Genotype comparison of *Plasmodium vivax* and *Plasmodium falciparum* clones from pregnant and non-pregnant populations in North-west Colombia

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**Abstract**

**Background:** Placental malaria is the predominant pathology secondary to malaria in pregnancy, causing substantial maternal and infant morbidity and mortality in tropical areas. While it is clear that placental parasites are phenotypically different from those in the peripheral circulation, it is not known whether unique genotypes are associated specifically with placent al infection or perhaps more generally with pregnancy. In this study, genetic analysis was performed on *Plasmodium vivax* and *Plasmodium falciparum* parasites isolated from peripheral and placental blood in pregnant women living in North-west Colombia, and compared with parasites causing acute malaria in non-pregnant populations.

**Methods:** A total of 57 pregnant women at delivery with malaria infection confirmed by real-time PCR in peripheral or placental blood were included, as well as 50 pregnant women in antenatal care and 80 men or non-pregnant women with acute malaria confirmed by a positive thick smear for *P. vivax* or *P. falciparum*. Five molecular markers per species were genotyped by nested PCR and capillary electrophoresis. Genetic diversity and the fixation index F⁹⁷ per species and study group were calculated and compared.

**Results:** Almost all infections at delivery were asymptomatic with significantly lower levels of infection compared with the groups with acute malaria. Expected heterozygosity for *P. vivax* molecular markers ranged from 0.765 to 0.928 and for *P. falciparum* markers ranged from 0.331 to 0.604. For *P. vivax* infections, the genetic diversity was similar amongst the four study groups and the fixation index from each pairwise comparison failed to show significant genetic differentiation. For *P. falciparum*, no genetic differentiation was observed between placental and peripheral parasites from the same woman at delivery, but the parasites isolated at delivery showed significant genetic differentiation compared with parasites isolated from subjects with acute malaria.

**Conclusions:** In North-west Colombia, *P. vivax* parasites have high genetic diversity that is equivalent in pregnant and non-pregnant populations as well as in symptomatic and asymptomatic infections. For *P. falciparum*, the overall genetic diversity is lower, with specific genotypes associated with asymptomatic infections at delivery.

**Keywords:** Malaria, Pregnancy, Colombia, *P. falciparum*, *P. vivax*, Placenta, Genotyping, Genetic diversity, Genetic differentiation

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Background

Malaria in pregnancy causes substantial maternal and infant morbidity and mortality in tropical areas due to increased risk of adverse pregnancy outcomes such as miscarriage, maternal anaemia and low infant birth weight [1]. Both Plasmodium falciparum and Plasmodium vivax infections cause adverse pregnancy outcomes, however only P. falciparum has been studied extensively [2-5]. The predominant pathology secondary to maternal P. falciparum infection is placental malaria. It is known that P. falciparum–infected red blood cells (Pf-iRBCs) accumulate in the placenta by binding to chondroitin sulfate A (CSA) through the ligand VAR2CSA, a 350-kD member of the P. falciparum erythrocyte membrane protein 1 (PfEMP1) family of surface adhesion antigens [3,6]. PfEMP1 proteins are encoded by ~60 members of the var multigene family and their expression appears to be controlled by allelic exclusion, whereby each parasite expresses a single variant on the surface of the iRBC. Antigenic variation is induced by switching expression of alternative PfEMP1 variants [7]. In pregnancy, the expression of different adhesion molecules can result in selection and segregation of parasite subpopulations in the peripheral and placental circulation [8]. Pf-iRBCs isolated from placenta bind to CSA but not to CD36, the primary receptor mediating Pf-iRBC binding to the microvasculature [3,6,8]. Plasmodium vivax–iRBCs also adhere to immobilized CSA and to fresh placental cells in vitro, but do not adhere to immobilized CD36 [9,10]. However, the identity of the parasite ligand that mediates binding to CSA is unknown.

