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High prevalence of *Plasmodium falciparum pfcrt* K76T mutation in pregnant women taking chloroquine prophylaxis in Senegal

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***Objectives*: The risk of malaria infection is increased during pregnancy, and many countries recommend chloroquine prophylaxis in pregnant women, despite *Plasmodium falciparum* chloroquine resistance. Chloroquine resistance is associated with the *pfcrt* gene K76T mutation. The aim of this study was to compare the prevalence rate of *pfcrt* T76 mutation in *P. falciparum* isolates from infected pregnant and non-pregnant individuals from Senegal.**

***Methods*: The study was conducted in the rural maternity hospital of Thiadiaye, Senegal, where malaria is seasonal. Sixty-nine *P. falciparum* isolates from infected women were collected at delivery. These women were part of a cohort study; they were followed from their first antenatal visit and advised to take chloroquine prophylaxis. For each woman, the earliest *P. falciparum*-infected blood sample was also used. A control group of 49 non-pregnant individuals with asymptomatic *P. falciparum* infection was enrolled.**

***Results*: During pregnancy, prevalence of T76 mutant parasites was higher than in the 49 non-pregnant controls ($P < 0.001$). Among pregnant women, this rate was highest at delivery ($P = 0.06$), and tended to be higher in women who had taken chloroquine prophylaxis, as assessed in urine samples ($P = 0.08$).**

***Conclusions*: Chloroquine prophylaxis is responsible for increased drug consumption and increased drug pressure that may lead to the selection of drug-resistant parasites. This is the first report showing that *P. falciparum*-infected pregnant women harbour *pfcrt* T76 mutant parasites more often than non-pregnant individuals, and that the prevalence of this mutation is higher at term than earlier during pregnancy.**

Keywords: malaria, pregnancy, drug resistance

Introduction

Malaria infection during pregnancy leads to placental infection and represents a substantial risk for the mother, her fetus and the neonate. In areas of limited *Plasmodium falciparum* transmission, pregnant women may experience severe disease, anaemia and abortion. The fetus may suffer from growth retardation, while the offspring may be born prematurely or be of low weight. In contrast, where transmission is stable and high, women have substantial acquired immunity, but *P. falciparum* infection during pregnancy may still be responsible for maternal anaemia and reduced birth weight of the offspring. Several epidemiological studies have demonstrated that primigravidae are at highest risk for malaria infection and that low birth weight babies may be born.[1]

For a long time, WHO advocated the use of chloroquine prophylaxis in pregnant women to prevent the adverse consequences of malaria during pregnancy. Although WHO is currently modifying its policy, now advocating the use of intermittent preventive treatment, the Ministries of Health in many African countries, including Senegal, are still currently recommending that pregnant women receive chloroquine chemoprophylaxis throughout

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pregnancy. However, the recent increase of chloroquine-resistant *P. falciparum* has become a major problem in Senegal, reaching rates of 50%,[2] and the Ministry of Health is considering a modification of its recommendations.

The spread of drug resistance owing to the use of malaria prevention drugs in pregnant women has never been assessed. However, the drug pressure occurring in pregnant women given chloroquine prophylaxis might induce a positive selection of chloroquine-resistant *P. falciparum* parasites. The effects of such prophylaxis may be even stronger given the high frequency of poor compliance.[3] Most notably, an allele of the *P. falciparum* chloroquine-resistance transporter gene (*pfcrt*) encoding threonine at position 76 (*pfcrt* T76) has been demonstrated to be strongly linked to chloroquine resistance.[4] Multiple studies in areas where *falciparum* malaria is endemic have demonstrated that mutant parasites presenting with the T76 point mutation in the *P. falciparum* chloroquine resistance transporter, encoded by the *pfcrt* gene, are linked to chloroquine resistance *in vitro* and *in vivo*.[5,6] The aim of this study was to compare the prevalence rate of the *pfcrt* T76 mutation in *P. falciparum*-infected pregnant and non-pregnant individuals from Senegal.

Materials and methods

Study site and subjects

This study was conducted in the rural maternity hospital of Thiadiaye, Senegal. Malaria is seasonally transmitted in this area, during the rainy season from August through December. Women delivering at the maternity ward between October 2001 and June 2002 were enrolled in the study, providing they presented with *P. falciparum* infection. Placental and peripheral blood were collected for thick smear preparation and filter paper blotting.

