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Molecular Detection of Malaria at Delivery Reveals a High Frequency of Submicroscopic Infections and Associated Placental Damage in Pregnant Women from Northwest Colombia

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Abstract. *Plasmodium* infection in pregnancy causes substantial maternal and infant morbidity and mortality. In Colombia, both *P. falciparum* and *P. vivax* are endemic, but the impact of either species on pregnancy is largely unknown in this country. A cross-sectional study was carried out with 96 pregnant women who delivered at their local hospital. Maternal, placental, and cord blood were tested for malaria infection by microscopy and real-time quantitative polymerase chain reaction (qPCR). A high frequency of infection was detected by qPCR (45%). These infections had low concentrations of parasite DNA, and 79% were submicroscopic. Submicroscopic infections were associated with placental villitis and intervillitis. In conclusion, the overall frequency of *Plasmodium* infection at delivery in Colombia is much higher than previously reported. These data prompt a re-examination of the local epidemiology of malaria using molecular diagnostics to establish the clinical relevance of submicroscopic infections during pregnancy as well as their consequences for mothers and newborns.

INTRODUCTION

Malaria in pregnancy causes substantial maternal and infant morbidity and mortality in tropical areas because of increased risk of maternal anemia and low birth weight (LBW) infants.[1] In 2007, an estimated 125.2 million pregnancies occurred in areas with malaria transmission.[2] In highly endemic areas, up to 50% of LBW deliveries can be attributed to malaria in pregnancy, leading to approximately 100,000 infant deaths.[2,3] All species of *Plasmodium* cause malaria infection in pregnant women, but only *P. falciparum* has been extensively studied in pregnancy, with few studies of *P. vivax*. However, both infections can cause adverse pregnancy outcomes.[4–7]

Women living in different geographical areas experience variable levels of exposure to *Plasmodium*, which influences the course of infection in pregnancy.[8] In areas of high, stable transmission, including sub-Saharan Africa, where *P. falciparum* predominates, pregnancy malaria is often asymptomatic but associated with severe maternal anemia and fetal growth retardation. In these areas, placental infection is most frequent in first-time mothers, because women develop immunity against placental parasites that provides protection over successive pregnancies. In areas of low, unstable transmission, malaria infection is typically symptomatic in women of all parities and associated with high rates of fetal loss and maternal death.[9]

In Latin America, both *P. falciparum* and *P. vivax* are endemic in areas of low, unstable transmission. According to the World Malaria Report (2011), malaria transmission occurs in 21 countries in the Americas, with Colombia and Brazil accounting for more than 60% of cases.[10] In Colombia, 115,884 cases of malaria were reported in 2010, and the Urabá-Altos Sinú-San Jorge-Bajo Cauca region accounted for 60% of all cases. There are no official reports on the prevalence of infection within the pregnant population in Colombia. In a recent study of 2,117 pregnant women in the Urabá region, 220 cases of malaria were diagnosed by microscopy.[11] In a subset of 79 pregnant women at delivery, the frequency of plasmodial infection in peripheral and placental blood samples detected by microscopy was 13% and 9%, respectively, whereas the frequency of infection detected by nested polymerase chain reaction (PCR) was 32% and 26%, respectively.[12] Importantly, the application of more sensitive molecular tests identified 2.5 times more infections than microscopy, classifying these infections as submicroscopic. Similar discrepancies between PCR and microscopy are reported from other endemic population surveys.[13,14] However, the epidemiology and clinical significance of submicroscopic malaria infections during pregnancy are not well-understood.[13,15–20] The present pilot study evaluated the frequency of submicroscopic plasmodial infections in parturient women in the Urabá-Altos Sinú-San Jorge-Bajo Cauca region of Colombia and their associations with clinical outcomes of pregnancy.