While it is clear that placental parasites are phenotypically different from those in the peripheral circulation, it is not known whether they also differ genetically. In several African studies, genotyping of matched peripheral and placental P. falciparum parasites from parturient women using the msp1 and/or msp2 genes showed that the majority of alleles were shared, yet some alleles were detected exclusively in one compartment [11-16]. In Latin America, it is not known whether plasmodial parasites in pregnancy have similar genetic characteristics to those in Africa; specifically, it is not known whether parasites in the placenta and peripheral blood of pregnant women are genetically related, or whether certain genotypes are specific to infections in pregnancy. To address these questions, genetic analysis was carried out of parasites from different population groups resident in the Urabá-Altos Sinú-San Jorge-Bajo Cauca region in North-west Colombia which accounts for 60% of all cases in the country [17]. Both P. falciparum and P. vivax cause gestational and placental malaria in this region [18-21]. Genotypes of P. vivax and P. falciparum parasites isolated from peripheral and placental blood in pregnant women were compared with strains causing acute malaria in non-pregnant populations resident in the same region.

Methods

Study population and design

Patients recruited in this study resided in the municipalities of Turbo (08°05’N,76°44’W) and Necoclí (08°25’N,76°47’W) of the Antioquia department, and Puerto Libertador (07°54’N,75°40’W) of the Córdoba department. Together, these departments comprise a high malaria transmission area of Colombia, termed Urabá-Altos Sinú-San Jorge-Bajo Cauca. This region has an estimated area of 43506 km², with 35 municipalities and a population of 2.5 million at risk of malaria. The epidemiological characteristics of this region have been described elsewhere [17,22,23]. The mean annual parasitic index (malaria cases/1000 inhabitants) during 2000–2009 was 46.6 in Turbo, 74.4 in Necoclí, and 23.4 in Puerto Libertador. Plasmodium vivax was reported in about 70% of malaria cases in the region by microscopic diagnosis [24,25].

This study included 57 pregnant women at delivery (“delivery group”) with a malaria infection confirmed by quantitative real-time PCR (qPCR) in peripheral and/or placental blood. For comparison, another group of 50 pregnant women with a positive thick smear for P. vivax (n = 30) or P. falciparum (n = 20) during antenatal care (“antenatal group”) were included. A third group included 80 men or non-pregnant women (“non-pregnant group”) with a positive thick smear for P. vivax (n = 20 men and 20 women) or P. falciparum (n = 20 men and 20 women). All subjects resided in the same three municipalities.

Inclusion and exclusion criteria

The general inclusion criteria for the study population were permanent residency in a malaria-endemic community of Turbo, Necoclí or Puerto Libertador, absence of serious general disease, complicated pregnancy or complicated malaria, and informed consent. The only exclusion criterion was consent withdrawal.

Specimen collection

Blood samples in the delivery group were collected in EDTA tubes within 8 hours of delivery. Maternal peripheral blood (delivery-periphery group) was obtained by venipuncture. Placental blood (delivery-placenta group) was collected from a pool formed on the maternal side when small sections of placenta were removed (approximately 1 cm³) after cleaning with saline. Peripheral blood from subjects in groups “antenatal” and “non-pregnant” was collected by venipuncture, prior to antimalarial treatment.
Thick smears were made for microscopic examination and dried blood spots were prepared on filter paper (Whatman 3MM) for DNA extraction. Blood spots were sealed in plastic bags, stored at 4°C and transported to the laboratory in Medellin.

**Malaria diagnostic tests**

Field-stained thick films were read by an experienced microscopist in the local research laboratory. Parasite density was measured by counting the number of parasites per 200 leukocytes, based on a mean count of 8,000 leukocytes/μL of blood. All subjects with a peripheral blood thick smear positive for malaria received anti-malarial treatment according to the guidelines of the Colombian health authorities [26].

