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Real-Time Quantitative PCR for Determining the Burden of *Plasmodium falciparum* Parasites during Pregnancy and Infancy

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Real-time quantitative PCR (RTQ-PCR) provides a quick, accurate, and reproducible quantification of parasites. However, the value of RTQ-PCR for predicting clinical outcomes of malaria is unknown. Here, we compared RTQ-PCR to microscopy of blood smears, nested PCR (nPCR), and parasite circulating-antigen (CAg) assays for detection of *Plasmodium falciparum* in pregnant Kenyan women and their infants and related these findings to parity and birth weights in their newborns ($n = 554$). nPCR was the most sensitive assay for detection of malaria in pregnancy, followed in decreasing order of sensitivity by RTQ-PCR, CAg assays, and blood smears. RTQ-PCR detected a higher frequency of malaria infection (46%) in maternal peripheral blood in primiparous than in multiparous women (35%; $P < 0.001$), with a >12-fold difference in parasite burden (geometric mean = 25,870 versus 2,143 amplicons/µl blood; $P < 0.0001$). Similarly, the presence of placental malaria determined by RTQ-PCR was approximately twofold higher in primiparous versus multiparous women (21% versus 13%; $P < 0.01$). The presence and intensity of malaria infection in pregnant women estimated by RTQ-PCR strongly correlated with low-birth-weight babies, especially in those with high amplicon numbers. RTQ-PCR identified malaria-infected women, missed by blood smear, who were at risk for having underweight offspring. By contrast, malaria detected by nPCR and CAg assay showed a much weaker association with parity or low birth weight. Thus, RTQ-PCR provides an estimate of parasite burden that is more sensitive than blood smear and is predictive of clinical outcomes of malaria infection in pregnant women and newborns.

Accurate, sensitive, and specific measures of malaria infection are the cornerstones for understanding malaria epidemiology. Malaria diagnosis has relied on the microscopic detection of *Plasmodium* on Giemsa-stained blood smears (BS) until recently, when circulating-antigen (CAg)- and PCR-based diagnostic methods were introduced. PCR-based assays have surpassed blood smears for sensitivity and specificity for detecting malaria but lack the ability to quantify the infection intensity (19). Nested PCR (nPCR) assays also require multiple steps that introduce the possibility for contamination. Assays that detect circulating antigens are rapid and do not require trained microscopists, yet they lack sensitivity, specificity, and the ability to quantify the intensity of malaria infection afforded by blood smear analysis (19).

Real-time quantitative (RTQ) PCR has the potential to overcome these limitations by providing a rapid, simple, sensitive, and quantitative option (8, 11). These assays use DNA binding dyes, such as SYBR green, or molecular probes labeled with fluorescent markers. By measuring the emitted signal, amplicons are quantified after each PCR cycle. Quantifiable results can be generated in less than 3 h on multiple samples simultaneously. No further handling of the samples is required once they are added to the reaction mixture, thereby reducing the possibility for contamination. Several RTQ-PCR assays have been developed in various laboratories for both human and murine malaria (1, 4, 5, 11, 23), and recently, Artus-Biotech has begun to market a standardized ready-to-use RTQ-PCR for all human malaria parasite species (8).

Initial studies suggest RTQ-PCR is sensitive and specific, and the amplicon number is correlated with parasitemia levels determined by blood smear analysis in either experimentally infected nonimmune individuals (10, 11), infected travelers (8), or patients presenting with clinical malaria to health centers in Africa (23). There are several factors that may limit the ability to directly correlate the amplicon number determined by RTQ-PCR with the number of parasites estimated by blood smear. First, the gene encoding the small-subunit rRNA (ssrRNA), which is amplified by RTQ-PCR, is a multicopy gene (13). Second, some individual erythrocytes will have multiple infections, and multinucleate schizonts will occasionally be present in peripheral venous blood and frequently in the placental intervillous blood (IVB). Third, it is possible that parasite DNA is released into the plasma by sequestered parasites. Thus, RTQ-PCR may provide a different overall estimate of the parasite burden compared to blood smears. RTQ-PCR has another potential advantage for quantifying malaria parasites compared to blood smear analysis because it does not rely on