These women were part of a cohort study, and had been followed up from their first antenatal visit (mean pregnancy term 19.3 ± 4.2 weeks) to delivery. During each antenatal visit, women were recommended to take prophylaxis with 300 mg of chloroquine twice weekly, and blood was collected for thick smear and filter paper blot, as well as urine samples for chloroquine detection,[7] to assess compliance with the prophylaxis regimen. For each woman, the first *P. falciparum*-infected blood sample (the earliest in pregnancy; mean pregnancy term 27.8 ± 6.4 weeks) was used for this study. Women were enrolled after informed consent was obtained. A control group of 49 non-pregnant individuals presenting with symptomless *P. falciparum* infection were also enrolled in the same area. Informed consent was obtained from all patients. Human experimentation guidelines of both French and Senegalese governments were followed in the conduct of clinical research. The study was approved by the ethics committee of the Ministry of Health, Senegal.

DNA extraction, PCR–restriction fragment length polymorphism (RFLP) of the pfcrt gene

Blood collected on filter papers was dried and conserved at room temperature until DNA extraction using chelex.[8] The oligonucleotides primers were designed from published sequences. The lower primer was 5′-AATAAAgTTgTgAgTTTCggA-3′, hybridizing from positions 280 to 300.[9] The upper primer was 5′-TgTgCTCATgTgTTTAAACTT-3′, hybridizing from positions 130 to 150 in the *pfcrt* sequence (Genbank accession number AF495378).[10] The PCR components, in a final volume of 25 μL, were 1.6 mM $MgCl_2$, 640 μM deoxynucleotide triphosphate, buffer 1×, 0.3 μM of each primer, 0.5 U of Ampli Taq polymerase (Ampli Taq Gold; Applied Biosystems, Foster City, CA, USA) and 4 μL of DNA samples. The cycling protocol was: 95°C for 7 min for initial denaturation; 40 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s; and a final extension of 72°C for 10 min.

After amplification, a volume of 20 μL of PCR product was incubated overnight at 55°C with the mutation-specific restriction enzyme *Apo* I to detect the *pfcrt* K76T mutation. In the PCR products, the DNA sequence was cleaved at the wild-type codon site (if present) into two fragments (98 and 72 bp), while the mutant allele was not cut (170 bp). The digested products were separated by electrophoresis in a 2% agarose gel containing ethidium bromide, and DNA was visualized by ultraviolet transillumination. DNA fragments were compared by size and with the PCR products generated from genomic DNA of the 3D7 and W2 strains (used as references for susceptible and resistant genotypes, respectively).

Statistical analysis

The χ^2-test for unpaired samples (or Fisher's exact test when required) was used to test for significant differences between categorical variables. When paired analysis was required, the Bowker test, an extension of the McNemar test, was used. A P value <0.05 was considered statistically significant.

Results

Sixty-nine women (mean age 24.3 ± 5.5 years, mean parity 3.0 ± 2.1) were included in the study on the grounds that they presented at delivery with a *P. falciparum* placental and/or peripheral blood infection. Among those, 19 were primigravidae and 50 multigravidae. They had been followed up from their first antenatal visit to delivery. Before delivery, 62 of these women had presented with at least one *P. falciparum* infection. At delivery, 67 women presented with *P. falciparum* placental malaria infection, and 58 with peripheral blood parasites. Median (interquartile range) parasite density was 5561 (7560) parasites per μL in peripheral blood and 2887 (4507) parasites per μL in placental blood. The control group included 49

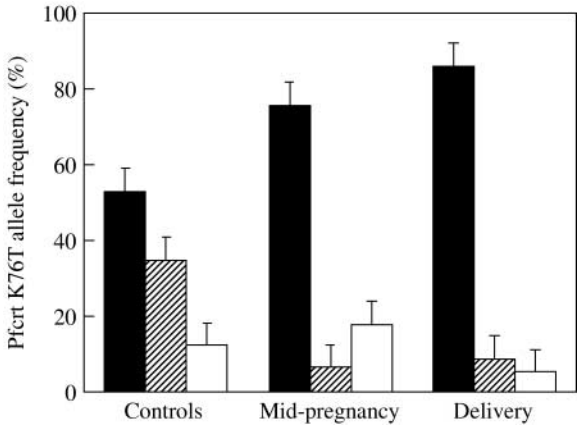

Figure 1. Prevalence rates of *pfcrt* K76T mutant (black bars), mixed (striped bars) and wild-type (white bars) parasites in *P. falciparum* infections from 49 non-pregnant controls and 69 women during the course of their pregnancy and at delivery in the peripheral blood. Error bars indicate standard deviations.