MATERIALS AND METHODS

Study site and population. Women were recruited at one hospital obstetric facility in each of the municipalities of Turbo (08°05′ N, 76°44′ W), Necoclí (08°25′ N, 76°47′ W; Antioquia), and Puerto Libertador (07°54′ N, 75°40′ W; Córdoba). The three hospitals are public institutions that provide equivalent primary-level healthcare, servicing populations with comparable demographics and socioeconomic status in the region. Both Antioquia and Córdoba comprise the malaria transmission area termed Urabá-Altos Sinú-San Jorge-Bajo Cauca, which has an estimated area of 43,506 km^2 and a malaria at-risk population of 2.5 million. Epidemiologic characteristics of this region are described elsewhere.[21–23] This region is homogeneous in terms of ecoepidemiology and malaria transmission. Transmission intensity is low and stable, with no marked fluctuations in the number of malaria cases during the year. The mean annual parasitic index (malaria cases/1,000 inhabitants) during 2000–2009 was 46.6 in Turbo, 74.4 in Necoclí, and 23.4 in Puerto Libertador. *P. vivax* is reported in approximately 70% of cases.[24,25] No national malaria control strategies specific for pregnant women have been implemented, and the use of bed nets is uncommon. Treatment guidelines for malaria in pregnancy include chloroquine for vivax malaria and quinine-clindamycin (first trimester) or artemether-lumefantrine (second and third trimesters) for falciparum malaria.[26]

Study and sample design. A cross-sectional pilot study was carried out to measure the frequency of submicroscopic infection at delivery in Urabá-Altos Sinú-San Jorge-Bajo Cauca.

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A sample size of 96 randomly selected women was calculated from a larger epidemiological study of 2,550 parturient women recruited between 2005 and 2011 using the formula $n = NZ^2p(1 - p)/[(Ne^2) + (Z^2p(1 - p))]$,[27] where N is the reference population (N = 2,550), Z is the confidence level (95%), p is the reported frequency of submicroscopic infection at delivery (18%),[12] and e is the sampling error (7.5%). From the entire list of 2,550 women, 96 women were selected using a simple random sampling without replacement method in accordance with the inclusion criteria (see below).

Inclusion and exclusion criteria. The inclusion criteria were permanent residence in the malaria-endemic community of Turbo, Necoclí, or Puerto Libertador, absence of serious general disease or complicated pregnancy, availability of filter papers with peripheral, placental, and cord blood, and signature on the informed consent form. The exclusion criterion was consent withdrawal.

Data and specimen collection. A questionnaire was completed with data on age, number of pregnancies, and number of malaria episodes during the pregnancy reported by the mother and based on her health card that documents the diagnosis (by microscopy) and treatment. Labor/delivery and infant outcomes data as well as maternal hemoglobin level at delivery were collected from the hospital chart.

Within 8 hours of delivery, a blood sample from each compartment was collected in EDTA tubes (BD Vacutainer®, USA) and used to prepare thick smears and dried blood spots on filter paper (Whatman™ Grade 3). Blood spots were prepared with approximately 100 μL blood (two drops). After drying at room temperature, spots were sealed in plastic bags (one bag per sample per woman) and stored at 4°C. Maternal peripheral blood was collected by venipuncture. Cord blood was collected by sectioning a fragment to expose a fresh segment and then draining the blood into the tube. After cleaning with saline, small (~1 cm^3) sections of placenta were removed from the maternal side, and the blood pooled was collected by pipet aspiration. Three tissue fragments were obtained by sectioning the placenta (2 cm^2 surface area through the entire thickness). Fragments were fixed in 10% neutral buffered formalin and paraffin-embedded within 48 hours. Sections of 5-μm thickness were stained with hematoxylin & eosin and read under 100× and 400× magnifications according to standard procedures.

Malaria diagnosis. Field-stained thick films were read by an experienced microscopist. Thick smears were defined as negative if 200 fields (1,000× magnification) were free of parasites.

