MAJOR ARTICLE

How Hidden Can Malaria Be in Pregnant Women? Diagnosis by Microscopy, Placental Histology, Polymerase Chain Reaction and Detection of Histidine-Rich Protein 2 in Plasma

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Background. Accurate diagnosis of malaria infection during pregnancy remains challenging because of low parasite densities and placental sequestration of *Plasmodium falciparum*. The performance of different methods to detect *P. falciparum* in pregnancy and the clinical relevance of undetected infections were evaluated.

Methods. *P. falciparum* infections were assessed in 272 Mozambican women at delivery by microscopy, placental histology, quantitative polymerase chain reaction (qPCR) and detection of histidine-rich protein 2 (HRP2) in plasma by enzyme-linked immunosorbent assay (ELISA) and a rapid diagnostic test (RDT). Association between infection and delivery outcomes was determined.

Results. Among the 122 women qPCR-positive for *P. falciparum* in peripheral and/or placental blood samples, 87 (71.3%) did not receive a positive diagnosis by peripheral microscopy, 75 (61.5%) by HRP2 ELISA, and 74 (60.7%) by HRP2 RDT in plasma. Fifty-seven of the 98 qPCR-positive placental infections (58.2%) were not detected by histology. Women who were qPCR-positive but negative in their peripheral blood by microscopy or HRP2 RDT in plasma (n = 62) were at increased risk of anemia, compared with negative women (n = 141; odds ratio, 2.03; 95% confidence interval, 1.07–3.83; $P = .029$).

Conclusions. Microscopy, placental histology and HRP2-based plasma diagnostic methods fail to identify the majority of the *P. falciparum* infections detected by qPCR in peripheral and placental blood. Undetected infections were associated with maternal anemia, highlighting the urgent need for more accurate malaria diagnostic tools for pregnant women to avoid the negative clinical impact that hidden infections can have during pregnancy.

Clinical Trials Registration. NCT00209781.

A major and not sufficiently recognized contributor to the burden of infectious diseases in developing countries is the lack of access to high-quality diagnostic techniques [1] for effective clinical management and monitoring of control interventions. In particular, early diagnosis and treatment of *Plasmodium falciparum* infection are key to reducing malaria-related morbidity and mortality [2] and are a fundamental aspect in the control and eradication of this disease [3]. Microscopy has been the cornerstone of malaria diagnosis and is recommended where its quality can be maintained. However, rapid diagnostic tests (RDTs) detecting *Plasmodium*-specific antigens have emerged during recent years as an attractive alternative to microscopy that is able to extend diagnosis to remote and poorly resourced areas.

Pregnancy poses specific challenges for the diagnosis of malaria, because *P. falciparum* parasites may be present in the placenta but absent or undetectable in peripheral blood [4]. This phenomenon is attributable to

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the accumulation of infected erythrocytes in the intervillous spaces of the placenta through specific adhesion to chondroitin sulphate A [5]. Even infections with low parasite densities undetected by standard microscopy can have severe effects on the pregnant woman and her fetus [6–11]. Thus, prompt and accurate detection of *P. falciparum* infection is fundamental for the control of malaria in pregnancy. Histological examination of placental tissue collected at delivery [12] has been shown to be more sensitive than peripheral and placental blood film examination [13, 14] and, although not applicable for routine diagnosis, is currently considered to be the gold standard for diagnosis of placental malaria. However, placental infection before delivery can only be inferred by detecting infection in peripheral blood samples from women.

Although polymerase chain reaction (PCR) methodology has the potential to provide more sensitive estimates of maternal infection [4], it is not feasible in limited-resource settings for routine diagnosis. The quantification in plasma of the *P. falciparum* histidine-rich protein 2 (HRP2) [15] has been shown to allow the estimation of total body parasite biomass in children with acute *P. falciparum* malaria [16]. This suggests that RDTs detecting HRP2 may have the potential to identify placental infection in the absence of peripheral infection [17]. A recent meta-analysis showed a sensitivity of RDTs on both peripheral and placental blood of 81%, compared with placental and peripheral microscopy [13], whereas sensitivities ranged from 56% to 92% compared with PCR [18, 19]. Of importance, only 1 study has reported a sensitivity of 57% for a RDT in peripheral blood using placental histology combined with HRP2 immunohistochemistry as gold standard [20]. However, the sensitivity of histology against PCR has not been evaluated in African settings, and no study has compared the performance of RDT and PCR to detect histologically confirmed placental infections.

