EVALUATION OF A MALARIA RAPID DIAGNOSTIC TEST FOR ASSESSING THE BURDEN OF MALARIA DURING PREGNANCY

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Abstract. Plasmodium falciparum infection during pregnancy may cause placental malaria and subsequently low birth weight, primarily through the placental sequestration of infected red blood cells. Measuring the burden of malaria during pregnancy usually involves determining the prevalence of placental malaria infection through microscopic examination of placental blood films, a difficult and error-prone process. A number of rapid diagnostic tests (RDTs) for malaria have been developed, most of them immunochromatographic dipstick assays. However, none have been tested for the direct determination of malaria antigen in placental blood. We undertook an evaluation of the Malaria Rapid Test (MAKR0med\textsuperscript{8} in determining placental malaria infection. The prevalence of placental parasitemia was 22.6% by microscopy, 51.0% by a polymerase chain reaction (PCR), and 43.1% by RDT. When the PCR was used as the gold standard, RDTs had a sensitivity of 89% and a specificity of 76%. The MAKROmed RDT was highly sensitive in the detection of placental malaria, but had lower than expected specificity.

INTRODUCTION

In areas of stable Plasmodium falciparum transmission, malaria infection during pregnancy may cause maternal anemia, placental malaria, and subsequently low birth weight.\textsuperscript{1–3} These adverse consequences put the health of the mother, her fetus, and the neonate at substantial risk. It is estimated that malaria infection during pregnancy causes 75,000–200,000 infant deaths each year.\textsuperscript{1}

Peripheral malaria parasitemia detected by microscopy in pregnant women does not always provide an accurate estimation of the prevalence of placental malaria parasitemia. In one study, detection of peripheral parasitemia by microscopy failed to identify one-fifth of women with placental malaria infections.\textsuperscript{4} Thus, previous assessments of malaria in pregnancy have used blood films made from blood from the maternal side of the placenta to determine whether placental malaria infection is present. However, placental slides are often difficult to read because of the presence of cellular debris, a greater number of white blood cells, and, in the case of P. falciparum, parasites from both early and late stages in the life cycle, as compared with peripheral slides where trophozoites are the predominate stage seen. Additionally, in less developed rural areas where malaria during pregnancy may have the greatest burden on the population, reliable microscopic examination of peripheral or placental blood films depends on the presence of adequate infrastructure and competent microscopists.

Two potential alternatives to microscopy are the polymerase chain reaction (PCR) and rapid diagnostic tests (RDTs). The PCR faces the same logistical and technical difficulties as microscopy and is more labor-intensive and costly compared with microscopy, and quality assurance is critical because contamination leading to false-positive results is common. Thus, the PCR is an unrealistic alternative for most in-country field studies. The RDTs, on the other hand, require no additional infrastructure and, theoretically, do not require extensive training for appropriate use. The tests are not labor-intensive, but are more costly than microscopy.

The RDTs produced by a variety of manufacturers have been evaluated as diagnostic tests for malaria; these tests were reviewed by Moody in 2002.\textsuperscript{5} Most of the tests are immunochromatographic dipstick assays that detect either histidine-rich proteins (HRP) produced by infected red blood cells or parasite lactate dehydrogenase, an enzyme present in the glycolytic pathway of the parasite. These assays have primarily been evaluated for diagnosis of peripheral malaria; most tests have generally performed better at densities >100 parasites/\textmu L.\textsuperscript{5} Recently, a few studies have assessed their ability to diagnose placental malaria.\textsuperscript{4,6,7} These placental diagnostic studies have focused on comparing the sensitivity of peripheral blood RDTs and peripheral blood microscopy for the detection of placental malaria.

None of these studies has assessed RDTs use in directly detecting placental parasitemia by testing placental blood. The potential use of placental RDTs include 1) epidemiologic tools in assessments of the burden of malaria during pregnancy; 2) research studies in which placental malaria is an outcome measure; and 3) program assessment (either determining the necessity of changes in drug policy or monitoring the impact of new policy). Therefore, as a part of a rapid assessment of the burden of malaria during pregnancy in Burkina Faso,\textsuperscript{8} we sought to determine the performance of RDTs when compared with microscopy in the direct (testing of placental blood to determine placental infection) or indirect (testing of peripheral blood to determine placental infection) detection of placental malaria.