TABLE 1. Frequencies of malaria infection in pregnant women and their newborns estimated by different assays

Sample	Frequency (% positive)[a]				
	BS	HRP-2	pLDH	Nested PCR	RTQ-PCR
Maternal infection	54/543 (9.9)	51/265 (19.2)	59/180 (32.8)	332/552 (60.1)	231/554 (41.7)
PB	49/542 (9.4)	18/248 (7.3)	49/167 (29.3)	301/523 (57.6)	200/528 (37.9)
IVB	9/526 (1.7)	38/202 (18.8)	23/116 (19.8)	188/451 (41.7)	80/460 (17.4)
Infant peripheral blood (ages 6–24 mo)	207/1,071 (19.3)	181/491 (36.8)	ND	581/965 (60.2)	458/1,021 (44.8)

[a] The numerator is the number of samples positive for the indicated assay; the denominator is the total number of samples tested. ND, not done.

the varying skills of microscopists. The present study aimed to determine whether RTQ-PCR can better predict clinical outcomes of malaria than other assays with respect to the impact of parity on the burden of malaria in pregnant women and the birth weights of their newborns.

MATERIALS AND METHODS

Study population. Healthy pregnant women residing in Kwale District, Coast Province, Kenya, where malaria is holoendemic, were recruited from the antenatal clinic at Msambweni District Hospital as previously described (12, 26). Within 24 h of delivery, maternal peripheral venous blood was collected in heparin. At delivery, intervillous placental blood was obtained by identifying the maternal side of the placenta, wiping away excess blood, careful cannulation of the intervillous space with a sterile 18-gauge needle, and removal of ~1 ml of free-flowing blood into a heparinized syringe. The offspring of these women, along with additional newborns, were examined for the presence of malaria infection in peripheral venous blood that was collected at 6-month intervals from birth to age 2 years. Thin and thick blood smears were made from the freshly collected whole blood. An aliquot of whole blood was frozen for subsequent measurement of *Plasmodium falciparum* antigens. DNA was extracted from 200 μl of whole blood collected from the placenta and from 200 μl of the red cell pellet following Ficoll-Hypaque centrifugation of peripheral blood (PB) using individual spin kits (QIAGEN, Valencia, CA) as previously described (12). Neonates were weighed using a triple-beam balance (±10 g). Informed consent was obtained from all study participants. Ethical approval for the study and the procedure for informed consent were obtained from the Human Investigations Institutional Review Boards of University Hospitals of Cleveland and the Kenya Medical Research Institute.

Determination of malaria infection status. For blood smear diagnosis, thick and thin films were stained with 4% Giemsa stain and subsequently examined for asexual *P. falciparum* in 100 oil immersion fields. Blood smears were defined as negative if no parasites were observed in ×200 oil immersion fields. Circulating antigen detection assays for *P. falciparum* histidine-rich protein 2 (HRP-2) was quantified by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Cellabs, Pty Ltd., Brookvale, NSW, Australia) on a randomly selected subset of samples for which blood smears were performed. This corresponded to approximately one-half of the total samples for HRP-2 and one-third for *P. falciparum*-specific lactate dehydrogenase (pLDH). None of the infant samples were tested for pLDH. Occasionally, we were unable to successfully collect intervillous blood samples from the placenta due to difficulties in delivery of the placenta. pLDH was measured using the OptiMAL kit (FlowLabs, Portland, OR) following the manufacturer's instructions. Nested PCR for the gene encoding *P. falciparum* ssrRNA was performed as described previously (12, 17) using a Peltier Thermal Cycler, PTC-100 (MJ Research, Watertown, MA). RTQ-PCR also amplified the gene encoding *P. falciparum* ssrRNA with different primers and probes as follows: forward primer, 5′-GTA ATT GGA ATG ATA GGA ATT TAC AAG GT-3′; reverse primer, 5′-TCA ACT ACG AAC GTT TTA ACT GCA AC-3′; turbo TaqMan probe, TGC CAG CAG CCG CGG TAA TTC as described by Hermsen et al. (11). The probe was labeled with 6-carboxy-fluorescein as a reporter and 6-carboxy-tetramethylrhodamine as a quencher. The sequences of the primers and probe are 100% homologous with the sequences of *P. falciparum* parasites 7G8 and 3D7, and the primers and probe did not cross-react with human DNA. The primers and probe are specific for *P. falciparum* and do not cross-react with *Plasmodium vivax*, *Plasmodium ovale*, or *Plasmodium malariae* DNAs (data not shown). The DNA was amplified in a thermal cycler (ABI 7500; Applied Biosystems, Foster City, CA). DNA (2.5 μl) was added to 47.5 μl of amplification mixture containing Taqman master mix

