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Microscopy Underestimates the Frequency of *Plasmodium Falciparum* Infection in Symptomatic Individuals in a Low Transmission Highland Area

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Abstract

In an area with unstable malaria transmission, detection of *Plasmodium falciparum* infection in 379 symptomatic individuals was assessed by microscopy and three polymerase chain reaction (PCR) methodologies. *P. falciparum* infection was detected in 25% of patients by microscopy, 37% by nested PCR, 41% by merozoite surface protein-2 (MSP-2) PCR, and 45% by a ligase detection reaction-fluorescent microsphere assay (LDR-FMA). Of the 64 individuals who were LDR-FMA positive, microscopy negative and did not receive treatment, 8 (12.5%) had persistent symptoms and returned for treatment. Malaria attributable fraction (MAF) in symptomatic individuals was 14.6% by microscopy (95% confidence interval [CI] = 6.6-21.8%) and 28.2% by nested PCR (95% CI = 17.9-37.2%). In this highland area, *P. falciparum* infection in symptomatic individuals is detected more frequently by PCR than microscopy, and most frequently by LDR-FMA. *P. falciparum* infection appears to resolve without treatment in most LDR-FMA-positive, microscopy-negative individuals, but is persistent in a subset of these individuals and requires treatment.

INTRODUCTION

The most commonly used test for malaria diagnosis is light microscopic examination of Giemsa- or Fields-stained thick and thin blood smears. However, microscopy may have low sensitivity when parasitemia is low.[1] In highland areas of Kenya with unstable, low transmission, we have shown that testing for *Plasmodium falciparum* infection by polymerase chain reaction (PCR) as compared with microscopy significantly increases estimates of asymptomatic *P. falciparum* infection, although frequencies detected by PCR remain low (5.9% to 14.5%).[2] Parasite density is typically lower in individuals with clinical malaria in

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areas of low as compared with high malaria transmission. If low-level parasitemia is common in an area of low transmission, microscopy might not detect all *P. falciparum* infections in individuals with symptoms of malaria, at least on initial visit. This study was conducted to evaluate whether PCR testing increases detection of *P. falciparum* infection in individuals in highland areas who have symptoms of malaria. Furthermore, a number of PCR-based methods have been used to detect *P. falciparum* infection in areas of stable transmission, but the optimal method to use in areas of unstable transmission is not clear. For this reason, presence of *P. falciparum* infection was assessed in this study by microscopy, nested *P. falciparum* PCR, merozoite surface protein-2 (MSP-2) PCR, and a multiplex PCR ligase detection reaction-fluorescent microsphere assay (LDR-FMA).

MATERIALS AND METHODS

Study participants were recruited from Kapsisiywa and Kipsamoite, highland regions in the Nandi Hills District of western Kenya prone to malaria epidemics. Malaria epidemics started in these areas in the 1980s, with severe outbreaks in 1997 and 1998. A total of 1,109 individuals ranging from 1 month to 83 years of age were followed weekly for development of clinical malaria over an 11-month period from January to November 2004. Individuals were asked about symptoms of malaria weekly (fever, chills, severe malaise, severe headache) and were requested to contact a study field assistant if they ever developed symptoms. These symptoms were chosen based on previous studies we performed in highland sites of Kenya documenting that all individuals with *P. falciparum* on a blood smear who were considered to have clinical malaria had one or more of these symptoms (C. John, unpublished data). Inclusion criteria were the presence of one or more of the above symptoms of malaria. Exclusion criteria were the presence of a clear alternative diagnosis as determined by a clinical officer. Axillary temperature was recorded, but a measured fever (temperature $\geq$ 37.5°C) was not required for a diagnosis of malaria. Specific individual symptoms were not recorded in this study. If symptoms of malaria developed, a blood sample was collected by finger prick with a lancet. Thick and thin blood smears were prepared for microscopic examination and ~200 μL of blood spotted onto Whatman FTA filter paper cards (Whatman, Florham Park, NJ) for DNA extraction and PCR assays. Microscopy was performed by senior microscopists from the Division of Vector Borne Diseases and readings were verified by a second reader, with discrepancies resolved by a third reader, as previously reported.[3] All study microscopists had extensive training at the Kenyan Ministry of Health Division of Vector Borne Diseases, and received certification of their expertise at the Walter Reed Army Institute for Research microscopy course.