A hole punch circle (~6 mm) of each filter paper was used for DNA extraction, corresponding to approximately 25 μL blood. DNA was extracted with the saponin-Chelex method.[28] The extracted DNA was resuspended in 50 μL water. Real-time quantitative PCR (qPCR) was performed as described elsewhere.[29] Samples were first tested for *Plasmodium* using genus-specific primers and a hydrolysis probe (Plasprobe). PCR was run on the ABI 7500 FAST platform. Samples with a Cycle Threshold (Ct) < 45 were tested in duplex species-specific reactions for *P. falciparum* and *P. vivax*.[29] Additional confirmation of mixed and weak infections (Ct = 42–45) was performed using newly designed reverse primers specific for *P. falciparum* (5′-AGCAATCTAAAAGTCACCTCGAA-3′) and *P. vivax* (5′-AGCAATCTAAGAATAAACTCCGAAGA-3′). These primers were combined with the forward Plasmo1 primer and the Plasprobe in singleplex reactions. Discordant samples in the three reactions (genus-specific, duplex species-specific, and singleplex species-specific reactions) were called negative. DNA copy number was quantified from the genus-specific reaction against a standard curve using a plasmid containing a fragment of the 18*S* gene from *P. falciparum*. The lower limit for quantitation with this plasmid is 10 copies per PCR (or 2 copies/μL template). The sensitivity of the qPCR assay for the detection of parasite DNA in clinical samples is limited by the input volume in the qPCR reaction, which corresponds to ~2.5 μL whole blood. Positive samples were further confirmed by nested and seminested PCR to amplify other sequences within the 18*S* gene[30] and other microsatellite and gene regions.[31–33]

Histopathology. A reader with experience in placental histology read an entire slide from each placenta. The reader established protocols and definitions based on her experience and data reported from other works.[8,34–39] Placental histological analyses included deciduitis, > 8 high-power fields (HPF) with immune cells in the decidua; villitis, > 10 immune cells/HPF in stromal villi; villi infarct, at least 1 HPF with an ischemic area with degenerative lesions; fibrin deposits, at least 1 HPF with accumulation of fibrin in the intervillous space; intervillitis, > 10 immune cells/HPF in the intervillous space; increased syncytial knots, > 3 HPF with aggregates of syncytial nuclei at the surface of villi; and malaria pigment deposits, at least 1 HPF with malaria pigment in fibrinoid or immune cells.

Definitions and statistical analysis. Malaria infection was defined by a positive diagnosis by microscopy and/or qPCR for *P. falciparum* and/or *P. vivax*. Submicroscopic infections were negative by microscopy and positive by qPCR. Definitions used were anemia, hemoglobin < 11 g/dL; LBW, birth weight < 2,500 g; and pre-term delivery, birth before 37 weeks of gestation.

According to the data distribution, the variable number of DNA copies per microliter was categorized in terciles as < 2 (below the linear range of the standard curve), 2–100, and > 100 DNA copies/μL. Data were analyzed using Excel and Epi Info 3.5.3. Kruskal–Wallis and χ^2 tests were used for comparison of continuous and categorical variables, respectively. Significance was set at $P < 0.05$.

Ethical considerations. Pregnant women or guardians signed a voluntary consent form. The study involved minor risk, and approval was granted by the Comité de Ética of Instituto de Investigaciones Médicas (ethical approval numbers: IIM2484, IIM2509, IIM2530, and IIM2557), Universidad de Antioquia, and the Health Research Ethics Board of the University of Alberta (ethical approval number: Pro00017660).

RESULTS

General characteristics of study women are shown in Table 1; 59% of women had at least one previous delivery. Frequencies of pre-term delivery and LBW were low (6% and 4%, respectively). However, the overall frequency of anemia was high; of 88 women with known hemoglobin levels at delivery, 52% were anemic.