(2×; Applied Biosystems), oligonucleotide primers (0.2 μM each for the *P. falciparum* ssrRNA gene), and TaqMan probe (0.2 μM; Applied Biosystems). After 10 min of preincubation at 95°C, PCR amplification was carried out for 40 cycles (95°C for 15 s, 60°C for 1 min). A plasmid containing a *P. falciparum* ssrRNA gene was used at multiple known concentrations to generate a standard curve. The plasmid was prepared using an Invitrogen TOPO II TA cloning kit (vector pCR 2.0).

Statistics. Copy number data were log-normally distributed. The values were therefore expressed as geometric means ± standard errors of the mean, and statistical tests were performed on log-transformed values. The significance of differences between groups was evaluated using Student's *t* test, and relationships between variables were examined by simple linear regression. Comparisons of the proportions of responders of various groups of donors were evaluated by chi-square analysis.

RESULTS

Comparison of different methods for detecting malaria infection in maternal peripheral and placenta intervillous blood. A total of 554 women were examined, of whom 143 (26%) were primiparous, 115 (21%) were secundiparous, and 296 (53%) were multiparous (three or more pregnancies). Table 1 compares the overall frequencies of *P. falciparum* infection in women based on five different assays for determining malaria infection subdivided by whether malaria was detected in maternal peripheral blood and/or in the placenta (intervillous blood). Blood smears estimated the lowest frequency for *P. falciparum* infection in maternal PB and placental IVB. Measurement of HRP-2 indicated a similar frequency of malaria infection in PB but estimated a greater frequency of infection in IVB than BS. The measurement of pLDH was similar in sensitivity to HRP-2 for detection of malaria in IVB. Parasite LDH, however, was more sensitive than BS or HRP-2 for detecting malaria in maternal peripheral blood. nPCR detected malaria parasite DNA in a much higher proportion of samples than the other assays. RTQ-PCR was less sensitive than nPCR but more sensitive than the other assays for detecting malaria. Infection rates among infants aged 6 months to 24 months ranged from 19% by BS to 60% with nPCR.

The four assays showed good concordance for the detection of malaria infection. This was accomplished by taking samples that were positive for the least sensitive of the assays compared and determining the proportion of the samples that were positive for the other assay. For example, the concordance between BS and CAg assays (HRP-2 and/or pLDH) was 0.80, that between BS and RTQ-PCR was 0.95, and that between nPCR and RTQ-PCR was 0.93. Infrequently, an individual that was BS and/or CAg positive failed to have detectable parasite DNA based on either PCR assay (*n* = 2).