Symptomatic individuals who were microscopy positive were treated by the clinical officer at the local health center with sulfadoxine-pyrimethamine, quinine, or amodiaquine, in accordance with the Kenya Ministry of Health guidelines for treating uncomplicated malaria at the time of this study. Individuals who were microscopy negative were evaluated and treated for other illnesses as appropriate by the health center clinical officer. The clinical officer had the option of treating for malaria even with a negative blood smear in case of a strong clinical suspicion of malaria. All individuals were followed weekly for symptoms of malaria for the 11-month period. The health center provided free evaluation, microscopy, and treatment for all study participants, and study participants were asked to seek an evaluation and treatment of malaria at the health center. This study was approved by the Kenya Medical Research Institute (KEMRI) National Ethical Review Committee and the Institutional Review Boards for Human Studies at University Hospitals of Cleveland, Case Western Reserve University, and the University of Michigan.

Nested *P. falciparum* PCR was performed with nest-one and nest-two primers as described.[4] The MSP-2 PCR reactions were performed using two sets of primers that detect two major allelic families, FC27 and ICI for the MSP-2[5] and scored positive for *P. falciparum* if there was a PCR product for both FC27 and ICI or for anyone of these alleles. The LDR-FMA was performed using genus-specific primers and species-specific probes to *P. falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae* as reported.[6,7] Briefly, the small subunit ribosomal RNA (ssu rRNA) gene common for the four human *Plasmodium* species was amplified by PCR[7] after optimizing the PCR for semi-quantitative detection of *Plasmodium* template concentrations by analysis of dilutions of genomic DNA of the four *Plasmodium* parasite species. Amplicons from genus-specific PCR were used for multiplex LDR with species-specific primers and subsequent hybridization with probes bound to fluorescent microspheres that emit unique fluorescent signals[6] for reading by a Bio-Plex array reader (Bio-Rad, Hercules, CA). A stringent microsphere fluorescent intensity (MFI) threshold of 200 units, determined as previously reported,[6] was used as a cutoff to declare LDR-FMA signals positive for *Plasmodium* detection. For nested and MSP-1 PCR assays, a positive control (DNA from a 3D7 malaria parasite strain, or for MSP-2 FC27, an FC-27 strain) and a negative control (DNA from an uninfected human) were run with each set of samples tested, whereas DNA samples from the four known *Plasmodium* species (positive controls) and DNA from uninfected human (negative control) were used for optimization and assays of LDR-FMA PCR. Laboratory personnel doing PCR assays were blinded to microscopy results. Multiplex LDR-FMA had the highest number of positive results (Table 1), and has previously detected lower level parasitemia than other PCR methods,[6,7] so sensitivity, specificity, positive predictive value, and negative predictive value of the other test methods were assessed as compared with LDR-FMA.[8] Because microscopy is still considered the gold standard for diagnosis of malaria, the sensitivity and specificity of LDR-FMA as compared with microscopy was also assessed. Concordance between test methods was calculated using Cohen's Kappa statistic. The malaria attributable fraction (MAF) in symptomatic patients in a subunit of the area was computed by logistic regression.[9]

RESULTS

P. falciparum infection as detected by microscopy and three methods of PCR testing

A total of 379 episodes of symptoms consistent with malaria (history of fever, chills, headache, or severe malaise) occurred in the study population over the 11-month study period. Axillary temperature was recorded in 313 of the 379 individuals. PCR-based methods detected 46% to 78% more cases of *P. falciparum* infection in symptomatic individuals than were detected by microscopy (Table 1). The LDR-FMA detected the largest number of positive results (Table 1) and enabled us to assess for *P. vivax*, *P. ovale*, and *P. malariae* infection from the same sample. We documented frequencies of infection of 0%, 0% and 0.8% for *P. vivax*, *P. ovale*, and *P. malariae* infection, respectively. No infections with *P. vivax*, *P. ovale*, and *P. malariae* were detected by microscopy. Because LDR-FMA has been previously shown in experimental conditions to detect levels of parasitemia lower than those detectable by microscopy[6] or other PCR methods,[7] sensitivity and specificity of microscopy and PCR methods were compared with LDR-FMA, using LDR-FMA results as true positives. Specificity was almost identical for microscopy and the two PCR methods as compared with LDR-FMA (~95%), but sensitivity increased from microscopy (50.9%) to nested PCR (75.1%) to MSP-2 PCR (85.8%) (Table 2). If microscopy was used to define true positives, LDR-FMA had a sensitivity of 90.5% and a specificity of 70.8% in comparison to microscopy. Concordance with LDR-FMA was 0.49 for microscopy, 0.71 for nested PCR, and 0.81 for MSP-2 PCR (Table 3). Individuals who were microscopy negative but LDR-FMA positive had a significantly lower level of *P. falciparum* parasitemia, as assessed by LDR-FMA MFI, than those who were positive by both microscopy and LDR-FMA (median MFI of 6,072 [range = 241-21,175] for smear-negative individuals versus median MFI of 15,877 [range = 219-20,412] for smear-positive individuals, $P < 0.001$). Mean age did not differ in microscopy positive/