Malaria infection at delivery. The frequency of malaria infection in the three compartments varied significantly between microscopy and qPCR (Table 2). Based on qPCR, 65% of women had at least one positive compartment, 25% of women had two positive compartments, and 23% of women were positive in all three compartments. Twelve women (13%) had infections only in the placenta, whereas five women (5%) were

TABLE 1
General characteristics of the study women

Variable	Value
Age (years) mean ± SD (range)	22.8 ± 6.4 (14–44)
Parity *n* (%)	1.7 ± 2.1 (0–11)
Primiparous	31 (33.4)
Secundiparous	24 (26.7)
Multiparous	35 (38.8)
Gestational age (weeks)	38.6 ± 2.1 (27–42)
Preterm delivery percent (*n*)	6 (5)
Birth weight (g) mean ± SD (range)	3,230 ± 473 (1,850–4,800)
LBW percent (*n*)	4 (4)
Hemoglobin (g/dL) mean ± SD (range)	10.8 ± 1.5 (7.6–18.3)
Anemia percent (*n*)	52 (46)

positive only in peripheral blood; no women had infection in the cord exclusively. The highest frequency of infection was in the placenta (57%) followed by peripheral blood (49%) and cord (29%). To confirm the specificity of these results, the positive samples were further genotyped by nested or seminested PCR and capillary electrophoresis of five molecular markers per species[40]; 74% of samples were also positive for at least one species-specific marker (Supplemental Table 1).

The distribution of *P. vivax* and *P. falciparum* in the infected samples differed according to the diagnostic method. By microscopy, the majority of infections (75%) were identified as *P. vivax*, whereas over 50% of the infections detected by qPCR were caused by *P. falciparum* (Table 2). Sixteen different species combinations were detected across the compartments. Of 46 women with infections in two or more compartments, 40 women (87%) had the same species, whereas the other 6 women had discordant species, mainly because of mixed infections in the placenta and monoinfections in peripheral or cord blood (Supplemental Table 2).

In general, the level of infection in these women was very low (Figure 1). Based on qPCR, 84 of 130 infections (65%) had < 2 DNA copies/μL template. Cord was the compartment with the lowest level of infection (Figure 1A). A comparison of the DNA concentrations by species revealed significantly higher DNA concentrations in *P. vivax* and mixed infections compared with *P. falciparum* ($P = 0.0082$). Across all three compartments, most of the *P. falciparum* infections (78%) had < 2 DNA copies/μL (Figure 1B).

Clinical and histological outcomes of infected women. Malaria infection was detected by qPCR but not by microscopy in 48 women. These submicroscopic infections account for 79% of the infections. The clinical characteristics were compared between women who were negative for malaria and women with microscopic and submicroscopic infections (Table 3). Age, hemoglobin level, gestational age, parity, and

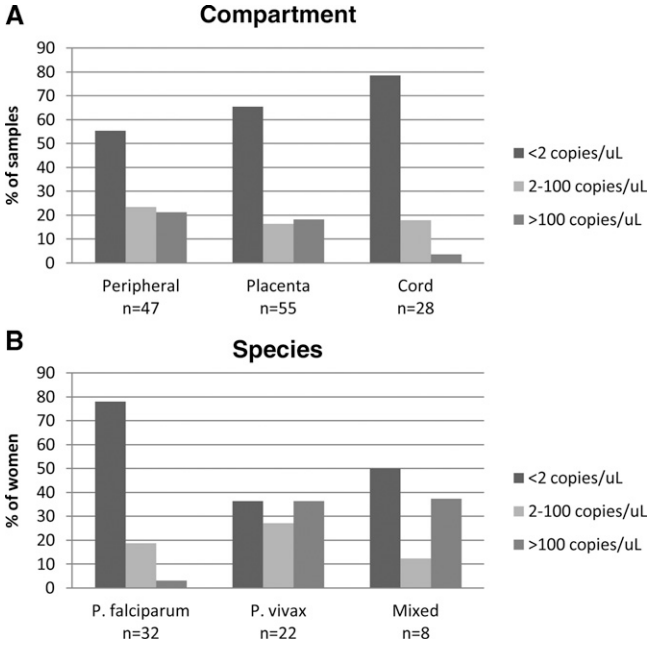

FIGURE 1. Quantitation of parasite DNA by qPCR. (**A**) Samples from each compartment were categorized into terciles based on quantitation of DNA copies by qPCR. (**B**) Quantitation of DNA copies according to the species of infection.