Despite the huge burden of malaria in pregnancy, tools to diagnose malaria in pregnant women, especially when hidden in the placenta, have not been evaluated in a systematic manner. This information is needed to guide patient treatment and for the evaluation of alternative preventive strategies, such as intermittent screening and treatment [21] and intermittent preventive treatment in pregnancy (IPTp) currently recommended by the World Health Organization, [22]. In the present study, we have compared the diagnostic performances among a *P. falciparum* HRP2-based enzyme-linked immunosorbent assay (ELISA), a HRP2 RDT in plasma, quantitative PCR (qPCR), microscopy, and histological placental examination for the diagnosis of malaria in pregnant women, and we have evaluated the association of the diagnostic results with maternal anemia and low birth weight.

METHODS

Study Population and Sample Selection

The present study was conducted retrospectively with samples collected at delivery from pregnant women participating in a placebo-controlled IPTp trial with sulfadoxine-pyrimethamine [23] that was performed at the Centro de Investigação em Saúde de Manhiça in southern Mozambique during 2003–2005 [24]. This area is characterized by perennial malaria transmission, with some seasonality mostly attributable to *P. falciparum* [25]. Thin and thick smears of maternal peripheral blood and placental impression smears were prepared from pregnant women at delivery. A tissue section was collected from the maternal side of the placenta and placed into 10% neutral buffered formalin. Fifty microliters of placental and peripheral blood were also spotted onto filter paper (Schleicher & Schuell 903TM). Newborn birth weight was measured on a digital scale, and the maternal hematocrit level was quantified in a microcapillary tube after centrifugation. Maternal venous blood samples were collected by venipuncture into EDTA Vacutainers, and plasma was stored at −70°C after blood centrifugation. Samples included in this study were obtained from 272 of the 1030 pregnant women participating in the IPTp trial. Inclusion criteria were availability of plasma samples, complete demographic information, and data of optical microscopy, qPCR, and placental histology [26].

In addition to these 272 women, 44 pregnant women not participating in the IPTp trial, with a positive HRP2 RDT result in placental blood were recruited at delivery. A placental plasma sample was frozen to allow the comparison of the performance of HRP2 RDT using blood and plasma.

Women with a positive blood smear result for malaria were prescribed antimalarial drug treatment according to national policies. The study protocol was approved by the national Mozambican ethics review committee and the Hospital Clínic of Barcelona ethics review committee.

Detection of *P. falciparum*

Thin and thick smears of maternal peripheral blood were Giemsa stained and examined for malaria parasites according to quality control procedures [27]. Slides were read by 2 independent microscopists and declared to be negative only after 2000 leukocytes had been counted. A third reading was performed if there was discrepancy in positivity between the 2 readings or the ratio of densities from the 2 readings was >1.5. Placental impression smears were stained with Giemsa and read in accordance with standard procedures [14]. Placental biopsy samples were processed for histological examination and stained as described elsewhere [28]. For quality control, 10% of the placental histologies were read in duplicate by independent pathologists. No differences were observed in

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terms of malaria parasites or pigment detection between both independent readings.

DNA was extracted from peripheral and placental blood samples collected onto filter paper with an ABI Prism 6700 automated nucleic acid work station (Applied Biosystems), and was resuspended in 200 µL of water, according to the manufacturer's instructions. Five microliters of DNA samples were screened in duplicate for *P. falciparum* DNA by qPCR targeting the 18S ribosomal RNA gene [9], and repeated a third time if the difference between duplicates was >0.5 cycle thresholds. Parasite densities (parasites/µL) were obtained by extrapolating cycle thresholds from a standard curve prepared with titrated samples containing known numbers of ring-infected erythrocytes diluted in whole blood. A sample with no DNA template was run in all reactions as negative control.

Five microliters of peripheral plasma samples were tested using the HRP2-based Malaria Antigen P.f. rapid diagnostic test from SD Bioline (Standard Diagnostics). The presence of HPR2 was also determined using the commercially available antigen-capture ELISA (Malaria Ag CELISA kit; Cellabs) [29] in 50 µL of peripheral plasma, defining a sample as positive if the absorbance value was above the negative control plus 0.1 units, as recommended by the manufacturer. Analysis was blinded to microscopic, histological, and qPCR results.

