

Diagnosis of *Plasmodium falciparum* Malaria at Delivery: Comparison of Blood Film Preparation Methods and of Blood Films with Histology

Stephen J. Rogerson,^{1,2,3*} Patrick Mkundika,¹ and Maxwell K. Kanjala¹

Malawi-Liverpool-Wellcome Trust Clinical Research Programme, College of Medicine, University of Malawi, Blantyre, Malawi¹; School of Tropical Medicine, University of Liverpool, Liverpool, United Kingdom²; and Department of Medicine (RMH/WH), University of Melbourne, Parkville, Victoria 3052, Australia³

Received 7 October 2002/Returned for modification 26 November 2002/Accepted 14 January 2003

We compared peripheral and placental blood films (made by different techniques) with placental histology for diagnosis of *Plasmodium falciparum* malaria in pregnancy. Samples from 464 women were examined, of whom 124 (26.7%) had active *P. falciparum* infection and 148 (31.9%) had past infection. Placental histology was more sensitive (91%) than peripheral blood film (47%) or placental blood film (63%) examination and also detected past infection. Few women had microscopically detectable infection without a positive histology. Infection detected by histology only and past infection were both associated with significantly lower infant birth weight and with lower hemoglobin concentrations compared to the results for uninfected women. Thick blood films were prepared with blood obtained by placental incision or scraping of the incision margin (263 samples) or by washing of placental tissue (235 samples). Each gave similar sensitivities (76 to 78%), specificities (98 to 99%), positive predictive values (92 to 98%), and negative predictive values (93 to 94%); but the median levels of parasitemia were lower for incision samples (840 parasites/ μ l) than scrapings (2,295 parasites/ μ l) ($P = 0.02$). Placental histology is the most sensitive method for the diagnosis of malaria in pregnancy. Methods for preparation of placental films may affect the density, but not the prevalence, of *P. falciparum* infection detected.

Plasmodium falciparum malaria is common in pregnancy and predisposes the mothers and their infants to mortality (6, 20). Infected erythrocytes (IEs) containing ring-stage parasite are found in peripheral blood, whereas trophozoite and schizont IEs accumulate in the placenta (2). Placental infection may be detected in the absence of peripheral blood parasitemia and may persist after initiation of antimalarial treatment (16).

Placental histology is the “gold standard” for the diagnosis of malaria at delivery, but it is relatively costly and labor-intensive and, hence, is frequently not available. The diagnosis of malaria in pregnancy is important for both operational and research purposes. There are few published data from studies that have compared examination of blood films (either peripheral or placental) with histology for detection of placental infection. While methods of preparing and examining peripheral blood smears appear to vary little, a number of different approaches to examination of placental blood have been used. Comparisons between different techniques have rarely been performed, and these approaches have rarely been validated against placental histology. The prevalence of malaria in pregnant women may differ according to the criteria used for diagnosis. To investigate this, we performed two related studies. First, the prevalence of *P. falciparum* as detected by microscopy of thick peripheral blood and placental blood films was compared with placental histology to determine the sensitivity and the specificity of each test. Second, we compared three

different methods of preparation of thick placental blood films for sensitivity and for the density of parasitemia found.

MATERIALS AND METHODS

Pregnant women attending the Queen Elizabeth Central Hospital in Blantyre, Malawi, were enrolled in a larger study of the pathogenesis of malaria in pregnancy, after written informed consent was obtained. Demographic information and obstetric histories were obtained. Peripheral venous blood was used to prepare thick blood films. Immediately after delivery, the placenta was placed maternal side up and cleaned with sterile normal saline, and a healthy paracentric area was incised. Thick blood films were prepared from a droplet of the blood welling into the incision (incision), from blood obtained by scraping the wall of the incision (scraping), and from a placental biopsy specimen approximately 0.5 cm³. The first two methods were used to make thick blood films immediately. Placental biopsy specimens were placed in 3 ml of phosphate-buffered saline with EDTA on a tube roller for 45 min, the tissue was removed, the solution was spun down, and the pellet was used for preparation of a blood film (washing). A larger biopsy specimen of placental tissue (2 by 2 by 1 cm) was placed into 10% neutral buffered formalin. After fixation it was embedded in wax; and sections were cut onto slides, processed, and stained with Giemsa and/or hematoxylin-eosin stain.

Each blood film was independently examined by two observers (P.M. and M.K.K.). The numbers of IEs per 200 leukocytes were counted, and the level of parasitemia was determined by using an assumed leukocyte count of 6,000 parasites/ μ l. The density was scored as 0, 1 (1 to 999 parasites/ μ l), 2 (1,000 to 9,999 parasites/ μ l), 3 (10,000 to 99,999 parasites/ μ l), or 4 (\geq 100,000 parasites/ μ l). In cases of disagreement in the scores, a third observer counted the numbers of parasites in the films. The average of two counts that agreed was used as the final level of parasitemia. Placental histology was examined by one of the investigators (S.J.R.) without knowledge of the blood film microscopy results. By using a standardized approach, 500 intervillous cells were counted to determine the level of parasitemia histologically (14). Past infection was defined as the presence of malaria pigment in fibrin or leukocytes without malaria parasites. Histology was reexamined in all cases in which histology and blood film results were in disagreement.

