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Diagnosis of Gestational, Congenital, and Placental Malaria in Colombia: Comparison of the Efficacy of Microscopy, Nested Polymerase Chain Reaction, and Histopathology

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Abstract. The technical capability of different methods to diagnose *Plasmodium* in maternal peripheral blood, placenta, and umbilical cord blood has not been assessed in Colombia and seldom explored in other malaria-endemic regions. We designed a study to compare the technical and the operational-economical performances of light microscopy (LM), nested polymerase chain reaction (nPCR), and histopathology (HP). In maternal blood, LM had 41% sensitivity and 100% specificity and in placental blood, 35% and 100%, respectively, compared with nPCR. In placental tissue, LM had 33% sensitivity and 95% specificity; and nPCR 47% and 77%, respectively; compared with HP. Light microscopy had the best operational-economical qualification. We concluded that nPCR and HP performed better compared with LM, but field implementation of these two techniques remains a problem. Therefore, LM is recommended as the gold standard for diagnosis of gestational malaria and placental blood infection in the field.

INTRODUCTION

Malaria during pregnancy is a major health problem in most endemic countries, particularly in Africa. In Colombia, where both *Plasmodium falciparum* and *Plasmodium vivax* are highly endemic, official information on the prevalence of the infection among the pregnant population is absent. The National health authority recommends monitoring, by microscopy, of all pregnant women attending the antenatal clinic, regardless of the presence of malaria symptoms. However, in practice, this is rarely done. The current recommendation for treatment of malaria during pregnancy includes combined therapy with artemether plus lumefantrine for the second and third trimester, and quinine plus clyndamicine for the first trimester of pregnancy. No current recommendation was done concerning application of intermittent preventive therapy.

For many years, the diagnosis of *Plasmodium* infection has been based on the examination of peripheral blood by thick smear using light microscopy (LM). This is a very specific test that performs well when parasitemias are > 1,000 parasites/L.[1–3] However, in America, a high percentage of patients exhibit low parasitemias; for instance, in Colombia, mean reported values of *P. falciparum* were 6,000 parasites/μL[4] and 7.700 parasites/μL in *P. vivax* malaria.[5] In particular, the regions of Uraba and Bajo Cauca, 18% of *P. falciparum* malaria patients had < 1,000 asexual parasites/μL.[6] Microscopy of peripheral blood allows species discrimination and parasite load measurement, both essential for adequate treatment and follow-up of malaria patients.[1–3] The efficacy of microscopy is affected by several factors, such as the quality of sample preparation, reader's experience, and the level of parasitemia, among others.[7]

Other techniques used in the diagnosis of malarial infection include rapid diagnostic tests (RDTs). In 2009, the World Health Organization (WHO) concluded that "RDTs performance varied between lots, and widely between similar products."[8] Recent studies on the performance of RDTs based on *P. falciparum* histidine-rich protein II (pfHRPII), confirmed that this is affected by concentration in the blood of the protein.[9] Furthermore, existence of *P. falciparum* isolates lacking pfHRPII has been confirmed in Peru.[10]

At the upper limit of sensitivity, nucleic acid-based amplification tests, such as nested polymerase chain reaction (nPCR), have been widely used in major laboratories,[11] but with limitations for field use in endemic areas.[12] Efforts are being made to design nucleic-based tests suitable for field application in rural settings, including isothermal loop amplification (LAMP).[13–16] The LAMP reactions are easy to set up, and results can readily be assessed by detection of turbidity or more importantly, simply through the naked eye.[17]

Histopathology (HP) is also suitable for diagnosis of *Plasmodium* infection in placental tissue. This is the gold standard in such cases.[18] Histopathology can detect parasites, malarial pigment (hemozoin), or both, any of which can establish the diagnosis. This enables classification of the placental tissue into acute active infection (presence of parasites with pigment scarce or absent), chronic active infection (presence of parasites and pigment relatively abundant), or past infection (exclusive presence of pigment) depending on the inflammatory process.[19,20] Additional classifications of placental malaria include aspects such as the analysis of infiltrating inflammatory cells.[21]

A review of the performance of LM, RDTs, and PCR to diagnose maternal and placental *P. falciparum* infection concluded that, in eight African countries, the mean sensitivities of these methods to detect maternal infection were 21.2%, 29.2%, and 49.6%, respectively, and the ratio of infections detected by PCR versus microscopy was 3.3:1.[22] Performance of RDTs in areas with a high prevalence of *P. vivax* infection, such as America and Asia, is expected to be lower than in African settings.[23–28]