MATERIALS AND METHODS

Study site and subjects. This study was conducted from June to November 2001 in Koupéla and Pouytenga delivery units in Koupéla District, approximately 120 km east of Ouagadougou, Burkina Faso. Samples were collected as a part of a rapid assessment of the burden of malaria during pregnancy; detailed clinical procedures are described elsewhere.\textsuperscript{8} Briefly, delivering women >15 years of age who had placentas collected and provided informed consent were enrolled. Enrolled women were administered a questionnaire and capillary blood was obtained by finger prick. Placental blood
samples were obtained by identifying the maternal side of the placenta, wiping away excess blood, cutting into the surface, and collecting pooled blood. For each maternal and placent al blood sample, malaria blood films were prepared for microscopic examination, a rapid diagnostic test was performed, and blood was dried on filter paper strips for later PCR analysis. Neonates were weighed (± 10 grams) using an electronic digital scale (Tanita Corporation, Tokyo, Japan). Informed consent was obtained from all study participants. Ethical clearance for this study was given by Human Subjects review committees at the Centers for Disease Control and Prevention, the Johns Hopkins University, and the Burkina Faso Ministry of Health.

**Microscopic examination.** All blood films were stained with Giemsa and examined for parasites. Thick films were examined under oil immersion (100× magnification) and parasites and leukocytes were counted in the same fields until 500 leukocytes were counted. Parasite densities were estimated using an assumed leukocyte count of 8,000 leukocytes/µL. Thin films were used to determine species when thick films were positive. All blood films were read twice by different microscopists at a reference laboratory. Discordant results were given a third reading (by a microscopist blinded to the results of the first two readings), the result of which was considered final.

**Rapid diagnostic testing.** All blood samples, maternal and placental, were tested in the delivery units using the Malaria Rapid Test (MAKROmed; MAKROmed Pty. Ltd., Johannesburg, South Africa) to detect soluble *P. falciparum* HRP-2. Tests were preformed according to the manufacturer’s instructions. Each test was examined and the results recorded by two independent observers in a blinded fashion. To minimize observation bias, the team member reading a woman’s placental and peripheral RDT was blinded to results of microscopic blood film examination, and *vice versa.* In Koupéla, tests were performed in duplicate on each sample.

**Polymerase chain reaction analysis.** Blood was collected onto filter paper strips, ISOCODE® STIX PCR template preparation dipsticks (Schleicher and Schuell, Keene, NH) and used for PCR testing at U.S. Centers for Disease Control and Prevention (Atlanta, GA). The DNA was extracted from filter paper strips by heating in distilled water. The tip of the filter strip was washed by vortexing three times in 500 µL of ultraviolet light (UV)–treated water, removing the wash water, and then heating for 30 minutes at 95°C in 50 µL of fresh UV-treated water. After brief micro-centrifugation, the DNA-containing supernatant was transferred to a clean sterile tube. Two microliters of sample was used for the first round of the nested PCR amplification. Primary- and secondary-round ribosomal RNA (rRNA) gene oligonucleotide primer pairs for *P. falciparum* were essentially the same as those described by Snounou and others.10 The conditions for amplifying the rRNA genes of *P. falciparum* using AmpliTaq Gold polymerase (Perkin Elmer, Boston, MA) were 95°C for 15 minutes; 30 cycles at 94°C for one minute, 60°C for two minutes, and 72°C for two minutes; 45°C for two minutes; and 72°C for 10 minutes. Products were identified after electrophoresis on 2% agarose gels, staining with ethidium bromide, and UV illumination for the detection of a 205-basepair band for *P. falciparum.* A subset of samples was checked for *P. ovale,* *P. malariae,* and *P. vivax* by the same method but using species-specific oligonucleotide primers. If maternal or placental blood films were positive and the initial PCR result was negative, the PCR was repeated using the original sample and a newly extracted sample. Repeat PCR results were used in the analysis. Given that the PCR is likely to detect submicroscopic infections, the PCR was not repeated when the result was positive but the blood film was negative. There were 14 instances in which the placental PCR result was negative and placental blood slide was positive. When those 14 were repeated, 9 repeat PCR results were positive. There were nine instances where the peripheral PCR result was negative and the peripheral blood film was positive. Five of the repeat PCR results were positive.

**Definitions.** Direct detection refers to using samples from one source to detect infection in that same source, e.g., using placental blood to detect placental infection. Indirect detection refers to using samples from one source to detect infection in another location, e.g., using peripheral blood to detect placental infection. Submicroscopic infections refer to those determined to be negative by placental microscopy but tested positive by either placental RDT or placental PCR.

**Statistical analysis.** Data were double entered and validated using Epi-Info version 6 (Centers for Disease Control, Atlanta, GA). SAS software version 8.02 (SAS Institute, Cary, NC) was used for statistical analysis. The chi-square or Fisher’s exact test were used to compare proportions of categorical variables. The Kruskal-Wallis test was used to compare birth weight between groups.