birth weight were similar among the three groups. Infant birth weight was significantly lower in primiparae and adolescents (≤ 18 years of age), but this finding was not associated with malaria infection. The mean infant birth weight in primiparae was 3,061 ± 467 g, and the mean infant birth weight in multiparous women was 3,333 ± 463 g ($P = 0.086$). Adolescent women had infants with a mean weight of 2,992 ± 284 g, whereas the infant birth weight in older women was 3,325 ± 486 g ($P = 0.0009$).

Placental histological analysis was available for 87 women (91%): 28 women were negative for plasmodial infection, 46 women had submicroscopic infections, and 13 women had microscopic infections. Placentas from women with both microscopic and submicroscopic infections had a higher frequency of villitis and intervillitis than uninfected women ($P = 0.04$). This inflammation was observed in women infected with either *P. falciparum* or *P. vivax*. In addition, malaria pigment deposits were more frequent in women with microscopic infection (62%) than women with submicroscopic infection (15%) or uninfected women (14%) (Table 3).

History of malaria in pregnancy. The history of malaria during the current pregnancy was known for 66 of 96 (69%)

TABLE 2
Number of microscopy- and qPCR-positive samples in each compartment

	Peripheral blood		Placental blood		Cord blood		All compartments	
	Microscopy ($N = 95$)	qPCR ($N = 96$)	Microscopy ($N = 95$)	qPCR ($N = 96$)	Microscopy ($N = 95$)	qPCR ($N = 96$)	Microscopy ($N = 285$)	qPCR ($N = 288$)
P. falciparum	1	23	2	28	0	16	3	67
P. vivax	11	22	6	20	0	10	17	52
Mixed	0	2	0	7	0	2	0	11
Total positive	**12**	**47**	**8**	**55**	**0**	**28**	**20**	**130**
Total negative	83	49	87	41	95	68	265	158

TABLE 3
Clinical characteristics and histological changes of women with or without plasmodial infection at delivery

Variable	Negative ($N = 34$)*	Submicroscopic ($N = 48$)*	Microscopic ($N = 13$)	P value
Age (years) mean ± SD (range)	22.5 ± 7.0 (14–44)	22.6 ± 5.8 (15–36)	24.9 ± 7.4 (14–38)	0.4647
Gestational age (weeks) mean ± SD (range)	38.7 ± 1.8 (32–41)	38.5 ± 2.4 (27–42.3)	38.6 ± 2.3 (32–40.8)	0.9201
Hemoglobin (g/dL) mean ± SD (range)	10.9 ± 1.8 (8.4–18.3)	10.7 ± 1.0 (9.0–13.3)	10.8 ± 1.6 (7.6–13.3)	0.8758
Birth weight (g) mean ± SD (range)	3,271 ± 469 (2,350–4,500)	3,186 ± 508 (1,850–4,800)	3,283 ± 348 (2,800–4,000)	0.5861
Parity percent (n)				
Primiparous	40 (13)	32 (14)	33 (4)	0.7254
Secundiparous	30 (10)	27 (12)	17 (2)	0.7254
Multiparous	30 (10)	41 (18)	50 (6)	0.7254
Deciduitis percent (n)	11 (3)	26 (12)	25 (3)	0.2691
Villitis percent (n)	7 (2)	28 (13)	39 (5)	**0.0397**
Increased villi infarct percent (n)	11 (3)	20 (9)	15 (2)	0.6018
Intervillitis percent (n)	7 (2)	33 (15)	31 (4)	**0.0382**
Fibrin deposits percent (n)	46 (13)	63 (29)	69 (9)	0.2605
Increased syncytial knots percent (n)	15 (4)	11 (5)	39 (4)	0.2214
Malaria pigment deposits percent (n)	14 (4)	15 (7)	62 (8)	**0.0009**