Definitions and Statistical Analysis

Pregnant women were classified as primigravidae if they were in their first pregnancy, or multigravidae if they reported having at least 1 previous pregnancy. Age was categorized as <23 or ≥23 years (median). Microscopic infection was defined as the presence of asexual parasites assessed by optical microscopy. Active placental infection was defined as the presence of infected erythrocytes in histological sections, either alone (acute infection) or in combination with hemozoin (chronic infection) [12]. Placentas showing hemozoin and no parasites were classified as past infections, and placentas negative for parasites and pigment were classified as uninfected. Maternal anemia was defined as an hematocrit <33%, and low birth weight as a birth weight <2500 g.

Statistical analysis was performed using Stata software, version 11.0 (StataCorp). Sensitivities and specificities of the different diagnostic methods were compared using McNemar test. Logistic regression models were used to estimate the association of infection at delivery with low birth weight and maternal anemia. *P* values <.05 were considered statistically significant.

RESULTS

Study Participants and Detection of Infection

Among the 272 women included in the study, median parity and age were 3 (interquartile range [IQR], 1–4) and 23 years (IQR, 19–28 years), respectively. One hundred thirty-nine women (51.1%) had received IPTp with sulfadoxine-pyrimethamine. The mean time of last dose receipt was 78.1 days before delivery (standard deviation [SD], 28.7).

Forty-two women (15.4%) were *P. falciparum* positive by microscopy in peripheral and/or placental blood, with 31 (73.8%) infected in both compartments, 4 (9.5%) only in the periphery, and 7 (16.7%) only in the placenta. Prevalence of qPCR-detected peripheral and placental infection was lower among women who received IPTp with sulfadoxine-pyrimethamine than among women who received placebo (Table 1).

Active placental infections were detected in 46 placentas (16.9%) by histology (30 chronic and 16 acute infections), past infections in 110 (40.4%), and 116 (42.6%) were negative for both pigment and parasites.

The subset of 272 women included in this study and the 1030 women participating in the IPTp randomized trial [23] were comparable in terms of IPTp received (*P* = .786), parity (*P* = .220), age (*P* = .533), peripheral infection by microscopy (*P* = .338), placental infection by histology (*P* = .283), and hematocrit level (*P* = .477).

By qPCR, 122 women were *P. falciparum* positive (44.8%), with 70 (57.4%) infected in both compartments, 24 (19.7%) only in the periphery, and 28 (22.9%) only in the placenta. Sensitivity of qPCR in peripheral blood to detect qPCR-positive placental infections was 71.4% (70 of 98; 95%

Table 1. Characteristics of Pregnant Women Participating in the Study, by Infection Detected by Quantitative Polymerase Chain Reaction of Peripheral and Placental Blood Samples

	qPCR-Detected Infection			
	Peripheral		Placental	
Characteristics	No. (%)	*P* Value	No. (%)	*P* Value
Parity				
PG (n = 78)	30 (38)	.401	30 (38)	.675
MG (n = 194)	64 (33)		68 (35)	
Age (y)				
<23 (n = 135)	50 (37)	.445	57 (42)	.043
≥23 (n = 137)	44 (32)		41 (30)	
IPTp				
Placebo (n = 133)	60 (45)	<.001	59 (44)	.006
SP (n = 139)	34 (24)		39 (28)	
Birth weight				
Normal (n = 243)	83 (34)	.684	85 (35)	.312
Low (n = 29)	11 (38)		13 (45)	
Maternal anemia				
No (n = 154)	43 (28)	.010	42 (27)	.001
Yes (n = 118)	51 (43)		56 (47)	

Abbreviations: IPTp, intermittent preventive treatment in pregnancy; MG, multigravidae; PG, primigravidae; qPCR, quantitative polymerase chain reaction; SP, sulfadoxine-pyrimethamine.

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confidence interval [CI], 62.5%–80.4%), with a specificity of 86.2% (150 of 174; 95% CI, 81.1%–91.3%).

P. falciparum HRP2 was detected in peripheral plasma samples from 49 of the 272 women (18.0%) by ELISA and in 51 (18.7%) by RDT. Both techniques were concordant in 264 of the 272 (97.1%) samples (218 negative and 46 positive results) with a κ score of 0.902 (SD, 0.061). The 8 discordant results corresponded to 3 negative samples by RDT but positive by ELISA and 5 positive samples by RDT but negative by ELISA.