LDR-FMA positive and microscopy negative/LDRFMA-positive individuals (mean age in years [SD] 17.4 [18.1] versus 18.9 [16.1], respectively, $P = 0.54$).

Fifty-one of the 84 microscopy-positive individuals (60.7%), in whom temperature was measured, had a measured fever (axillary temperature ≥ 37.5°C) as compared with 72 of 154 individuals LDR-FMA positive (46.7%) and 63 of 124 (50.8%) nested PCR positive individuals. Among LDRFMA-positive individuals, frequency of measured fever was significantly higher in microscopy-positive individuals as compared with microscopy-negative individuals (50 of 77, 64.9% versus 22 of 77, 28.6%, $P < 0.0001$).

The MAF in symptomatic patients for blood smear microscopy and nested PCR was computed by logistic regression.[9] The MAF was calculated using previous data on the prevalence of asymptomatic parasitemia by blood smear microscopy and nested PCR from a subunit of the highland area in this study that comprised approximately half the population studied,[2] and data from the symptomatic individuals from this subunit area. The MAF was 14.6% for microscopy (95% CI = 6.6-21.8%) and 28.2% for nested PCR (95% CI = 17.9-37.2%). In patients with measured fever (axillary temperature > 37.5°C), the MAF was 38.2% for microscopy (95% CI = 17.7-53.5%) as compared with 44.9% for nested PCR (95% CI = 22.5-60.9%). The MAF could not be calculated for LDR-FMA because previous samples from asymptomatic individuals were not tested by LDR-FMA.

Frequency of recurrent infection or persistent symptoms in individuals with infection detected by LDR-FMA versus microscopy

During the study period, 95 *P. falciparum* infections were detected by microscopy. Seventy-six individuals were infected a single time (80% of all infections), 8 individuals had infection twice (16.8% of all infections), and 1 individual had infection three times (3.2% of all infections). In contrast, of the 169 cases of *P. falciparum* infection detected by LDRFMA, 109 individuals were infected a single time (64.5% of all infections), 19 individuals had infection twice (22.5%), and 5 individuals had infection three or more times (13%). The frequencies of individuals with more than one infection did not differ significantly between the testing methods (10.6% for microscopy, 18.0% for LDR-FMA, $P = 0.13$). In 19 of the 83 episodes in which *P. falciparum* infection was detected by LDR-FMA but not microscopy (22.9%), anti-malarial medication was given based on clinical concern for malaria despite the negative blood smear results. In the remaining 64 episodes (77.1%), no anti-malarial medication was given. Eight of the 64 individuals not treated with anti-malarial medication (12.5%) returned to the health center within 45 days with persistent or recurrent symptoms of malaria. Of these 8 individuals, 3 were positive (37.5%) for *P. falciparum* infection on a return visit by microscopy but all 8 were positive by LDR-FMA. Mean age of individuals who were LDR-FMA positive, microscopy negative who returned for treatment (mean age in years [SD] 23.1 [16.2]) did not differ significantly from that in individuals who did not return for treatment (mean age in years [SD] 17.6 [16.0], $P = 0.21$)

DISCUSSION

The study findings demonstrate that in this area of unstable, low malaria transmission, microscopy consistently underestimated the frequency of *P. falciparum* infection in symptomatic individuals. The PCR methods detected from 46% to 78% more *P. falciparum* infections than microscopy, with LDR-FMA detecting the highest frequency of infection. The majority of individuals who were LDR-FMA positive but microscopy negative appear to have resolved the infection without treatment. However, 12.5% of individuals had persistent symptoms, and all of these individuals remained LDRFMA positive on repeat testing, so it appears that there is at least a subset of symptomatic individuals with clinical malaria who have low-level parasitemia undetected by microscopy but detected by LDR-FMA.