Comparison of the sensitivity of the different tests to detect placental infection by *P. falciparum* has been cumbersome. As reviewed by Uneke in 2008,[22] microscopy, RDTs, PCR, and HP detected, in African countries, an average 22.7%, 35.2%, 52.1%, and 50.3%, respectively. In two of these reports, HP had 38% sensitivity against 27% of microscopy, whereas five of the studies showed a gross sensitivity of 52% for PCR against 24% for microscopy. Four studies compared the sensitivity of microscopy against a HRP2-based RDT resulting in gross values of 23% of the former against 36% of the later. It is important to point out that, although microscopy and HP can provide a quantitative result based on the number of parasites

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per μL or 100 × field, conventional PCR gives a qualitative result (i.e., species detection but not parasites quantification) and only real time-based assays can provide information about the number of parasites. For microscopy, some authors set up a threshold at 50 parasites/μL[29] versus the estimated 1 parasite/μL for PCR, and 1 parasite/100× field. Both microscopy and PCR allow species discrimination, but microscopy also identifies different parasite stages.[29]

In America, there are no reports on the performance and operational-economic aspects of LM compared with PCR and HP to detect *Plasmodium* infection in the placenta, the mother's blood, or cord blood. Such studies should be based on the evaluation of the sensitivity and specificity as well as the predictive values (PV) and likelihood ratio of a positive test or negative test.[30,31] In addition, operational-economic evaluation (see below for definition of these terms) is essential to identify the optimal diagnostic approach in these settings.[32]

This report summarizes the results of a study carried out in parturient women of a malaria-endemic region of Colombia. We evaluated 1) the technical capacity of two tests (LM versus nPCR) for the diagnosis of plasmodial infection in the blood of three compartments: peripheral maternal, placental, and umbilical cord; 2) the technical capacity of three tests (LM, nPCR, HP) to diagnose plasmodial infection in placental tissue; and 3) we performed a comparison of the operational-economic (see Methods section for definition of the term) capacity of the tests.

METHODS

Study area. The study was carried out in three municipalities of the Uraba region of Colombia: Turbo (8°5′42″N, 76°44′123″W), Necocli (8°25′53.36″N, 76°47′5.90″W), and Carepa (7°45′34.02″N, 76°39′25.44″W). Epidemiologic characteristics of this highly endemic region have been described in detail elsewhere.[33–35] The mean annual parasite indexes (cases/per 1,000 inhabitants) during 2004–2007 were 41.32 in Turbo, 71.35 in Necocli, and 30.11 in Carepa (Local health authorities, personal communication). Throughout the Uraba region, *P. vivax* and *P. falciparum* are responsible for 65–70% and 30–35% of malaria cases, respectively.

Study design and sample size. A prospective, parallel (tests were applied simultaneously but in an independent manner), and closed (not open) (examiners were not aware of results obtained with other tests) study design for the evaluation of a diagnostic test was applied.[36]

In a previous study of 2,117 pregnant women in the Uraba region, the frequency of gestational malaria was 10% by microscopy.[35] A sample size $N = 79$ was calculated based on $n = N\,Z2\,p\,(1\text{-}p)/(N\,e2) + Z2\,p\,(1\text{-}p)$,[37] where $N = 2{,}117$ pregnant women, $Z = 1.96$ units for a 95% confidence level, p = prevalence of gestational malaria 10%, sampling error = 0.065. Therefore, 79 blood samples of each: mother, placenta, and cord, and 79 placental tissue samples were required.

All samples, blood and tissue, were collected from parturient women attending their local hospital, regardless of the presence of acute febrile syndrome compatible with malaria. Subjects were recruited in a sequential fashion until the sample size was reached.

Inclusion criteria for the study and malaria term definition. The inclusion criteria for this study were permanent residency in the endemic area, absence of serious general disease, delivery at one of the local hospitals, and signature of the informed consent form. The only exclusion criterion was consent withdrawal. The study protocol was reviewed and approved by the Ethics Committee of the Sede de Investigacion Universitaria, Universidad de Antioquia (Medellín, Colombia, Act 07-32-126).