Peripheral Blood Microscopy Versus qPCR to Detect Peripheral Malaria

Of the 94 women with peripheral infection detected by qPCR, 35 were positive by peripheral blood microscopy (sensitivity, 37.2%; 95% CI, 27.4%–47.0%). All the women with no parasites detected by peripheral qPCR were found to be negative by microscopy (100% specificity). Women negative by microscopy but qPCR positive in peripheral blood had lower qPCR-quantified parasite densities (median, 1 parasite/µL; IQR, 0.5–9 parasites/µL) than did women positive by microscopy and qPCR (2456 parasites/µL; IQR, 320–23,657 parasites/µL; $P < .001$).

Microscopy, HRP2 Detection, and Histology Versus qPCR to Detect Placental Malaria

Sensitivities and specificities of microscopy, HRP2 detection in plasma and histology, compared with qPCR, are detailed in Table 2. Placental impression smear and peripheral microscopy showed similar sensitivities (37.7% [95% CI, 28.1%–47.3%] and 35.7% [95% CI, 26.2%–45.2%]) but were lower than the sensitivity of HRP2 RDT (48.9%; 95% CI, 39.1%–58.9%; $P < .033$). Sensitivity of histology referred to qPCR was 41.8% (95% CI, 32.0%–51.6%) when infection was defined as the histological observation of parasites (active infection), and it increased to 72.4% (71 of 98; 95% CI, 63.6%–81.3%) when infection included both the presence of parasite and/or pigment (active or past infections). In contrast, specificity was high in the former, compared with the latter definition of placental infection by histology (97.1% [95% CI, 94.6%–99.6%] vs 48.9% [95% CI, 41.4%–56.3%], respectively).

qPCR-quantified placental parasite densities were lower in women qPCR positive but negative by placental histology (median, 1 parasite/µL; IQR, 0.5–27 parasites/µL), HRP2 RDT (1 parasite/µL; IQR, 0.5–5.3 parasites/µL), and HRP2 ELISA (1 parasite/µL; IQR, 0.5–6.2 parasites/µL), than in women qPCR positive with histologically detected active infections (278 parasites/µL; IQR, 15–9289 parasites/µL; $P < .001$), positive by HRP2 RDT (1730 parasites/µL; IQR, 99–25 229 parasites/µL; $P < .001$), or positive by HRP2 ELISA (1916 parasites/µL; IQR, 133–26 300 parasites/µL; $P < .001$).

Table 2. Performances of Microscopy, Histidine-Rich Protein2–Based Plasma Diagnostics and Placental Histology, Compared With Placental Quantitative Polymerase Chain Reaction

Test	Placental qPCR Negative (n = 174)	Placental qPCR Positive (n = 98)	Sensitivity (95% CI)	Specificity (95% CI)
Placenta				
Impression smear			37.7 (28.1–47.3)	99.4 (98.2–100)
Negative	173	61		
Positive	1	37		
Histology			41.8 (32.0–51.6)	97.1 (94.6–99.6)
Negative	169	57		
Positive[a]	5	41		
Peripheral blood				
Microscopy			35.7 (26.2–45.2)	100
Negative	174	63		
Positive	0	35		
HRP2 ELISA			47.9 (38.1–57.8)	98.8 (97.2–100)
Negative	172	51		
Positive	2	47		
HRP2 RDT			48.9 (39.1–58.9)	98.3 (96.4–100)
Negative	171	50		
Positive	3	48		

Abbreviations: CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; HRP2, histidine-rich protein 2; qPCR, quantitative polymerase chain reaction; RDT, rapid diagnostic test.

[a] Active infections.

If detection of *P. falciparum*–infected erythrocytes by placental histology (active infection) was considered as the reference standard (Supplementary Table 1A), placental impression smear had a sensitivity of 67.4% (95% CI, 53.8%–80.9%) and peripheral microscopy a sensitivity of 65.2% (95% CI, 51.5%–79.0%). Specificity was high for both techniques (96.9% [95% CI, 94.6%–99.2%] and 97.8% [95% CI, 95.9%–99.7%], respectively). Similarly, sensitivity of HRP2 ELISA was 76.1% (95% CI, 63.7%–88.4%) and 78.3% (95% CI, 66.3%–90.2%) for HRP2 RDTs (high specificities in both cases: 93.8% [95% CI, 90.7%–96.9%] and 93.4% [95% CI, 90.1%–96.6%]). Sensitivities decreased when detection of both parasite and pigment by placental histology was considered as the reference standard (Supplementary Table 1B).