*The histological study was available for 26 women with negative results of placental infection and 46 women with submicroscopic placental infection.

women. A total of 48 women (73%) had at least one recorded episode of malaria; 34 women had a history of *P. vivax*, and 6 women had a history of *P. falciparum*. In 8 women, the species was not recorded. All women received treatment at the time of diagnosis. Of these women with a history of malaria in the pregnancy, 73% had at least one positive compartment at delivery by qPCR; *P. vivax* was detected in 24 of 34 women with a history of *P. vivax*, whereas *P. falciparum* was detected at delivery in 4 of 6 women with a history of *P. falciparum*.

A comparison of the clinical characteristics of women with and without a history of malaria in the pregnancy revealed a significant association between malaria in pregnancy and lower birth weight (LBW) infants (Table 4). In addition, the species detected during the pregnancy influenced the birth weight; infants born from women with a history of *P. vivax* had a mean birth weight of 3,053 ± 427 g, whereas the infant birth weight from women with a history of *P. falciparum* was 3,300 ± 379 g. Infant birth weight from women without a history of malaria was 3,578 ± 559 g ($P = 0.010$). In contrast, the species detected at delivery by qPCR was not statistically associated with birth weight ($P = 0.254$); however, the mean infant birth weight in women with *P. vivax* infection at delivery was lower (3,059 ± 459 g) than the mean infant birth weight in women with *P. falciparum* infection (3,320 ± 512 g) and the mean infant birth weight in women without infection (3,271 ± 469 g). Furthermore, deposits of malaria pigment on placental tissue were only detected in women with a history of malaria in pregnancy, consistent with a past infection. The other placental histological variables were similar among women with and without history of malaria during the pregnancy (Table 4).

DISCUSSION

This pilot study is the first in Latin America to evaluate malaria in pregnancy using qPCR diagnostics. The frequency of infection detected by qPCR was far greater than previously reported; 65% of pregnant women in our study had at least one positive compartment. The few previous reports in Colombia on the frequency of malaria infection during pregnancy or at delivery were carried out in municipalities of Urabá; according to microscopy, the frequency of malaria infection in maternal peripheral blood was 11–14%, the frequency of malaria infection in placental blood was 9–12%, and the frequency of malaria infection in cord blood was 2–4%.[11,12,41,42] One study used nested PCR in addition to microscopy to diagnose malaria infection in the three compartments, and the frequency of infection increased markedly from 13% to 32% in peripheral blood, from 9% to 26% in placenta, and from 2% to 13% in cord blood.[12]

TABLE 4
Clinical characteristics of women with or without history of malaria in the pregnancy

Variable	History of malaria in pregnancy		P Value
	No ($N = 18$)	Yes ($N = 48$)	
Age (years) mean (range)	24.3 (16–32)	22.2 (14–38)	0.1014
Gestational age (weeks) mean (range)	39.1 (37–41.3)	38.5 (27–42.3)	0.4211
Hemoglobin (g/dL) mean (range)	11.4 (9.4–18.3)	11.0 (7.6–13.3)	0.8003
Birth weight (g) mean (range)	3,578 (2,800–4,800)	3,132 (1,850–4,000)	**0.0081**
Parity percent (n)			
Primiparous	22 (4)	38 (18)	0.2256
Secundiparous	17 (3)	25 (12)	0.2256
Multiparous	61 (11)	38 (18)	0.2256
Deciduitis percent (n)	11 (2)	31 (12)	0.0987
Villitis percent (n)	22 (4)	30 (12)	0.3908
Increased villi infarct percent (n)	11 (2)	23 (9)	0.2609
Intervillitis percent (n)	22 (4)	35 (14)	0.2559
Fibrin deposits percent (n)	83 (15)	76 (31)	0.4485
Increased syncytial knots percent (n)	17 (3)	20 (8)	0.5365
Malaria pigment deposits percent (n)	0	48 (19)	< **0.001**