Gestational malaria was defined as any parturient women with a peripheral blood test positive for *Plasmodium*, regardless of the presence of symptoms of malaria. Similarly, as congenital malaria was considered the neonate with an umbilical cord blood test positive for *Plasmodium*, also regardless of the presence of symptoms, it should be noticed that, for this study, neonates were not followed up afterward. Finally, placental malaria was defined as any test positive for *Plasmodium*, either from blood or tissue, and regardless of the presence of symptoms in the mother or the neonate.

Sample preparation. Whole blood samples were obtained at delivery from mother's peripheral blood, cord blood, and placenta. The mother's sample was obtained by finger prick and cord's sample was obtained after cleaning the cord with saline 0.9% and sectioning a fragment to expose a fresh segment of the cord. Placental blood was obtained from the lake formed after a wash with saline (0.9%) and removal of a 3 cm × 3 cm × 3 cm section in the area of the cord's insertion on the maternal side of the organ. These samples were used to perform thick and thin smear examination and nPCR as detailed later. In addition, placental tissue samples were collected immediately after delivery. A total of five fragments 1 cm × 2 cm and 3-cm depth were collected from the maternal side of the placenta (one from cord insertion and four from periphery). Tissue was preserved in 10% buffered formaldehyde and processed within the following 48 hours.

Microsocopy. Giemsa-Field stained thick/thin blood films were examined with a 100× objective to identify presence of parasites, *Plasmodium* species, parasite density, and schizontaemia. Parasite density was measured by counting the number of asexual parasites per 200 leukocytes, based on a mean count of 8,000 leukocytes per microliter of blood (theoretical value). A slide was considered negative after examination of at least 300 microscopic fields.[1] In placental tissue, presence of parasites was assessed in hematoxylin-eosin (H-E) stained sections per 100× field.

Nested PCR. Whole blood collected onto Whatman Grade 3 filter paper was stored at –20°C until DNA extraction with Chelex®100 (Sigma). Amplification was carried out using a nested PCR assay to detect the 18s ribosomal RNA (rRNA) gene of *P. falciparum* and *P. vivax*, according to previously published procedures.[11] Positive reaction controls consisted of DNA from the laboratory-adapted strain *P. falciparum* 3D7 and from a well-characterized field isolate of *P. vivax*. Amplification products were resolved in a 2% agarose gel using ethidium bromide and visualized under UV light. Samples were processed once as long as controls were confirmed to operate in optimal conditions, otherwise, the assay was repeated until successful performance of controls.

Histopathology. All five samples of placental tissue were analyzed. A negative result was defined after evaluation of the total number of samples collected from each placenta. Fixed placental tissue was embedded in paraffin following standard procedures and 5-μm sections were stained using H-E. Slides were read by a medical pathologist who was aware of the clinical history of each patient but blind to the results of the

other malaria diagnostic tests. The placental histopathology classification had four categories: 1) acute infection, 2) chronic infection, 3) past infection, and 4) uninfected.[19,20]

Operational and costs assessment. Information on several aspects concerning this evaluation was recorded on a precoded form. Data were gathered after interviewing experts in this field (Delphi method). The aspects evaluated to perform the tests were 1) personnel related: degree of formal education required, level of specific training needed, degree of experience of the technician, and personnel cost; 2) sample collection and processing: supplies and reagents needed to collect, storage requirement, and process the sample; 3) type and cost of reagents: availability from local suppliers, need to import supplies or reagents, transport requirements, and reagents storage (cold chain, ambient condition, or any other particular), cost; 4) type and cost of the equipment: local distribution, need to import, technical assistance, local distribution of spare parts, and local availability of technical support; 5) time to produce a result, cost-effectiveness when applied individually, automation possibility, feasibility of application in field conditions in malaria endemic areas, final production cost of the test, final sale cost of the test, etc.

The Delphi method (or method of consensus among experts) is a structuring procedure applied to group communication processes addressing complex problems. This is based on individual interviews to experts using sequential questionnaires to evidence opinion convergence and elucidate consensus. These sequential questionnaires are applied individually and supported on the results of a previous round of results.[38]

Statistical analysis. Point values and confidence intervals of 95% (CI95%) as indicators of the diagnostic capacity were calculated using EpiInfo 6.04 (CDC, Atlanta, GA): sensitivity, specificity, positive predictive value, and negative predictive value. Likelihood ratio of a positive test or negative test was calculated manually. Kappa coefficients (K) were obtained using Epidat 3.1 to assess concordance.