**RESULTS**

We enrolled a total of 853 women in the study. Complete results of the study concerning the burden of malaria during pregnancy in this population, specifically associations with adverse outcomes and the impact of chloroquine chemoprophylaxis, have been previously reported by Sirima and others.8 The prevalence of placental parasitemia as detected by placental microscopy was 22.6% (188 of 832). When detected by placental RDT, the prevalence was 43.1% (310 of 719). In Koupéla, each peripheral and placental RDT was repeated in duplicate; there were no discordant results. By microscopy two women showed mixed *P. falciparum* and *P. malariae* infections; one in the placenta only and one in the periphery only. The prevalence of placental parasitemia was 51.0% (247 of 484) when detected by the PCR. Of the subset of samples tested by the species-specific PCR, all parasites detected were *P. falciparum*; no *P. ovale* or *P. malariae* were found.

**Direct detection of placental infection.** For direct detection of placental malaria, 693 placentas were tested by both microscopy and RDT. Of the 151 placentas where parasites were detected by microscopy, 143 were positive by RDT (sensitivity = 95%, Table 1). Of the 542 placentas in which no parasites were detected by microscopy, 151 were positive by RDT (thus, 391 placentas were negative by both tests, specificity = 72%, Table 1). However, parasites were detected in 59% of these false-positives samples (i.e., blood film negative, RDT positive) that were tested by the placental PCR. Compared with PCR detection of placental malaria, placental RDTs and placental microscopy have sensitivities of 89% and 58% and specificities of 76% and 96%, respectively (Table 2).

Placental RDTs and the placental PCR had sensitivities of 99% and 97%, respectively, in placental samples containing
Sensitivity and specificity of peripheral and placental microscopy and rapid diagnostic test (RDT) for the detection of malaria infection, using microscopy as the reference standard

<table>
<thead>
<tr>
<th>Peripheral parasitemia</th>
<th>Placental parasitemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (%)</td>
<td>Specificity (%)</td>
</tr>
<tr>
<td>Peripheral microscopy</td>
<td>183/275 (67)</td>
</tr>
<tr>
<td>Peripheral RDT</td>
<td>202/224 (90)</td>
</tr>
<tr>
<td>Placental RDT</td>
<td>–</td>
</tr>
</tbody>
</table>

≥100 parasites/μL as detected by microscopy. The placental PCR had a slightly higher sensitivity than placental RDTs at parasite densities <100 parasites/μL (86% versus 80%).

**Indirect detection of placental infection.** For indirect detection of placental malaria, 690 women were tested using peripheral RDT and placental microscopy. Of the 153 placentas in which parasites were detected by placental microscopy, 146 were positive by peripheral RDT (sensitivity = 95%, Table 1). Of the 537 women with placentas in which no parasites were detected by microscopy, 208 women had positive peripheral RDTs (thus, 329 women were negative by both tests, specificity = 61%, Table 1). However, parasites were detected in 47% of the false-positives samples that were tested by the placental PCR.

Peripheral microscopy had a sensitivity and specificity of 82% and 86%, respectively, when compared with detection by placental microscopy (Table 1). Peripheral RDTs and peripheral microscopy had sensitivities of 92% and 67% and specificities of 59% and 88%, respectively, when compared with placental PCR detection of placental malaria (Table 2).

Peripheral RDTs and peripheral PCR had sensitivities of 99% and 98%, respectively, in placental samples containing ≥500 parasites/μL as detected by microscopy. Peripheral RDTs had slightly lower sensitivity in placental samples with 100–499 parasites/μL (92% versus 100% for the peripheral PCR). Both peripheral RDTs and the peripheral PCR were less sensitive at placental parasite densities <100 parasites/μL (89%).

**Direct detection of peripheral infection.** To place the results of this study in context regarding direct detection of peripheral parasitemia, 690 women were tested by peripheral RDT and peripheral microscopy. Two hundred four (30%) women had peripheral parasitemia as detected by peripheral microscopy. Of these, 196 were positive by peripheral RDT (sensitivity = 96%, Table 1). Of the 486 peripheral samples where parasites were not detected by peripheral microscopy, 160 were positive by peripheral RDT (specificity = 67%, Table 1). Forty-nine percent of the false-positives samples that were tested by peripheral PCR were positive. When compared with peripheral PCR detection of parasitemia, peripheral RDTs were more sensitive (90% versus 67%), but less specific (65% versus 98%) than peripheral microscopy (Table 2).

