC27	FCR3
156*	K1/Ro33/Mad20	FC27	FCR3/CAMP	K1	3D7	FCR3
217	K1	FC27	FCR3	K1	FC27	FCR3
271*	K1	FC27	FCR3	K1	3D7	FCR3
329	K1/Mad20	3D7	FCR3/CAMP	K1	3D7	FCR3
873	K1	3D7	FCR3	K1	3D7	FCR3
266*	K1/Ro33	-	CAMP	Ro33	unassigned	FCR3
363	Ro33	3D7	-	Ro33	FC27	-
714*	Mad20	unassigned	FCR3	Mad20	FC27	FCR3
88	Ro33	3D7	FCR3	K1	unassigned	FCR3
129	K1	FC27	-	unassigned	3D7	-
201*	Unassigned	unassigned	CAMP	Mad20	3D7	FCR3
245	K1/Mad20	3D7	CAMP	-	-	FCR3
337*	Unassigned	-	FCR3/CAMP	K1	-	FCR3/CAMP
875	Unassigned	3D7	CAMP	Ro33	-	FCR3

*, aparasitemic patients at pretreatment stage.

Isolates and corresponding genotypes in bold type indicate isolates with identical allele patterns pre- and posttreatment.

In summary, the occurrence of *P. falciparum* SMI was investigated in 278 individuals before and after sulfadoxine-pyrimethamine-artesunate treatment. The results confirmed both the usefulness of such combined treatment and the persistence of SMI in endemic areas.

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