The Delphi method of consensus among experts was used to analyze economic and operational aspects. Two readings were done for each test: 1) a descriptive qualification was carried out in which simple/low complexity was applied to the easiest, cheapest, and least complex, and very high/very complex to indicate the more difficult, costly, and demanding test; high/complex was used to indicate an intermediate situation; 2) a points rating included a scale ranging from simple/low = 1 point, complex/high = 2 points, and very complex/very high = 3 points. For feasibility of application in the field, automation and immediate individual application, a score of two was assigned to negative answers and zero points to positive ones. The variable "time of test completion" was qualified according to the number of hours required to produce a result, i.e., 1 point for a 1 hour test, 24 points for a 24 hours test, and 48 points if the result was produced > 24 hours. This system of qualification allowed an approximate qualification of the diagnostics test to be obtained in which the one with the lowest score can be ranked as the "best" and the highest as the "worst."

RESULTS

Mean age of the parturient mothers was 23.1 ± 5.2 years; the mean number of previous pregnancies was 1.9 and 32% were primiparous. Among the subjects aged < 25 years, 44% were in their first pregnancy.

Frequency of *Plasmodium* infection according to diagnostic test. Frequencies of maternal blood infection were 13% by LM and 32% by nPCR (p[X2] = 0.003171), in placental blood 9% with LM and 26% with nPCR (p[X2] = 0.002851), and in cord's blood 2% by LM and 13% by nPCR (p[X2] = 0.009357). When samples were positive for *Plasmodium* infection, a predominance of *P. vivax* infection was detected in all compartments evaluated (mother, placenta, and cord's blood), both by microscopy and by nPCR (Table 1); frequency of detection of the later species ranged from 65% to 100% according to the source of the sample and the test applied. Therefore, the results herein presented mainly reflect data on *P. vivax*.

The main difference between parasitemia by nPCR and by LM is the so-called "submicroscopic parasitemia," which in maternal blood was 19% (32% by nPCR minus 13% by LM), in cord blood was 11% (13% minus 2%), and in placental blood was 17% (27% minus 10%), in our study this was not significant (p[X2] = 0.306328).

Coincidence of infection according to nPCR. This analysis was based on the comparison of the results of nPCR, as this exhibited the highest sensitivity. Infections in maternal blood were significantly associated with placental blood infection in 20% (17/84) of the samples ($P < 0.0001$) (Table 2); in 4% (3/84) both mother and cord's blood had infection ($P = 0.980$); and in 5% (4/84) infection of both placenta and cord's blood was detected ($P = 0.723$). A significant relationship could only be established between mother and placental infection, whereas maternal or placental blood infection might not necessarily result in cord blood infection.

TABLE 1
Frequency of *Plasmodium* spp. blood infection according to diagnostic test and compartment*

	Light microscopy			Nested PCR		
	Mother	Placenta	Cord	Mother	Placenta	Cord
P. vivax	9	6	2	20	15	8
P. falciparum	2	2	0	7	7	3
Both (mixed)	0	0	0	0	1	0
Negative	73	76	82	57	61	73
Total	84	84	84	84	84	84
	p[X2]† < 0.142 excluding mixed infections			p[X2] < 0.059 excluding mixed infections		
	mother 13% (11/84) LM vs. 32% (27/84) nPCR; (p[X2]. < 0.004)					
	placenta 9% (8/84) LM vs. 26% (22/84) nPCR; (p[X2]. < 0.003)					
	umbilical cord 2% (2/84) LM y 13% (11/84) nPCR; (p[X2]. = < 0.010)					

* PCR = polymerase chain reaction; nPCR = nested polymerase chain reaction.
† p(X2) = probability for χ^2 test.

TABLE 2

Coincidence of infection in mother, placenta, and cord's blood, according to nested polymerase chain reaction (nPCR)

	P. vivax	*P. falciparum*	Both (mixed)	Negative	Total	Summary (n = 84)	
Mother			Placenta				
P. vivax	11	0	1	8	20		
P. falciparum	0	5	0	2	7	(+)	(−)
Both (mixed)	0	0	0	0	0 (+)	17	10
Negative	4	2	0	51	57 (−)	6	51
Total	15	7	1	61	84	23	61
						p(X2)* < 0.00	
Mother			Cord				
P. vivax	2	0	0	18	20		
P. falciparum	0	1	0	6	7	(+)	(−)
Both (mixed)	0	0	0	0	0 (+)	3	24
Negative	6	2	0	49	57 (−)	8	49
Total	8	3	0	73	84	11	73
						p(X2)* < 0.99	
Placenta			Cord				
P. vivax	1	1	0	13	15		
P. falciparum	1	1	0	5	7	(+)	(−)
Both (mixed)	0	0	0	1	1 (+)	4	19
Negative	6	1	0	54	61 (−)	7	54
Total	8	3	0	73	84	11	73
						p(X2)* < 0.73	

* Probability for χ^2 test.