Sensitivities and specificities of the different assays using RTQ-PCR as the referent standard. Because the sensitivity for

TABLE 2. Relationship of parasite density determined by blood smear to RTQ-PCR, nested PCR, and HRP-2 in children ages 6 to 24 months

Parasite density[a]	RTQ-PCR geometric mean amplicon no./μl[a]	Frequency (% positive)[b]		
		% Positive	nPCR (% positive)	HRP-2 (% positive)
0	8.7×10^2	307/849 (36)	420/796 (53)	117/416 (28)
0–99	2.9×10^5	28/29 (97)	28/31 (90)	11/17 (65)
100–499	6.6×10^5	25/29 (86)	27/29 (93)	10/11 (91)
500–999	9.1×10^5	29/30 (97)	28/30 (93)	12/13 (92)
1,000–4,999	9.0×10^6	43/43 (100)	41/42 (98)	12/13 (92)
>5,000	8.6×10^7	26/26 (100)	28/28 (100)	13/13 (100)

[a] Per microliter of peripheral blood of PCR-positive individuals assuming a packed red blood cell volume of 0.3.
[b] Number of samples positive for the assay indicated over the total number of samples tested at that particular density of parasites.
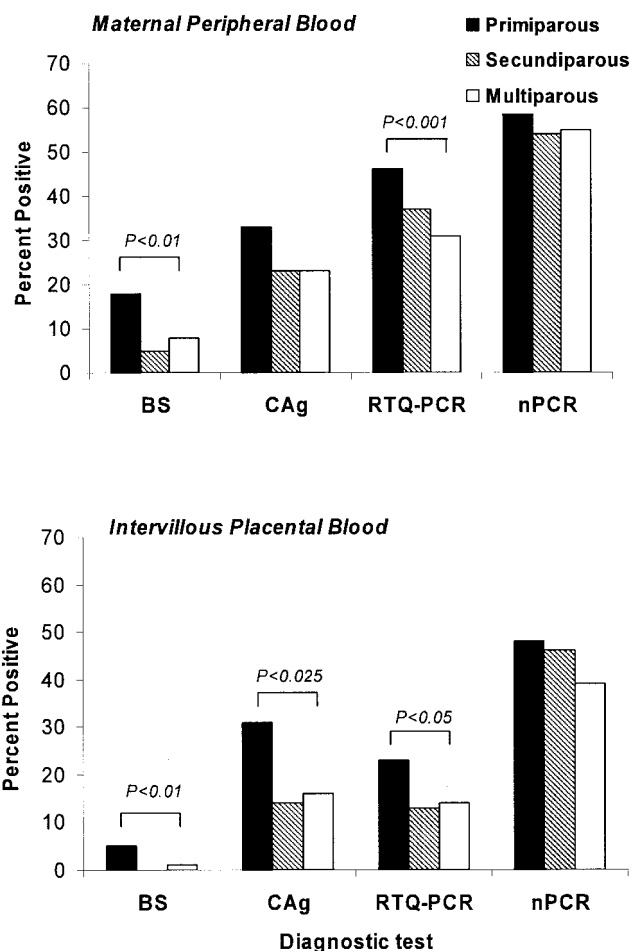

FIG. 1. Proportion of women infected with *P. falciparum* parasites stratified by parity. The presence of malaria infection was detected by either BS, RTQ-PCR, nPCR, or malaria CAgs from peripheral venous blood (upper panel) or intervillous placental blood (lower panel) from the same women. Significant differences between groups by the chi-square test are shown ($P < 0.05$). The sample size is shown in Table 1.

BS was low and that for nPCR was high and potentially subject to contamination, we used RTQ-PCR as the reference standard to examine the sensitivities and specificities of the other tests with respect to peripheral and placental blood diagnosis. Blood smear, pLDH, and HRP-2 showed 18%, 40%, and 15.3% sensitivity, respectively, in peripheral blood samples. Specificities for these same samples were 97%, 85%, and 97% for BS, pLDH, and HRP, respectively. For intervillous placental blood samples, sensitivities for BS, pLDH, and HRP-2 were 3%, 48%, and 55%, respectively. Specificities for these same placental samples were 99%, 89%, and 90% for BS, pLDH, and HRP, respectively.