P. falciparum-infected non-pregnant individuals with a median parasite density of 6400 (14 050) parasites per μL.

Genotyping of the *pfcrt* gene of all infected samples was achieved by PCR–RFLP. During pregnancy, 47 of the 62 infected pregnant women harboured mutant parasites, 11 women wild-type parasites, while four presented with a mixed infection with both wild-type and mutant parasites (Figure 1). In the non-pregnant control group, infection with mutant, wild-type and mixed genotypes was observed in 26, six and 17 patients, respectively. Therefore, the prevalence of mutant parasites was higher in pregnant women than in controls, while that of mixed infections was lower ($P<0.001$).

At delivery, infections with mutant, wild-type and mixed genotypes were observed in 50, three and five, respectively, of the 58 peripheral blood samples. As during pregnancy, the prevalence of mutant parasites was higher in delivering women than in controls, while the rate of mixed infections was lower ($P<0.001$). In placental blood, mutant, wild-type and mixed genotypes were observed in 57, two and eight, respectively, of the 67 infected samples. The distribution of the various types of infections was similar in placental and peripheral blood. However, among the 11 women presenting with pure wild-type infection during their pregnancy, seven presented with pure mutant genotypes at delivery in the peripheral samples, while three presented with a mixed infection. Conversely, among the 38 women presenting with pure mutant genotypes during their pregnancy, two isolates presented a wild-type genotype at delivery and one presented with a mixed genotype. Lastly, in all four women presenting with mixed infection during their pregnancy, pure mutant genotypes were observed in peripheral blood at delivery (Bowker test for paired samples $=7.57$, df 3, $P=0.06$). A similar picture was observed in the placental blood. This shows an increasing trend in prevalence rates of mutant parasites in the peripheral blood at term than earlier during the course of pregnancy. The prevalence rates of isolates with mutant genotypes were similar in primigravidae and multigravidae, both in placental (16 of 19 for primigravidae and 40 of 48 for multigravidae; $P=0.62$) and peripheral blood (11 of 15 versus 38 of 43; $P=0.29$). This prevalence rate of mutant parasites in the placental blood tended to be higher when the woman had correctly followed chloroquine prophylaxis, as assessed by urine tests (31 of 33), than when she had not (26 of 35; $P=0.08$).

Discussion

The aim of our study was to assess the prevalence of chloroquine-resistance-associated mutation of the *pfcrt* gene in pregnant women presenting with a *P. falciparum* malaria infection in comparison with non-pregnant individuals. *P. falciparum*-infected pregnant women presented *pfcrt* T76 mutant parasites more often than non-pregnant women. At delivery, the prevalence of mutant parasites in placental and peripheral blood was similar, in line with previous results demonstrating a high level of homology in parasite populations from the peripheral and placental blood from the same woman.[11,12] Similar homology has also been indicated in studies using non-PCR methods.[13–15]

Numerous studies have demonstrated that pregnant women are at a higher risk of suffering from clinical malaria, and drug prevention has been advocated for years, using either weekly chloroquine prophylaxis or, more recently in several countries, intermittent presumptive treatment.[1] The Senegalese Ministry of Health recommends a weekly chloroquine prophylaxis for each pregnant woman from the beginning of pregnancy to delivery. One possible drawback of such increased drug consumption is an increased drug pressure that may lead to the selection of drug-resistant parasites. There is some evidence in favour of this hypothesis. Women from Thiadiaye presented a lower prevalence level of *pfcrt* T76 mutant parasites at an early stage of pregnancy than at delivery. Moreover, women who were assumed, by urine sample, to have taken their prophylaxis correctly presented with a higher prevalence rate of *pfcrt* K76T mutant parasites. This is the first report showing that *P. falciparum*-infected pregnant women harbour *pfcrt* T76 mutant parasites more often than non-pregnant individuals, and that the prevalence rate of this mutation is higher at term than earlier during pregnancy.

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