Peripheral Blood Microscopy and HRP2 Detection in Plasma Versus qPCR to Detect Malaria in Pregnant Women

Of the 122 women qPCR positive for *P. falciparum* in peripheral and/or placental blood, 35 (28.7%; 95% CI, 20.7%–36.7%) received a positive diagnosis on the basis of peripheral blood microscopy, 47 (38.5%; 95% CI, 29.9%–47.2%) by HRP2 ELISA, and 48 (39.3%; 95% CI, 30.6%–48.0%) by HRP2 RDT on plasma (Table 3). Peripheral microscopy did not detect any of the 28 women with qPCR-confirmed placental

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Table 3. Performances of Histidine-Rich Protein 2–Based Plasma Diagnostics, Compared With Quantitative Polymerase Chain Reaction Detection of *Plasmodium falciparum* in Pregnant Women (in Placental and Peripheral Blood)

Test	Infection by qPCR[a] Negative (n = 150)	Positive (n = 122)	Sensitivity (95% CI)	Specificity (95% CI)
Peripheral blood microscopy				
Negative	150	87	28.7 (20.7–36.7)	100
Positive	0	35		
HRP2 ELISA				
Negative	148	75	38.5 (29.9–47.2)	98.7 (96.8–100)
Positive	2	47		
HRP2 RDT				
Negative	147	74	39.3 (30.6–48.0)	98.0 (95.8–100)
Positive	3	48		

Abbreviations: CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; HRP2, histidine-rich protein 2; qPCR, quantitative polymerase chain reaction; RDT, rapid diagnostic test.

[a] Placental and/or peripheral infection.

infection who were negative by qPCR on peripheral blood, and only 2 (7.1%) were detected by HRP2 ELISA and HRP2 RDT.

HRP2 RDT on Whole Blood Versus Frozen Plasma Samples

To compare the performance of RDT between plasma and whole blood samples, 44 frozen placental plasma samples from women who were HRP2 RDT positive in fresh placental blood were also tested with the HRP2 RDT. Among them, 43 (97.7%) were also RDT positive in placental plasma. The only discrepancy corresponded to an infection of very low parasitemia by qPCR (mean parasite load ± SD, 1.19 ± 0.20 parasites/μL).

Delivery Outcomes

The risk of anemia was increased among women with parasites detected by peripheral or placental blood microscopy, qPCR, HRP2 ELISA, or HRP2 RDT on plasma (Table 4). Women who were positive by qPCR in peripheral blood but negative by peripheral microscopy or RDT (n = 62) were at increased risk of being anemic, compared with women negative in placental and peripheral blood by all the techniques used in the study (n = 141; odds ratio [OR], 2.03; 95% CI, 1.07–3.83; $P = .029$, adjusted for age, parity, and intervention group). Similarly, women who were qPCR positive in the placenta but negative by histology and placental smear (n = 66) were at increased risk of anemia, compared with the fully negative women (n = 141; OR, 2.12; 95% CI, 1.10–4.08; $P = .025$, adjusted analysis).

DISCUSSION

This study reveals a marked underestimation of malaria infection in pregnant women when diagnosed by standard microscopy in peripheral and placental blood, compared with qPCR, as previously reported [9, 30]. Although detection of HRP2 on peripheral plasma by ELISA or RDT is more sensitive than peripheral blood microscopy, it still fails to identify 61% of the infections detected by qPCR, including 26 of the 28 women who were qPCR positive in the placenta but negative in their peripheral blood. Moreover, qPCR also shows a substantial number of women with parasites undetected by histology (58%). These results suggest that, similar to optical microscopy, placental histological examination also fails to detect a considerable proportion of *P. falciparum* infections, possibly because of a limitation of histology to differentiate between chronic, true past infections, and artefacts due to the accumulation of pigment other than hemozoin.