For diagnosis by qPCR, an alcohol-sterilized hole punch was used to cut a circle (approximately 6 mm) from each filter paper and DNA was extracted using the Saponin-Chelex method described by Plowe et al. [27]. The qPCR was performed as described by Shokoples et al. 2009 [28]. Samples were first tested for Plasmodium using a genus-specific set of primers and hydrolysis probe (Plasprobe). Real-time PCR was performed on the ABI 7500 FAST platform, under universal cycling conditions. Samples with a Cycle Threshold (Ct) value under 45 were tested in a duplex species-specific reaction for P. falciparum and P. vivax [28]. Parasite DNA concentration was quantified in the genus-specific screening reaction against a plasmid standard curve of known copy number included in each run. Concentrations are reported as the number of copies of the 18S rRNA gene per microliter of purified DNA.

**Molecular genotyping**

All molecular markers were amplified by nested or seminested PCR, using 3 μL of extracted DNA as template in the first amplification step and 1 μL of the first PCR product for the second amplification. For samples positive for P. vivax, the microsatellites 1.501, 3.502, 3.27, and MS16, as well as the msp3α gene were analyzed based on the protocol described by Koepfli et al. 2009 [29]. The PCR reaction was performed in a final volume of 20 μL containing 1× PCR buffer (Qiagen), 2 mM of MgCl₂ (Qiagen), 200 μM of each dNTP (Takara Bio), 0.25 μM of each primer, and 1.5 units of HotStar Taq DNA polymerase (Qiagen). The cycling program was as follows: initial denaturation for 5 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 56°C–60°C (according to the marker analysed [29]), 1 min at 72°C; and a final step for 5 min at 72°C.

Samples positive for P. falciparum were genotyped based on the microsatellites ARA2, TA1, Polya and PFPK2 and the gene msp2. Amplification of microsatellites was according to the protocol described by Anderson et al. [30]. The reaction volume was 15 μL containing 1× PCR buffer, 2 mM of MgCl₂, 200 μM of each dNTP, 0.25 μM of each primer, and 0.2 units of HotStar Taq DNA polymerase. The cycling conditions were as follows: initial denaturation for 2 min at 94°C; 45 cycles of 30 sec at 94°C, 30 sec at 42°C, 30 sec at 40°C, and 40 sec at 65°C for the first reaction. The nested reaction consisted of 40 cycles of: 30 sec at 94°C, 30 sec at 45°C and 30 sec at 65°C. The final elongation step in both reactions was 5 min at 65°C. The msp2 gene was amplified based on the protocol described by Felger and Beck [31]. The reaction volume was 20 μL containing 1× PCR buffer, 1.5 mM of MgCl₂, 200 μM of each dNTP, 0.25 μM of each primer for the first reaction and 0.4 μM for the nested reaction, and 0.6 units of HotStar Taq DNA polymerase. The cycling program was: initial denaturation for 2.5 min at 94°C; 40 cycles of 30 sec at 94°C, 45 sec at 42°C for the first reaction or 50°C for the nested reaction, and elongation for 1.5 min at 70°C with a final elongation step of 10 min at 70°C.

All PCR analyses were performed in an Applied Biosystems 2720 Thermal Cycler. Amplification was confirmed by visualization in 2% agarose gels and PCR products were stored at 4°C in the dark. Product sizes were resolved by capillary electrophoresis in an ABI Prism 3100 Genetic Analyzer (Perkin Elmer Applied Biosystems), using GS500 LIZ as internal size standard and the microsatellite conditions as the default settings. The results were analysed using GeneMapper software (version 3.5; Applied Biosystems). All electropherograms were visually inspected; peaks above a cut-off of 300 relative fluorescent units (RFU) were considered true amplification products. Alleles were grouped manually based on their repeat length: 3-bp bins for all the P. falciparum microsatellites, PvMS16 and Pvmsp3α; 4-bp bins for Pv3.27; 7-bp bins for Pv1.501 or 8-bp bins for Pv3.502. Multiple alleles per locus were scored if minor peaks were >33% of the height of the predominant allele present for each locus. All mixed infections were genotyped with molecular makers of both species.