Data were entered into Microsoft Access software and transferred to Stata software (version 6.0; Statacorp, College Park, Tex.) for analysis. Normally distributed variables were compared by Student's *t* test, and nonnormally distrib-

* Corresponding author. Mailing address: Department of Medicine (RMH/WH), University of Melbourne, Post Office Royal Melbourne Hospital, Parkville, Vic 3050, Australia. Phone: 61 3 9342 7701. Fax: 61 3 9347 1863. E-mail: sroger@unimelb.edu.au.

TABLE 1. Comparison of placental histology and thick peripheral and placental blood film examination

Methods compared and result	No. of samples with the following result by the other method being compared:	
	Negative	Positive
Placental film and histology (n = 464)		
Histology negative	346	5
Histology positive	40	73
Peripheral film and histology (n = 463)		
Histology negative	342	8
Histology positive	63	50
Peripheral and placental films (n = 463)		
Placental film negative	376	9
Placental film positive	29	49

uted variables were compared by the Wilcoxon rank sum test. Sensitivities, specificities, and positive and negative predictive values were determined for each method of diagnosis of *P. falciparum* malaria.

RESULTS

Samples from 464 women were available for analysis. Of these women, 124 (26.7%) had active *P. falciparum* infection, defined as the presence of malaria parasites on the thick peripheral blood film or the thick placental blood film or as detected by placental histology. Histology was the most sensitive method, detecting 113 infections (91.1%), and was significantly more sensitive than detection on thick peripheral blood films (58 infections; 46.8%) or thick placental blood films (78 infections; 62.9%) (Table 1). Of the placental infections, 37 (32.7%) were not detected in peripheral or thick placental blood films. Six women had malaria on peripheral blood examination only, two had parasites on both thick peripheral and placental blood film testing but not by histology (suggesting that histology failed to detect a true infection), and three had thick placental blood films but not thick peripheral blood films showing infection and were negative by histology. The last three women had very low parasite densities (60 to 180 para-

sites/ μ l) and may have had either false-positive blood films or low-density placental infections not apparent by histology. Among the women in whom malaria was detected only by histology, the median (range) placental level of parasitemia was 0.4% (0.2 to 7.8%). This was significantly lower than that for women with positive thick peripheral blood smears (2.3%; range, 0 to 96%; $P < 0.0001$).

Women with malaria detectable microscopically had babies who were, on average, 314 g lighter than those of uninfected women, and low birth weight (LBW; weight, <2,500 g) was common (23.0% of the infected women had babies with LBWs, whereas 9.6% of the uninfected women had babies with LBWs) (Table 2). Past infection (malaria pigment in fibrin) was seen on histology in 148 women (31.9%). Women with malaria on histology only or with past infection had babies with significantly lower mean birth weights than women without malaria, but the prevalences of babies with LBWs were similar for both groups of women (Table 2). Hemoglobin concentrations were significantly lower and anemia (hemoglobin concentration, <11 g/dl) was more common in women with malaria in whom malaria was detected by microscopic examination than in uninfected women (Table 2). The hemoglobin concentration was also lower in women with malaria as determined by histology only or women with past infection than in women without malaria, but these differences were not statistically significant (Table 2).

P. falciparum infection as determined with blood films was more common in primigravid women (26.2%) and secundigravid women (25.0%) than in multigravid women (10.1%). Of the women negative by microscopy, past infection was seen in 40.2% of primigravid women, 34.9% of secundigravid women, and 36.4% of multigravid women; but no infection was found in 46.5% of primigravid women, 54.0% of secundigravid women, and 56.1% of multigravid women (the χ^2 test for trend was not significant for any comparison).