The present study shows that estimates of the sensitivity of HRP2-based tests performed in plasma depend on the reference standard used [31–33]. RDT and ELISA to detect HRP2 in peripheral plasma identified as positive 76%–78% of the women who had a histologically confirmed active placental infection, which is in agreement with previous studies [17, 18]. However, when qPCR of placental blood was set as gold standard, the sensitivity of HRP2 ELISA and RDT in plasma decreased to 48% and 49%, respectively. The limited sensitivity of HRP2 plasma diagnosis is consistent with the high prevalence of infection detected only by qPCR [26]. In agreement with this, parasite densities quantified by qPCR were found to be lower in women with infections detected only by qPCR, compared to women with infections detected both by qPCR and microscopy, HRP2 ELISA or RDT. Other factors, such as antigenic variation [34] or gene deletions of HRP2 [35], and the presence of blocking antibodies [36] may also contribute to the limited sensitivity of HRP2-detection tests. These results highlight the limitations of RDTs for clinical management during pregnancy and for the monitoring of new preventive strategies, such as intermittent screening and treatment [21].

The present study has limitations. First, the use of HRP2 diagnostics did not follow manufacturers' standards, because whole blood was not used for the tests. RDT was performed on archived plasma samples, which may have decreased its sensitivity. However, the high concordance between HRP2 ELISA and HRP2 RDT on plasma, together with the good agreement between RDT performed on placental blood and on the corresponding plasma, suggests the reliability of the estimates obtained in this study. Of importance, RDT uses a much lower amount of plasma than does ELISA (5 μL vs 50 μL). However, the degree of concordance between RDTs performed using peripheral blood and plasma remains to be determined. Second, viability of parasites cannot be confirmed by qPCR and HRP2 detection, in contrast to

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Table 4. **Risk Estimates for Low Birth Weight and Maternal Anemia According to Diagnostic Methods of *Plasmodium falciparum* in the Placenta and Peripheral Blood**

	Low Birth Weight			Anemia		
Test	OR	95% CI	*P* Value	OR	95% CI	*P* Value
Peripheral blood						
Microscopy						
Negative (n = 237)	1			1		
Positive (n = 35)	1.93	.72–5.12	.190	2.49	1.19–5.17	.015[a]
qPCR						
Negative (n = 178)	1			1		
Positive (n = 94)	1.18	.53–2.61	.686	1.96	1.18–3.26	.009[a]
HRP2 ELISA						
Negative (n = 223)	1			1		
Positive (n = 49)	1.88	.78–4.53	.161	2.42	1.28–4.57	.006[a]
HRP2 RDT						
Negative (n = 221)	1			1		
Positive (n = 51)	1.77	.74–4.26	.202	2.39	1.28–4.45	.006[a]
Placenta						
Histology						
Negative (n = 226)	1			1		
Positive[b] (n = 46)	2.06	.85–4.98	.111	2.63	1.36–5.06	.004[a]
Impression smear						
Negative (n = 234)	1			1		
Positive (n = 38)	1.72	.65–4.55	.274	2.55	1.26–5.19	.010[a]
qPCR						
Negative (n = 174)	1			1		
Positive (n = 98)	1.51	.69–3.29	.299	2.41	1.45–3.99	.001[a]

Abbreviations: CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; HRP2, histidine-rich protein 2; OR, odds ratio; qPCR, quantitative polymerase chain reaction; RDT, rapid diagnostic test.

[a] $P < .05$ after adjustment for parity, age group, and intervention.

[b] Active infections.

microscopy and histology, because of the potential presence of trace amounts of DNA and HRP2 even after life parasites have been cleared from blood. PCR techniques able to detect RNA, which is eliminated faster than DNA after the death of the parasite, may be an alternative for molecular detection of viable parasites. Regardless of this potential shortcoming, the clinical relevance of infections only detected by qPCR have been evidenced in previous studies showing that submicroscopic infections are associated with maternal anemia and low birth weight [7, 9–11, 18, 37]. In accordance with these previous reports, results of this study show that women with infections exclusively detected by qPCR have an increased risk of anemia, although such association was not found for low birth weight, possibly because of limitations in the sample size.

Overall, our results show that microscopy, placental histology, and HRP2-based plasma diagnostic methods failed to identify 60% of the malaria infections detected by qPCR on peripheral and/or placental blood samples from Mozambican pregnant women, including the majority (93%) of placental infections in the absence of peripheral infection. Moreover, our findings indicate that qPCR, although not feasible for routine diagnostics, may be useful as reference standard to evaluate new diagnostic methods, or alternatively, that statistical methods avoiding the use of a reference standard should be applied [38]. The identification of peripheral blood biomarkers for placental malaria infection when peripheral parasitemia is absent remains a priority for malaria control in one of the groups most vulnerable to the infection.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/cid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

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Notes

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