**Statistical analysis**

Microsatellite analyzer version 4.05 [32] was used for calculating allele frequency, expected heterozygosity (He) and the FST index. He was defined as the probability that two clones selected from the population at random carry different alleles, and was calculated with the formula $He = \frac{n}{(n - 1)} [1 - \Sigma p_i^2]$, where n is the number of isolates analyzed and $p_i$ is the frequency of the $i^{th}$ allele in the population. The FST index [33] with pairwise comparisons was used to evaluate the genetic differentiation between subpopulations of parasites isolated from the different groups of patients (delivery-periphery vs. delivery-placenta vs. antenatal vs. non-pregnant). Each
monoinfection was confirmed by qPCR in 38 of 40 samples, *P. vivax* monoinfection in 39 of 40, and there were three mixed infections. Individuals in both antenatal and non-pregnant groups had symptomatic malaria and received the treatment recommended by the Colombia health authorities based on the thick smear result.

The infection level quantified by thick smear (parasites/μL) or qPCR (DNA copies/μL template) was significantly lower in the delivery group, particularly for *P. falciparum* infections (Table 1). The low concentration of parasite DNA influenced the success in genotyping; in the delivery group, genotyping at any marker failed in 11 out of 97 samples (two peripheral and seven placental samples), 28 samples were genotyped at one marker (12 peripheral and 16 placental) and 58 were successfully genotyped at two or more markers (28 peripheral and 30 placental). In the antenatal and non-pregnant groups, which had higher levels of parasite DNA, 88% (118/134 infections in both groups) of samples were genotyped for the five genetic markers while four markers were genotyped in the remaining samples. Overall, successful genotyping for each marker varied from 78% to 91% for *P. vivax* samples and from 62% to 79% for *P. falciparum* (Table 2).

### Genetic diversity and parasite differentiation

*Plasmodium vivax* showed significantly higher genetic diversity than *P. falciparum* (Table 2). The number of different alleles detected for each marker ranged from 8 to 24 for *P. vivax* and from 2 to 6 for *P. falciparum*. Furthermore, the probability that two clones selected at random from the population carry different alleles at each marker varied from 77% to 93% for *P. vivax* and from 33% to 60% for *P. falciparum* (Table 2). Another distinction between the species is that polyclonal infections were more common in *P. vivax* than *P. falciparum* infections. Multiple alleles were detected in 62% (76/122) of *P. vivax* samples, and only 17% (19/113) of *P. falciparum* samples.

The mean He was similar for *P. vivax* in all study groups yet for *P. falciparum*, the delivery samples had a higher He than the antenatal and non-pregnant groups (Table 3). Interestingly, infections with either species at

### Table 1 Level of plasmodial infection according to the study groups

| Study group       | *Plasmodium vivax* | *Plasmodium falciparum* |
|-------------------|--------------------|-------------------------|
|                   | n                  | Parasites/μL (mean±SD)  | DNA copies/μL (mean±SD) | n | Parasites/μL (mean±SD) | DNA copies/μL (mean±SD) |
| Delivery-periphery| 22                 | 960 ± 2134              | 382 ± 732*               | 21 | 208 ± 843              | 16 ± 33*               |
| Delivery-placenta | 25                 | 89 ± 247                | 118 ± 234                | 29 | 5 ± 17                 | 88 ± 288               |
| Antenatal         | 31                 | 6464 ± 6467             | 1825 ± 2326              | 20 | 6124 ± 8245            | 1222 ± 2052            |
| Non-pregnant      | 42                 | 7000 ± 7585             | 3020 ± 5372*             | 41 | 6852 ± 13273           | 1521 ± 2715*           |

* p < 0.05 for comparison between *P. vivax* and *P. falciparum*.
delivery showed a significantly lower frequency of multiple alleles compared with the samples from acute infection. Within the delivery group, there was no statistical difference in the \( H_e \) and the frequency of polyclonal infections between peripheral and placental samples. Likewise, there were no differences in \( H_e \) and polyclonal infections between antenatal and non-pregnant groups.