Parasite burden determined by RTQ-PCR compared to microscopy. Quantification of parasites by RTQ-PCR showed a weak correlation with that observed by microscopy in the peripheral blood of pregnant women ($r^2 = 0.11$; $P < 0.001$). Peripheral blood was also obtained from their infants (ages, 6 to 24 months), and the presence of malaria was determined by the different assays (Table 1). When the sensitivities of the assays are stratified by parasite density determined by blood smear (Table 2), RTQ-PCR quantification correlated with BS in these infants (Table 2) ($r^2 = 0.23$; $P < 0.001$).

Relationship of parity with frequency and intensity of infection. In areas of continuous malaria transmission, it has been well established that primiparous women are more likely to be infected with *P. falciparum* parasites, develop episodes of symptomatic malaria, and have involvement of the placenta than are multiparous women based on BS analysis (3, 16, 21). Figure 1 shows the relationship of parity with malaria infection in PB and IVB using the different assays to detect malaria parasites. Primiparous women were twofold more likely to be peripheral blood smear positive for malaria (18%) than multiparous women (8%). Primiparous women also had higher mean parasite densities for blood smear-positive individuals than multiparous women (geometric mean = 922/μl versus 381/μl; $P = 0.04$). More primiparous women than multiparous women were also RTQ-PCR positive in the peripheral blood ($P = 0.001$). Among infected individuals, the amplicon number determined by RTQ-PCR was 12-fold higher in primiparous than multiparous women (Fig. 2). Although more primiparous women were infected, as determined by nPCR or circulating antigens, than multiparous women, these differences were

smaller than that observed for RTQ-PCR and BS and were not significant.

Placental malaria occurred with greater frequency in primiparous than multiparous women based on diagnosis by RTQ-PCR and the CAg assay (Fig. 1, lower panel). Few placentas showed the presence of parasites by BS. The amplicon numbers determined by RTQ-PCR were similar in the placentas of primiparous and multiparous women (Fig. 2). The overall detection of malaria infection by RTQ-PCR showed a strong and consistent association with parity.

Comparison of different assays for detecting malaria for predicting low-birth-weight babies. An important clinical outcome of malaria infection in pregnant women is giving birth to underweight babies as a consequence of intrauterine growth retardation or premature delivery (2). This analysis examined only newborns from term deliveries. The presence of maternal malaria infection assessed by blood smear or RTQ-PCR was more closely associated with low-birth-weight infants than were nPCR or CAg assays (Table 3). To determine whether the intensity of infection assessed by RTQ-PCR also correlated
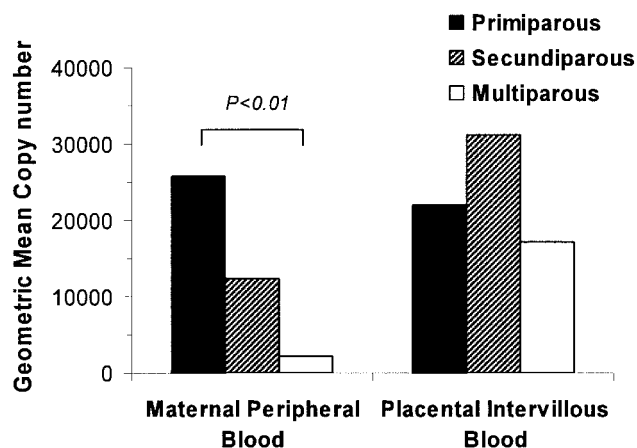
FIG. 2. Geometric mean amplicon numbers measured by RTQ-PCR for maternal peripheral and intervillous placental blood are shown for primiparous, secundiparous, and multiparous women. The values represent mean amplicon numbers for RTQ-PCR-positive individuals. The number of positive individuals is shown in Table 1.