The human immunodeficiency virus (HIV) infection status was known for 425 women, of whom 114 (26.8%) were HIV infected. Leukocyte counts did not differ significantly by HIV infection status (data not shown). The prevalence of malaria by microscopy was similar between HIV-infected women (19.3%) and uninfected women (17.8%) (the difference was not signif-

TABLE 2. Comparison of birth weight and maternal hemoglobin concentrations according to presence or absence of malaria by microscopy and histology

Patient status ^a	Infant birth wt (g) ^b	No. (%) of babies with LBW	Maternal hemoglobin concn (g/dl) ^b	No. (%) of patients
Malaria on microscopy	2,787 \pm 436 (87) ^c	20 (23.0) ^d	11.9 \pm 2.0 (87) ^e	29 (33.3) ^f
Malaria by histology only	2,923 \pm 423 (38) ^g	4 (10.5)	12.0 \pm 1.9 (36) ^h	11 (30.6)
Past malaria	2,978 \pm 425 (141) ⁱ	16 (11.3)	12.2 \pm 1.7 (137) ^j	31 (22.6)
No malaria	3,101 \pm 455 (198)	19 (9.6)	12.6 \pm 1.7 (190)	35 (18.4)

^a Malaria on histology only, parasites identified by histology but not microscopy; past malaria, malaria pigment identified by histology with negative blood film microscopy; no malaria, no parasites by blood film microscopy or placental histology.

^b The values are means \pm standard deviations.

^c $P < 0.001$ compared with placentas with no *P. falciparum* infection.

^d $P = 0.02$ by χ^2 test. Other comparisons are not significant unless indicated otherwise.

^e $P = 0.0045$ compared with placentas with no *P. falciparum* infection.

^f $P = 0.003$ by χ^2 test.

^g $P = 0.026$ compared with placentas with no *P. falciparum* infection.

^h $P = 0.092$ compared with placentas with no *P. falciparum* infection.

ⁱ $P = 0.012$ compared with placentas with no *P. falciparum* infection.

^j $P = 0.073$ compared with placentas with no *P. falciparum* infection.

TABLE 3. Comparison of three different methods of placental smear preparation with placental histology

Histology result	No. (%) of samples with the indicated result:					
	Incision sample		Washing sample		Scraping sample	
	Negative	Positive	Negative	Positive	Negative	Positive
Positive	13 (5.4)	42 (17.4)	11 (5.1)	35 (16.3)	12 (5.0)	43 (17.8)
Negative	185 (76.8)	1 (0.4)	166 (77.2)	3 (1.4)	183 (75.6)	3 (1.2)

icant). Histologic findings did not differ significantly between HIV-infected and uninfected women (data not shown).

Comparison of methods of placental film preparation. An incision sample and a scraping sample were available for 263 patients, and histology results were available for 241 of those patients. A washing sample was available for 235 patients, and histology results were available for 215 of those patients. There was little difference in the yields of the three techniques, which varied between 17.7 and 19.1% for all women (Table 3). Each technique had similar rates of failure compared to placental histology (5.1 to 5.4% for all women).

Among the infected placentas, the levels of parasitemia varied by the testing approach. The results for 36 placentas for which observations by all three methods were available were compared. The median level of parasitemia with the incision sample was 840 parasites/ μ l, that for the washing sample was 2,182 parasites/ μ l, and that for the scraping sample was 2,295 parasites/ μ l. The level of parasitemia for the incision sample was significantly lower than that for the scraping sample ($P = 0.02$ by the rank sum test). Other comparisons were not significantly different statistically. When placental histology was used as the standard, the three techniques had very similar sensitivities (76 to 78%), specificities (98 to 99%), positive predictive values (92 to 98%), and negative predictive values (93 to 94%).

DISCUSSION

Approximately 50 million pregnancies occur each year in countries where malaria is endemic, half of which are in Africa (20); but our tools for diagnosing placental malaria—peripheral and placental microscopy, placental histology, and more recently, antigen detection tests and PCR—have rarely been evaluated in a systematic manner. We compared microscopy (including different techniques for preparation of placental blood films) and histology as tools to detect *P. falciparum* infection and for their associations with maternal hemoglobin concentrations and infant birth weight.

Placental histology was considerably more sensitive than microscopy with peripheral or placental blood in detecting malaria, and microscopy with placental blood was more sensitive than that with peripheral blood (Table 1), consistent with previous observations (10, 18, 22). *P. falciparum* infection of peripheral blood in the absence of placental infection was rare and was detected in 1.4% of women. Pregnancy-associated malaria is believed to be due to the presence of parasite variants capable of sequestering in the placenta through adhesion to ligands such as chondroitin sulfate A and hyaluronic acid (4), explaining the high prevalence of placental infection seen relative to the prevalence of peripheral blood infection. A

minority of women may be infected with variants that also circulate in the wider population; these variants adhere to CD36 cells and form erythrocyte rosettes (4, 12) and may sequester elsewhere in the body. These variants may not be detected in placental smears. Placental pathological changes associated with malaria are reported to be uniformly spread throughout the placenta, although no data were presented (24). Our samples for placental histology were collected after initial examination of the placental film, sometimes from a different paracentric site. Systematic comparison of the detection and the density of parasitized erythrocytes at different sites in single placentas would be of interest.