Exclusive analysis for *P. vivax* infection confirmed 20 cases in maternal blood and 55% (11/20) of them were associated with placental blood infection. On the other hand, placental blood infection was detected in 15 cases and the frequency of associated maternal blood infection was 73% (11/15). A total of 51 mother and placenta paired samples were negative for the presence of *Plasmodium* ($P < 0.0001$).

Diagnostic capability of microscopy versus nPCR for detection of *Plasmodium* spp. in blood. In mother's peripheral blood, the comparison of the distribution of frequencies obtained by LM against nPCR for the diagnosis of infection, was statistically significant ($P < 0.00001$); in placental blood this comparison was also significant ($P < 0.0001$). However, in cord blood significance could not be established because of the reduced frequency of infection in these samples.

In all types of blood samples (maternal, cord, and placenta), microscopy exhibited high specificity but very low sensitivity (Table 3). The positive predictive value (PPV) was 100% and the positive likelihood ratio (PLR) was high (> 35%). For *P. vivax* infection, microscopy had a sensitivity of 33%, specificity of 99%, PPV of 83%, and negative predictive value (NPV) of 82%, PLR of 1.1%, and 1.5% negative likelihood ratio (NLR).

Statistical measures were not applied to *P. falciparum* and mixed infections caused by the reduced number of cases.

For *Plasmodium* infections in placenta, nPCR detected 18% (15/84) and microscopy detected 7% (6/84), giving a positivity ratio of nPCR/microscopy of 2.5. Out of the 15 *P. vivax* positive infections diagnosed by nPCR, microscopy missed 10 (67%) and in 7 cases of *P. falciparum* infection diagnosed by nPCR, microscopy missed 5 (71%). All 61 negative samples by nPCR were also classified as negative by microscopy. In six cases, microscopy indicated a *P. vivax* positive result, whereas nPCR detected a mixed infection. The two cases with unique infection by *P. falciparum* were detected by both tests.

Diagnostic capability of microscopy and nPCR versus histopathology for detection of infection in placental tissue. Diagnosis by HP was based on the finding of pigment, parasites, or both. Meanwhile, diagnosis by microscopy or nPCR was based on the exclusive detection of parasites.

Comparison of the distribution of frequencies obtained by LM and nPCR, against HP for the diagnosis of placental infection confirmed significant differences between LM and HP ($P < 0.0046$.), but not between nPCR and HP ($P < 0.14$).

Sensitivities for diagnosis of placental infection by microscopy or nPCR compared with HP were very low: 33% and 47%, respectively. In addition, microscopy showed higher specificity (95%) compared with nPCR (77%). Both tests exhibited high NPV (86% each), but their PPV was low (Table 4).

Further analysis according to the type of lesion observed by HP confirmed infection of the placenta in 19% (15/79) of specimens, of which 33% were acute, 7% were chronic, and 60% had past infection (Table 5). Complete concordance was observed between the tests in cases of acute or chronic malaria infection and this dropped to 89% in cases of past infection. The HP diagnosed the absence of placental infection in 64 placentas, whereas nPCR confirmed the presence of parasites in 23% (15/64) of them. In 18% of the 15 HP positive cases, microscopy was positive for *Plasmodium*.

Comparison of the operational and economic aspects of the tests. In field conditions in malaria-endemic areas of Colombia, microscopy performed exceedingly well in several

TABLE 3

Comparison of the diagnostic capability of microscopy (LM) versus nested polymerase chain reaction (nPCR) (as gold standard) according to compartment (maternal blood, cord blood, and placental blood)

		nPCR						
		Positive	Negative	Total	Percentages*/values†			
A. Maternal blood					S	E	PPV	NPV
Microscopy	Positive	11	0	11	41	100	100	78
	Negative	16	57	73	PLR	NLR	K	
	Total	27	57	84	> 41	1.6	0.4827	
B. Cord blood					S	E	PPV	NPV
Microscopy	Positive	0	2	2	0	97	0	87
	Negative	11	71	83	PLR	NLR	K	
	Total	11	73	84	0	1.03	–0.042	
C. Placental blood					S	E	PPV	NPV
Microscopy	Positive	8	0	8	35	100	100	80
	Negative	15	61	76	PLR	NLR	K	
	Total	23	61	84	> 35	1.5	0.4365	

* S = sensitivity; E = specificity; PPV = positive predictive value; NPV = negative predictive value; PLR = positive likelihood ratio; NLR = negative likelihood ratio; K = Kappa coefficient.
† For PLR and NLR.