TABLE 4. Relationship of *P. falciparum* ssRNA amplicon numbers with mean birth weights

Blood sample	Birth weight (n)		Significance
	Upper quartile[a]	Lower quartile[a]	
Maternal PB	2,821 ± 435 (49)	3,078 ± 420 (49)	0.008
Placenta IVB	2,763 ± 462 (18)	2,982 ± 430 (18)	0.06

[a] Women whose amplicon number determined by RTQ-PCR fell into the top or bottom quartile for blood samples from maternal PB or IVB. Birth weights are in grams. Sample size (n) is in parentheses.

with low-birth-weight babies, infected women were placed in either the highest or lowest quartile based on amplicon number. Women in the highest quartile for amplicon number had lower-birth-weight babies than mothers of newborns in the lowest quartile (Table 4).

DISCUSSION

The introduction of nested PCR for detection of malaria in populations has revolutionized our understanding of malaria epidemiology. It is now understood that *P. falciparum* malaria often produces chronic, persistent, and usually asymptomatic infections in partially immune individuals. These chronic infections are punctuated by bursts of increased parasitemia, usually from new infections that are more likely to produce disease. The drawback of nPCR is that it cannot distinguish very light infections with little clinical consequence from greater parasite burdens that might be more clinically important.

Blood smear analysis better estimates the infection burden, but it has a number of drawbacks, including variability in the skills of microscopists and underestimation of the parasite bur-

den due to sequestered parasites. The development of RTQ-PCR, which directly amplifies DNA from a multicopy gene without a second round of PCR, provides a more quantitative estimate of parasite burdens that blends specificity and increased sensitivity and potentially bridges periods of parasite sequestration. Here, we show that RTQ-PCR measurement of the *P. falciparum* infection burden in pregnant women is more predictive of clinical outcomes of malaria than nPCR or CAg assays. The detection of malaria in the peripheral blood and placenta by RTQ-PCR correlated more closely with parity among pregnant women than nPCR, suggesting that it better estimates the malaria burden based on host differences known to affect susceptibility to malaria. Partially immune women become more susceptible to malaria following pregnancy than in the time immediately before pregnancy. Pregnancy itself is immunosuppressive, and the developing placenta provides a niche for new parasite strains to sequester and avoid elimination (7, 22). Indeed, the parasite burden estimated by RTQ-PCR in the peripheral blood of primiparous women was 12-fold higher than in multiparous women (Fig. 2). This contrasts with a 2.5-fold difference in parasite numbers estimated by blood smear between primiparous and multiparous women. This highlights how susceptible primiparous women are to malaria. Although primiparous women were more likely to have placental malaria determined by RTQ-PCR, the amplicon numbers obtained from intervillous blood were similar in primiparous and multiparous women. This could reflect a sampling error where a small amount of blood obtained from a single cotyledon may correlate poorly with the total parasite burden in the placenta. This single placental blood sample may also account for the low number of positive blood smears observed from this source. Impression smears or biopsies of

TABLE 3. Comparison of infant birth weights among women positive for malaria by blood smear, real-time quantitative PCR, nested PCR, and circulating-antigen test

Assay	Sample	Birth wt (g)			No. (%) LBW[a] (<2,500 g)		
		Positive test result (mean ± SD)	Negative test result (mean ± SD)	P[b]	Positive test result	Negative test result	P
Bs	PB	2,885 ± 443	3,079 ± 465	*0.006*	49 (16.3)	453 (8.2)	0.1
	IVB	2,855 ± 637	3,088 ± 441	*0.05*	10 (30)	412 (6.6)	*0.01*
CAg	PB	2,942 ± 522	3,017 ± 479	>0.2	62 (16.1)	177 (10.2)	>0.2
	IVB	2,910 ± 450	3,048 ± 505	0.1	46 (13.0)	155 (9.7)	>0.2
RTQ-PCR	PB	2,926 ± 455	3,092 ± 467	*0.009*	197 (10.2)	308 (8.1)	>0.2
	IVB	2,903 ± 450	3,090 ± 460	*0.001*	70 (14.3)	371 (7.0)	*0.05*
nPCR	PB	3,049 ± 482	3,074 ± 438	>0.2	300 (10.9)	221 (8.3)	>0.2
	IVB	3,038 ± 459	3,106 ± 440	0.07	186 (9.1)	261 (7.3)	>0.2

[a] LBW, low-birth-weight babies.
[b] Statistically signficant (P ≤ 0.05) values are in italics.

the placenta, which were not performed in this study, are much more sensitive for the detection of parasitized erythrocytes (20).