Microscopically detectable *P. falciparum* infection was more common in women with their first and second pregnancies than in multigravid women; but infection detected by histology only and past infection did not differ with gravidity, and similar numbers of women were uninfected. Previous studies with Malawian women show parasite prevalences and densities as determined by microscopy to be higher in women with their first or second pregnancies than in multigravid women (13, 15, 22). Gravidity appears to influence the detection of infection by microscopy but not the prevalence of low-grade *P. falciparum* infection in pregnancy.

Birth weights differed significantly with a diagnosis of malaria, and the prevalence of LBW babies was significantly higher for women with microscopically detectable infection (Table 2). Recent analysis suggests that malaria in pregnancy predisposes the infant to death through its effects on birth weight and may be responsible for 75,000 to 200,000 infant deaths each year (20). Women with microscopically detectable malaria were more likely to be anemic and had a mean hemoglobin concentration 0.7 g/dl lower than that for uninfected women. Malaria in pregnancy is responsible for an estimated 400,000 cases of severe anemia and 10,000 maternal deaths each year (6). Successful antimalarial drug interventions with increased birth weight and the maternal hemoglobin concentration as the key endpoints have been reported to result in changes in birth weight of 100 to 200 g and changes in hemoglobin concentration of about 0.5 g/dl (9, 11, 13, 17). The differences in infant birth weights and maternal hemoglobin concentrations between women with present or past infection with *P. falciparum* identified by histology alone and uninfected women are within this range. While microscopy identified the women at highest risk, women with malaria identified by histology alone form a significant, and often neglected, proportion of those with malaria-related morbidity in pregnancy.

Apart from histology and microscopy of peripheral and placental blood, other methods for detection of placental malaria have recently been evaluated. Antigen detection tests (rapid immunochromatographic tests) measure parasite-derived his-

tidine-rich protein 2 or parasite-specific lactate dehydrogenase secreted into blood. In our hands, the Optimal test for measurement of lactate dehydrogenase was no better than microscopy for detection of placental parasitemia (8), whereas others reported that the test for measurement of parasite-derived histidine-rich protein 2 detected more placental infections than peripheral blood microscopy (7, 10). PCR amplifies *P. falciparum*-specific DNA for detection of low-level parasitemia or circulating genetic material (19) and was significantly more sensitive than microscopy with peripheral blood for detection of placental parasitemia (8, 10). Women in whom malaria was detected by PCR but not microscopy were more likely to be anemic than uninfected women (10) but did not have babies with LBWs (8). In each of these studies microscopy with placental blood rather than histology was used as the comparator, and further evaluation of these techniques in comparison to histology is indicated.

HIV infection has been associated with an increased prevalence and density of malaria in pregnancy in a number of studies (21, 23). In our study, HIV infection was not significantly related to the likelihood of malaria as detected by microscopy or placental histology. This may reflect our relatively small sample size compared to those in the epidemiological studies cited.

Microscopy performed with blood from a placental incision had a relatively low sensitivity for detection of malaria in comparison to that of histology. To determine whether the method of preparation of placental blood films affected the likelihood of a positive film, we compared three different methods for preparation of placental blood films. The placental incision is commonly used. The blood welling into the incised placenta is collected. Cells which are free in the intervillous space or fetal vessels are preferentially sampled. Incision gave the lowest parasite densities, suggesting that parasitized erythrocytes may be preferentially retained in the intervillous space through adhesion to host receptors or other processes. The washing approach is analogous to the methods that we have used to extract parasitized erythrocytes from the intervillous space (3). In the present series, by using small pieces of tissue, the washing approach gave a modest increase in the level of parasitemia, but it may not greatly facilitate the detachment of cells from the syncytiotrophoblast (5) or from masses in the intervillous space (1). Scraping may detach some of these adherent cells and gave the highest parasite densities of any approach tested. Interestingly, all approaches were very similar in their specificities, sensitivities, and predictive values compared to the results of histology. Over 20% of samples positive by histology were negative by use of blood films prepared by all three methods. The mode of preparation of placental blood films appears to influence the level of parasitemia but not the likelihood of detection of malaria.

Blood film microscopy identifies a subset of women with malaria who have a high risk of anemia and of having LBW infants, but malaria in pregnancy is most sensitively diagnosed by placental histology. The diagnosis of present and past infections by histology alone is also associated with decreased infant birth weight and decreased maternal hemoglobin concentration. Use of histological diagnosis of placental malaria in epidemiological studies will allow more detailed characteriza-

tion of the burden of morbidity attributable to malaria in pregnancy.

ACKNOWLEDGMENTS

This work was supported by the Wellcome Trust (Career Development Fellowship 046012 and Senior Fellowship 063215 to S.J.R.).

We thank Malcolm Molyneux and members of the Department of Obstetrics and Gynaecology, College of Medicine, University of Malawi, for support and Ebbie Chaluluka and Labes Njiragoma for assistance with sample collection.

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