TABLE 4

Comparison of the diagnostic capability of microscopy (LM) and nested polymerase chain reaction (nPCR) versus histopathology (HP) in *Plasmodium* infection in placental tissue

		HP						
		Positive	Negative	Total	Percentages*/values†			
A. LM vs. histopathology					S	E	PPV	NPV
LM	Positive	5	3	8	33	95	62	86
	Negative	10	61	71	PLR	NLR	K	
	Total	15	64	79	6.6	1.4	0.3488	
B. nPCR vs. histopathology					S	E	PPV	NPV
nPCR	Positive	7	15	22	47	77	32	86
	Negative	8	49	57	PLR	NLR	K	
	Total	15	64	79	1.4	1.5	0.1971	

* S = sensitivity; E = specificity; PPV = positive predictive value; NPV = negative predictive value; PLR = positive likelihood ratio; NLR = negative likelihood ratio; K = Kappa coefficient.
† For PLR and NLR.

TABLE 5
Sub-classification of histopathology results according to Bulmer,[19,20] compared with nPCR

		Histopathology: type of placental malaria				
		Acute	Chronic	Past	Uninfected	Total
		Parasites and scarce or absent pigment	Parasites and relatively abundant pigment	exclusive presence of pigment	No parasites or pigment	
nPCR	*Plasmodium* positive	5	1	1	15	22
	Plasmodium negative	0	0	8	49	57
	Total	5	1	9	64	79

aspects as summarized in Table 6. A major difference between nPCR and HP is based on the lengthy period of the latter to produce a result.

DISCUSSION

Frequencies of maternal/gestational, congenital, and placental malaria observed in this work were high and confirmed the serious public health problem in Colombia. Microscopy detected maternal/gestational malaria in 13% of women from the Uraba región, but if nPCR is applied, this proportion rises to 32%. All data supports previous reports in the same population.[35]

If, as suggested by this study, one out of three parturient women is infected with *Plasmodium*, urgent actions to tackle this problem are required. In Colombia, the sanitary law addresses the diagnosis of malaria during pregnancy by recommending examination of blood by microscopy at each antenatal check out. However, in practice, this is rarely accomplished. An early and precise diagnosis of malaria ensures adequate treatment and reduction of morbidity and mortality, and this is also essential to prevent the emergence of drug resistance as a consequence of irrational use of antimalarials.[39]

The frequency of submicroscopic parasitemia was high in all compartments (19% mother, 11% umbilical cord, 17% placenta). Submicroscopic parasitemia corresponds to 5–20 parasites/μL or 0.001–0.004%.[29] Submicroscopic infections by *P. falciparum* during pregnancy are very common and they might be important in developing immunity against gestational malaria besides causing adverse effects to the mother (such as anemia) and the fetus (such as low birth weight).[40,41] Moreover, in areas of high genetic diversity of *P. falciparum*, infected individuals might exhibit polyclonal infections with a relatively high frequency.[40–43] The relationship between these polyclonal infections and clinical disease is complex and depends on factors such as age, transmission intensity and prior immunity.[44]

Submicroscopic parasitemia was very common in this study and, because this negatively affects birth outcome and maternal anemia,[45] is an urgent issue that should be addressed by the health authorities of the region with measures such as intermittent preventive treatment,

The frequency of umbilical cord blood infections detected by nPCR was higher than by LM (13% versus 2%, respectively). However, the frequency diagnosed by LM was similar to previous studies in the region where a 7-day follow-up period was included (2.7%).[35] This finding should encourage health authorities to establish a particular program of surveillance of congenital malaria in endemic regions to make opportune diagnosis of this condition.