As with direct detection of placental parasitemia, both peripheral RDTs and the peripheral PCR are highly sensitive at directly detecting peripheral parasitemia at densities ≥100 parasites/μL as detected by microscopy (sensitivity = 99% and 100%, respectively).

At parasite densities between 1 and 99 parasites/μL, both tests have lower sensitivities (81% for peripheral RDTs and 88% for peripheral PCR).

**Associations with birth weight.** By all methods of direct detection, placental infection (detected by placental microscopy, peripheral RDT, and placental PCR) was significantly associated with birth weight. Delivery of low birth weight infants (<2,500 grams) was significantly more common in women who had placental infection detected by any of the direct testing methods (Table 3). Placental infection as detected indirectly by peripheral RDT was significantly associated with lower median birth weight (P = 0.05), but not with the prevalence of low birth weight. Maternal infection as identified by peripheral microscopy or peripheral PCR was not associated with either median birth weight or the prevalence of low birth weight (Table 3). Women with submicroscopic infections delivered infants that weighed significantly less than those delivered by women with no placental infection (i.e., negative by all direct measures) (mean ± SD = 2,281 ± 431 grams versus 2,974 ± 391 grams; P = 0.02 for submicroscopic placental PCR and 2,880 ± 428 grams versus 2,959 ± 413 grams; P = 0.03 for submicroscopic placental RDT). However, delivery of low birth weight neonates was not more common among women with submicroscopic placental parasitemia.

**DISCUSSION**

We evaluated a rapid diagnostic test for malaria (MAKROmed) as an epidemiologic tool in the context of assessing the burden of malaria during pregnancy. The test
Comparison of birth weights and proportions of low birth weight (LBW) among women positive for malaria by microscopy, rapid diagnostic test (RDT), or polymerase chain reaction (PCR)*

<table>
<thead>
<tr>
<th>Test (no.)</th>
<th>Birth weight (grams)</th>
<th>Low birth weight (grams)</th>
<th>PR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive test result Mean ± SD</td>
<td>Negative test result Mean ± SD</td>
<td>Positive test result Total (% LBW)</td>
<td>Negative test result Total (% LBW)</td>
</tr>
<tr>
<td>Peripheral microscopy (788)</td>
<td>2,868 ± 487</td>
<td>2,940 ± 432</td>
<td>0.11</td>
<td>235 (16.6)</td>
</tr>
<tr>
<td>Placental microscopy (787)</td>
<td>2,821 ± 534</td>
<td>2,949 ± 417</td>
<td>0.01</td>
<td>179 (20.7)</td>
</tr>
<tr>
<td>Peripheral RDT (681)</td>
<td>2,868 ± 483</td>
<td>2,955 ± 427</td>
<td>0.05</td>
<td>350 (17.7)</td>
</tr>
<tr>
<td>Placental RDT (683)</td>
<td>2,860 ± 494</td>
<td>2,951 ± 415</td>
<td>0.02</td>
<td>292 (18.5)</td>
</tr>
<tr>
<td>Peripheral PCR (456)</td>
<td>2,886 ± 485</td>
<td>2,947 ± 404</td>
<td>0.24</td>
<td>271 (15.1)</td>
</tr>
<tr>
<td>Placental PCR (454)</td>
<td>2,854 ± 512</td>
<td>2,960 ± 393</td>
<td>0.02</td>
<td>232 (16.8)</td>
</tr>
</tbody>
</table>

*PR = prevalence ratio; CI = confidence interval.

had high sensitivity but low specificity when compared with placental microscopy, the current standard field method for detection of infection. The RDTs were particularly sensitive at parasite densities >100 parasites/µL. When a PCR was used as the reference, placental RDTs had high sensitivity, but low specificity, whereas placental microscopy had low sensitivity and high specificity. Overall, direct detection (i.e., use of placental samples to detect placental infection) always detected infection more accurately than indirect detection (i.e., the use of peripheral samples to estimate placental infection) by the same method. All direct detection methods (i.e., microscopy, RDT, and PCR) demonstrated the associated between placental malaria and the adverse outcome of low birth weight.