Given the unexpectedly high frequency of malaria in our study, consideration of false-positive results is critical. In the data reported here, false positives are unlikely based on the inclusion of controls and rigorous criteria to call positives. (1) Each run included at least two negative controls. (2) Only samples that were positive in two independent reactions with different sets of primers and probes (genus and species) were considered positive. (3) Mixed and weak infections were confirmed using a third set of primers. (4) Samples with discordant results among the three different reactions were considered negative. Positive results were further confirmed by nested PCR for genotyping in 74% of samples. Given that the majority of infections had < 2 DNA copies/μL, detection by nested PCR was limited by the sensitivity of the assay and Poisson distribution of template in solution.

In addition to the high overall frequency of infection, more cases of *P. falciparum* (56%) than *P. vivax* (44%) were detected by qPCR in this study. These data contradict findings based on microscopy and nested PCR, where the majority of infections in pregnant women from Urabá were caused by *P. vivax*.[11,12,41,42] However, there are reports from the Brazilian and Peruvian Amazon that pregnant women are at higher risk of *P. falciparum* infection compared with the general population.[43–45] The Brazilian study showed that the ratio of *P. falciparum* to *P. vivax* changed in pregnant women from 1:5.6 to 1:2.3,[43] and in Peru, pregnant women were 2.3 times more likely to be infected with *P. falciparum* than non-pregnant women.[44]

Consistent with these findings, we detected the highest frequency of infection in the placenta, and the majority of infection was caused by *P. falciparum*. Furthermore, 24% of infected women had placental infection without peripheral infection, suggesting that placental sequestration of *P. falciparum* may be more common than previously reported.[12] This hypothesis is further supported by the high rates of villitis and intervillitis detected in infected women. Although we did not observe an association between placental infection and LBW, we found that even submicroscopic infections cause significant placental inflammation. It is, therefore, important to design expanded studies to investigate the clinical outcomes that may result from these infections in this transmission setting. Based on a systematic review, the frequency of maternal anemia and LBW was significantly lower in uninfected women compared with women with a submicroscopic infection.[16] Other reports also show increased risk of maternal anemia in submicroscopic infections compared with uninfected women[46,47]; however, those studies were carried out in Africa, where *P. falciparum* is dominant and the intensity of transmission is very high, unlike in Latin America.

We also observed that the levels of infection quantified by qPCR are significantly lower with *P. falciparum* than *P. vivax*. One hypothesis is that the strains of *P. falciparum* in Colombia are less virulent, and parasitemia is controlled by immune mechanisms, consistent with the fact that the women in our study are all asymptomatic. Alternatively, qPCR may be detecting gametocytes or free parasite DNA circulating in the blood rather than intact, pathogenic asexual parasites. This distinction is critical when considering sensitive methods such as qPCR as diagnostic tools to predict clinical malaria in pregnancy.

One of the limitations of this study is the small sample size. Nevertheless, the study was sufficiently powered to detect significant findings in the study population, notably a high frequency of submicroscopic malaria that is associated with placental injury at delivery. Although subsequent, larger studies are required to broaden the scope of these findings, the results of our pilot study warn that the magnitude of pregnancy-associated malaria in Colombia may be far greater than anticipated based on insensitive diagnostic tests. Importantly, our data prompt a re-examination of the local epidemiology of malaria in this lower transmission setting using molecular diagnostics to establish the clinical relevance of submicroscopic infections during pregnancy as well as their consequences for mothers and newborns.

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