Some very relevant findings of this study were the high frequency of concomitant infection of mother and placenta (20% by nPCR) and when the maternal blood was infected, 63% of their placentas were also infected. Likewise, when the placentas had parasites, 74% of the mothers were also infected. Further research should investigate this relationship and the hypothesis that surveillance of maternal blood may be a good reflection of placental infection, because access to placental tissue before delivery is practically impossible. On the other hand, concomitant infection of mother and cord blood, or placenta and cord blood was relatively low, suggesting a low neonatal risk compared with placenta and mother. Of major interest to us is the finding that the relationships previously described and commented in the general context of *Plasmodium* spp. infection, are also generally valid for *P. vivax*. Gestational and placental infections by *P. vivax* have been rarely studied, not only worldwide, but also in America and Southeast Asia where this species is dominant.

TABLE 6
Description of operational and economic aspects of the diagnostic tests in field conditions in malaria endemic areas*†

		Description			Score		
	Feature	Microscopy	nPCR	Histopathology	Microscopy	nPCR	Histopathology
	Infrastructure	Simple	Complex	Complex	1	2	2
	Equipment cost	Low	High	Very high	1	2	3
Personnel	Training	Simple	Complex	Very complex	1	2	3
	Experience	Very high	High	Very high	3	1	2
	Cost	Low	High	Very high	1	2	3
	Cost per test (US$)	1.5	2.5	7.5	1	2	5
	Sample preparation	Simple	Complex	Complex	1	2	2
	Time to a result	1 hour	24 hours	> 24 hours	1	24	48
	Automation	No	Yes	No	2	0	2
	Field application	Yes	No	No	0	2	2
	Single sample testing	Yes	No	Yes	0	2	0
Total Score					12	41	72

*See Methods section (Operational and costs assessment) for term definition.
†The lowest score allows ranking as the "best" and the highest as the "worst" test.

The first objective of this study was the evaluation of the diagnostic capability of LM against nPCR, to diagnose *Plasmodium* infection in blood. This allowed confirmation that LM had very high specificity in the three compartments but sensitivity, in all cases, was below 50%. Most malaria-endemic countries lack financial and operational capacity to carry PCR-based tests. In any case, introduction of a PCR-based method in daily laboratory practice is still uncommon, especially in rural-endemic regions. In addition, as recently reviewed by Ndao,[46] these PCR-based methods suffer by the lack of standardization of DNA extraction, choice of primer sets, and use of a unified amplification protocol.

The second objective of this study was the evaluation of the diagnostic capability of LM and nPCR against HP to diagnose *Plasmodium* infection in placental tissue. Although specificities of LM and nPCR were high (77–95%), sensitivities were low (< 50%).

Taking into account the economic and operational requirements of HP, this test may only be used in a few and very specific cases, but not for public health studies and epidemiology studies, in which mass diagnosis is usually required. A further setback of HP is that only around 40% (33% acute and 7% chronic infection) of the cases diagnosed with this technique are susceptible to intervention by antimalarial treatment.

As stated before, considering the economic and operational conditions, LM was superior in specificity, but not in sensitivity, than nPCR for the diagnosis of *Plasmodium* infection in this region of the country. Therefore, LM diagnosis should remain as the main test for routine exploration of malaria infection in large groups, including pregnant women. There is an urgent need for research on new viable tests, at a reasonable cost and of easy application, in which sensibility to diagnose each species, or at least *P. vivax* and *P. falciparum*, affecting humans is not jeopardized. Until such a test is available, the most important recommendation in malaria-endemic areas of Colombia is the monthly examination by thick smear to every pregnant woman to establish early diagnosis and specific treatment.

Several biases can be implicated in the evaluation of a diagnostic test, including the selection of patients, independence between the test and the gold standard, and closed (blinded) interpretation of the results. We controlled such biases by inclusion of subjects from the general population of pregnant women resident in the municipalities delivering at the local hospital, and exclusion of those with low frequency diseases or those modifying the clinical picture of malaria. This allowed us to study a consecutive sample of the general population of pregnant women with the highest similarity to the reference population.[47] To guarantee independence between the gold standard and the tests applied, a parallel study design was applied. Finally, closed design was guarantee because the tests were applied by different researchers.

CONCLUSIONS

The nPCR and HP had better technical performance but major operational and financial disadvantages than LM. Therefore, we recommend the use of LM in malaria-endemic areas for exploration of pregnancy-associated malaria. The LM must be performed in optimal conditions to guarantee adequate performance.

The high prevalence of *Plasmodium* infection blood in mother, umbilical cord, or placenta, confirmed by any of the methods here applied, confirms the serious public health problem in Colombia.

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