Comparison of the FST index per pair of study groups (delivery-periphery vs. delivery-placenta vs. antenatal vs. non-pregnant) for \( P. \) vivax infections did not show significant genetic differentiation (Table 4). However, for \( P. \) falciparum samples, the global FST based on all loci was 0.154 (\( p = 0.0001 \)) and significant differentiation was detected when parasites isolated from peripheral and placental blood of pregnant women at delivery were compared with parasites isolated from antenatal and non-pregnant subjects. The highest genetic differentiation was observed between parasites from the delivery (periphery or placenta) and non-pregnant groups. The predominant alleles detected in both the antenatal and non-pregnant groups were exactly the same for all five markers. Moreover, of 14 women with falciparum infection in peripheral and placental blood simultaneously, 8 (57%) had the same parasite genotype in both compartments. One woman had different genotypes in these compartments and in five women it was not possible to compare the genotypes because the molecular markers failed to amplify.

**Discussion**

The current study compared the genotypes of \( P. \) vivax and \( P. \) falciparum clones isolated from pregnant and non-pregnant infected subjects in North-west Colombia,

### Table 2 Genetic diversity of each molecular marker

| Plasmodium vivax (n = 120) | 1.501 | 3.502 | 3.27 | MS16 | MPS3a |
|---------------------------|-------|-------|------|------|-------|
| Successful genotyping (%) | 91    | 89    | 84   | 79   | 78    |
| No. different alleles detected | 8     | 8     | 23   | 24   | 19    |
| Polyclonal samples (%)    | 12    | 13    | 55   | 12   | 25    |

### Table 3 Expected heterozygosity and polyclonal infections in each study group

| Study group               | \( P. \) vivax | \( P. \) falciparum |
|---------------------------|---------------|---------------------|
|                           | \( H_e \)     | Polyclonal infections | \( H_e \)     | Polyclonal infections |
| Delivery-periphery        | 0.876 ± 0.072 | 47                  | 0.554 ± 0.120 | 5                  |
| Delivery-placenta         | 0.869 ± 0.093 | 33                  | 0.629 ± 0.145 | 8                  |
| Antenatal                 | 0.847 ± 0.073 | 84                  | 0.481 ± 0.103 | 25                 |
| Non-pregnant              | 0.874 ± 0.046 | 73                  | 0.353 ± 0.215 | 25                 |
| \( p \)                   | 0.3373        | 0.0004              | <0.0001       | 0.1462             |

### Table 4 Pairwise FST index of \( P. \) vivax and \( P. \) falciparum isolated from each study group

| Study group               | \( P. \) vivax | \( P. \) falciparum |
|---------------------------|---------------|---------------------|
|                           | Delivery-placenta | Antenatal | Non-pregnant |
| Delivery-periphery        | −0.1014       | −0.0394            | −0.0344     |
| Delivery-placenta         | −0.0496       | −0.0464            |             |
| Antenatal                 | −0.0229       |                   |             |

*Number of samples successfully amplified for at least one molecular marker in each species.
using five genetic markers per species. Consistent with previous reports from non-pregnant populations, the genetic diversity of *P. vivax* is much greater than that of *P. falciparum* [34-36], which is explained by the different origins of both species and their distant phylogenetic relationship [37,38]. Other studies in Colombia have confirmed high genetic diversity for *P. vivax* infections [39-41] and low diversity for *P. falciparum* [42-45] in naturally infected, non-pregnant patients.