Malaria infection in pregnant women can result in intrauterine growth retardation, which results in the birth of underweight babies, thereby increasing their risk for infant death (18, 25). The mechanisms causing intrauterine growth retardation remain incompletely understood but correlate with the presence of placental malaria and the intensity of malaria infection in pregnant women (6, 25). Therefore, the ability to detect clinically significant malaria infection would lead to more effective testing and management of women in pregnancy. Using peripheral blood samples, RTQ-PCR detected clinically significant malaria, which may lead to low-birth-weight babies, better than nPCR or circulating-antigen assays. This relationship was most striking for those women with high amplicon numbers in the peripheral or intervillous blood (Table 4). Moreover, RTQ-PCR was much more likely to identify malaria-infected women during pregnancy: 38% of women examined compared to 9% by BS. Indeed, of 49 pregnant women identified in the top quartile of gene copy numbers by RTQ-PCR, only 18 were also blood smear positive. This suggests that RTQ-PCR detects an additional population of women at risk for low-birth-weight babies that were otherwise BS negative.

These studies raise the question of exactly what RTQ-PCR measures in peripheral blood. The amplicon numbers shown are the raw values and do not correspond to the number of parasitized red cells. Factors such as the presence of multiple copies of the gene encoding ssrRNA (13), multinucleate schizonts, and parasite DNA released from lysing cells into the plasma may cause substantial overestimation of the number of infected red cells. Recently, it has been shown that parasite DNA encoding *P. falciparum* rRNA can be amplified in plasma from acutely infected individuals (9), suggesting that DNA released from sequestered parasites may contribute to the parasite burden estimated by RTQ-PCR. This is supported by observations that parasite DNA can be amplified for several days following successful eradication of parasites and infected erythrocytes in experimentally infected individuals (11). This indicates that parasite DNA may briefly linger in the blood following eradication of infected erythrocytes. Overall, RTQ-PCR provides an additional estimate of the intensity of infection that complements, but may differ from, blood smear detection.

Using RTQ-PCR as the referent standard, we showed that circulating-antigen assays provide a rapid, sensitive, and specific method for detection of placental malaria relative to blood smears. This is of particular value, since examination of placental blood films for infected erythrocytes is often much more difficult and error prone than examination of peripheral blood because of tissue debris, large numbers of white cells, and multiple stages of the parasites found in placental tissues (24). Importantly, we also showed that circulating-antigen assays are more specific for detection of placental malaria than previously reported (14, 15, 24). Our findings differ from the previous studies because we used RTQ-PCR as the referent standard for placental malaria rather than blood or impression smears of the placenta, where the presence of malaria may have been missed or misclassified.

Overall, the results show that detection of *P. falciparum* by RTQ-PCR in peripheral blood and placental blood, along with microscopy, may improve diagnosis. Real-time PCR is useful for quantitative detection of *P. falciparum* in pregnant women, and it is a good predictor of adverse outcome. The major drawbacks for RTQ-PCR are its cost and the requirement for a real-time PCR machine and trained individuals to run it. Currently, we estimate the reagent costs for running a single sample by RTQ-PCR to be approximately US$2.50. This contrasts with pennies for a blood smear. However, reading blood smears is time-consuming and requires highly trained microscopists. We estimate that it would take four to five times as long to prepare and read the same number of blood samples for malaria compared to evaluation by RTQ-PCR. As reagent costs decline and RTQ-PCR technology improves, this assay will become a more economically viable option in developing countries. Thus, RTQ-PCR could be successfully used in a central laboratory for surveillance work and would provide a valuable tool for epidemiologic studies of malaria that avoids the problems of inconsistent precision and sensitivity associated with blood smear analysis.

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