Given that there are no other published studies assessing the use of RDTs for the detection of malaria infection in placental samples, we are unable to compare our findings to those of others. However, we are able to compare our results on the indirect detection of placental malaria to the results of other studies. In this setting, the MAKROmed malaria rapid test had higher sensitivity, but lower specificity than ICT pF™ (Amrad ICT, Sydney, New South Wales, Australia) and OptiMAL® (Flow Inc., Portland, OR) tests for the indirect detection of placental malaria compared with microscopy. Our findings with MAKROmed are similar to reports that the sensitivities of ICT and OptiMAL tests are increased with increasing parasite density.5,6,7

For direct detection of peripheral parasitemia in our study, MAKROmed had high sensitivity and low specificity compared with both microscopy and PCR. The specificity of MAKROmed in our study was substantially lower than that of the only other published study of MAKROmed, in which the MAKROmed test was compared with microscopy and PCR for detection of malaria in febrile returned travelers.9 In our hands, MAKROmed also did not perform as well as the manufacturer stated in the product literature: >99.5% sensitivity and >94% specificity compared with the PCR.11 A potential reason for this is that the manufacturer states that the product should be stored between 8°C and 30°C and kept away from sunlight and temperature extremes. These conditions are difficult to achieve in many field settings in malarious countries. It is important that future research be conducted on the stability of rapid diagnostic tests in various climatic conditions.

In general, comparisons of microscopy, RDTs, and PCR are challenging to interpret because they detect infection in different ways: microscopy detects viable whole parasites in red blood cells, RDTs detect specific parasite proteins, and PCR detects parasite DNA. The RDTs and PCR potentially have lower thresholds of detection of viable parasites than microscopy because they are able to detect parasite debris present in the blood when viable parasites may be sequestered or at very low densities (below the threshold of visible detection). However, both parasite proteins and DNA can persist after successful treatment, making the results of RDTs and PCR potentially clinically confusing. One study of a similar HRP-2 rapid diagnostic test, the ParaSight™ F (Becton Dickinson, Franklin Lakes, NJ) test, showed that HRP-2 positivity persisted 28 days after treatment.12 Kain and others demonstrated that PCR remains positive longer than does microscopy after the initiation of treatment.13

These comparisons are also challenging because it is unclear which type of detection is best correlated with adverse outcomes. Theoretically, microscopy, since it detects only viable parasites, should be the most relevant measure. However, some research has demonstrated the clinical importance of submicroscopic infections during pregnancy. Although all use indirect detection of placental malaria, some studies have shown that submicroscopic infections detected by a PCR are associated with mild maternal anemia and inflammation,14 and submicroscopic infections detected by rapid diagnostic test are associated with maternal anemia4 and low birth weight.7 In our study, infants born to mothers with submicroscopic placental infections directly detected by either PCR or RDT tended to weigh less that those with no infection. One limitation of this result is that we were not able to separate true submicroscopic infections from recently treated infections. In general, all direct detection methods performed better than their indirect counterparts in demonstrating an association of infections with adverse outcomes, such as low birth weight. To aid in the interpretation of our results and of future results of similar studies, we need a better understanding of the clinical relevance of infections detected by each mechanism.

In addition to findings concerning the performance of this RDT, we also made some operational observations that may be helpful to future users. Rather than using a traditional capillary tube, this test employs a much larger tube (similar to the diameter of a small straw) in which the user must collect a thin film of blood to be dispensed onto the test strip. Our field staff was accustomed to using capillary tubes to collect blood. There was a tendency among study staff to collect more blood into the wider tube and apply too much blood to the test strip. The excess blood did not completely wash out during the test procedure, turning the strip to a pink hue that made the results more difficult to read. Also, we saved each
RDT as documentation that each test had been done. Initially, some staff later re-read saved tests that had changed color over time, and results were changed in laboratory notebooks; none of these altered results were used in the analysis. It is essential that used RDTs be discarded promptly after initial reading.

For direct detection of placental malaria, the low specificity of the MAKROmed RDT is likely due to a combination of picking up some infections missed by microscopy, and some treated infections with residual plasmodial protein. The MAKROmed RDTs are therefore likely to provide a higher estimate of the burden of placental malaria infection when compared with microscopy, and a slightly lower estimate when compared with the PCR. Given the logistic and financial difficulties of the PCR in most field settings, only microscopy and RDTs are viable options at the present time. Since RDT results were also correlated with adverse outcome (low birth weight), it is reasonable to consider future use of the MAKROmed or other RDTs as an epidemiologic tool. The cost of the MAKROmed test (approximately 1 U.S. dollar) should be affordable within the context of epidemiologic studies. Different RDTs with different properties may be useful for different settings, including the rapid assessment of the burden of malaria during pregnancy, research studies, and program evaluation. In the context of program assessment, it is important that the same testing methodology, whether microscopy or RDT, be used to determine both baseline and post-intervention measurements to ensure consistency. No matter which method is used, our study confirms that peripheral malaria parasitemia is an imperfect proxy measure of placental infection, and most importantly is not as strongly associated with one of the main outcome measures of interest, low birth weight.

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