The number of different alleles detected and *He* were high in all *P. vivax* infections, regardless of the population (delivery, antenatal, non-pregnant) or the compartment from the same subjects (delivery-periphery, delivery-placenta). However, polyclonal infections were significantly more common in antenatal and non-pregnant than in delivery in both peripheral and placental infections. A similar finding was observed for *P. falciparum* infections; higher genetic diversity and fewer polyclonal infections were detected in the delivery group compared with the antenatal and non-pregnant groups. This can be explained by the low level of parasitaemia and parasite DNA copies detected in women of the delivery group, consistent with asymptomatic infection. Other authors also reported an association between low parasitaemia and low complexity infections [46-48].

The selection criteria used in this study likely explains the differences in parasite density observed across the study groups; all parurient women included here (delivery, antenatal, non-pregnant) were part of a larger prevalence study on malaria infection at delivery, whose plasmodial infection was detected only by PCR (submicroscopic infection) in most cases. In contrast, selection of subjects included in the other two groups (antenatal and non-pregnant) was based on a positive thick smear (microscopic infection).

Interestingly, in both groups with microscopic infection (antenatal and non-pregnant), the level of parasitaemia was similar for *P. falciparum* and *P. vivax* infections. This finding agrees with previous reports from Colombia that both *P. vivax* and *P. falciparum* uncomplicated malaria patients have low parasitaemia (<10,000 parasites/μL) [49-51]. However, reports from other endemic regions where both species co-circulate generally describe lower parasitaemias for *P. vivax* infections [5,52-54] attributed to the fact that *P. vivax* exclusively invades reticulocytes [55,56].

One of the most significant findings in this study is that *P. falciparum* infections showed specific parasite genotypes associated with delivery vs. non-pregnant populations. Two hypotheses could explain this finding. First, different parasite genotypes can be associated with low vs. high levels of parasitaemia. Previous studies in non-pregnant populations have reported association of specific genotypes with low parasitaemic and asymptomatic infections [57], as well as genotypes associated with severe malaria [58]. Second, genetically distinct subpopulations of *P. falciparum* may segregate differentially to peripheral and placental blood. Other authors have compared *P. falciparum* clones isolated from matched peripheral and placental blood using genes that encode surface proteins such as *msp1* and *msp2* [11-16] or genes associated with antimalarial drug resistance such as *dhfr*, *dhps* [59,60], and *pfcr* [61,62]. Although the *dhfr*-dhps studies failed to find an association of specific genotypes per compartment, the *msp1-msp2* and *pfcr* studies did identify specific genotypes present exclusively in the placenta.

The genetic diversity and differentiation of placental parasites in *P. falciparum* infections suggests that different strains or parasite genotypes share the phenotypic
property of adherence to placental tissue. Genetic characterization of placental parasites should therefore be considered in the development of a pregnancy-associated malaria vaccine to ensure maximum cross-protection against genetically different strains.

Conclusions

This study confirms that in North-west Colombia \textit{P. vivax} parasites have high genetic diversity, which is conserved in pregnant and non-pregnant populations as well as in symptomatic and asymptomatic infections. For \textit{P. falciparum}, the genetic diversity is lower compared with \textit{P. vivax} with specific genotypes that are associated with low-level, asymptomatic infections at delivery.

Abbreviations

PF-ABCs: \textit{P. falciparum}-infected red blood cells; CSA: Chondroitin sulfate A; PFMPI: \textit{P. falciparum} erythrocyte membrane protein 1; qPCR: Quantitative real-time polymerase chain reaction; Ct: Cycle threshold; RFU: Relative fluorescence units; He: Expected heterozygosity; bp: Base pairs.

Competing interests

The authors affirm that they have no commercial or other association that might pose a conflict of interest.

Authors' contributions

EM participated in the study design, carried out the molecular genotyping and statistical analyses and wrote the manuscript. RS participated in the molecular genotyping. OMA participated in the collection of field samples. JCF participated in the study design, statistical analyses and preparation of the manuscript. AM and SK participated in the study design, coordination and preparation of the manuscript. All authors read and approved the final manuscript.

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