Currently, microscopy is the most widely used diagnostic test for malaria infection, but it has a number of significant limitations.[1,10,11] Low parasite densities, which are common in low malaria transmission areas, and secondary *Plasmodium* species may not be detected by microscopy.[1] Parasites may also be washed off or lysed during staining. The limitations of blood smear light microscopy are of practical relevance in the Kenyan highlands as presumptive diagnosis and selftreatment of malaria often lead those who are ill to use herbal remedies, to visit traditional healers, or use over-the-counter anti-malarials, all of which are associated with underdosing.[12,13] When efficacious or partially efficacious medication is taken prior to a health center visit, resulting parasitemia below the limit of microscopy detection can confound proper malaria treatment.

It is unlikely that the low rate of microscopy positives in our study was the result of sub-standard microscopy reading. Slides were confirmed independently by two readers, with any discrepancies resolved by a third reading. All study microscopists had extensive training at the Kenyan Ministry of Health Division of Vector Borne Diseases, and received certification of their expertise at the Walter Reed Army Institute for Research microscopy course. The high specificity of our microscopy readings when compared with the PCR methods further demonstrates the quality of these readings. Thus, we believe that the increased detection by PCR reflects an increase in detection from the most rigorous microscopy detection, and that even higher increases in detection by PCR as compared with microscopy might be seen when microscopy is performed in standard field settings as opposed to research settings.

Most studies assessing PCR methods for detection of parasitemia in malaria-endemic areas have been conducted in areas of higher transmission than the highland area in this study, and have usually been conducted to assess asymptomatic parasitemia. The increased detection of infection by PCR noted in the present study (1.5- to 1.8-fold increase over microscopy) is in the range of those seen in studies of asymptomatic parasitemia in areas of mid-level transmission (2- to 2.5-fold increase) [14] and low-level transmission (~1.4- to 5-fold increase). [15] Studies of PCR in symptomatic individuals would not be useful in high transmission areas, where most children have parasitemia even when asymptomatic. We are not aware of such studies being conducted in low transmission areas, where they would be useful, so the present study provides important new information. The increased detection of parasitemia by PCR methods is unlikely to reflect background asymptomatic parasitemia in the present study area. Our earlier studies in this population documented low levels of asymptomatic parasitemia (range by microscopy, 2.9-7.9%, by nested PCR, 5.9-14.5%).[2] Assessment of the MAF in symptomatic individuals using the current data and prior nested PCR data documented that MAF almost doubled when assessed by nested PCR results (28.2%) as compared with microscopy results (14.6%). The MAF remained higher, though less markedly so, when assessed only in patients with measured fever (microscopy, 38.9% versus nested PCR, 44.2%). Although the use of measured fever is more clinically rigorous than the use of symptoms for calculation of MAF, many individuals with malaria may not have measured fever at the time of clinical evaluation, so calculation of MAF in both groups of individuals appears appropriate. The pronounced increase in MAF when assessed by nested PCR as compared with microscopy suggests that the symptoms in a substantial proportion of PCR-positive, microscopy-negative individuals in this highland area are indeed attributable to malaria.

The PCR-based tests used in this study detected more *P. falciparum* infections than microscopy but also showed variations in performance with respect to LDR-FMA. Nested *P. falciparum* PCR detected fewer infections than MSP-2 PCR or LDR-FMA and had a slightly lower concordance with MSP-2 PCR and LDR-FMA. We cannot definitively determine whether LDR-FMA detected more infections because it detected false positives or because it is truly more sensitive than other PCR methods, because there is still no final “gold standard” for diagnosis of *P. falciparum* parasitemia. However, concordance between all three PCR methods was very high (kappa score 0.71-0.81), although concordance between microscopy and any of the PCR methods was lower (kappa score 0.49-0.54). The strong concordance between the three different PCR methods, with each method using a set of primers that targets a different area of the *P. falciparum* genome, suggests that the increased detection by the PCR methods, including LDR-FMA, is unlikely to be caused by false positives. The detection of dead parasite forms present after earlier treatment cannot be absolutely excluded, but the individuals tested did not report receiving prior treatment, and the availability of immediate, free access to evaluation and treatment make it unlikely that a large proportion of them would seek treatment elsewhere initially. In an earlier finding, LDR-FMA had greater sensitivity than sequence-specific oligonucleotide probe hybridization (SSOPH) PCR for detection of low-level parasitemia.[7] This supports the idea that LDR-FMA may detect more infections than other PCR methods in areas where parasite densities are typically low. The LDR-FMA method allows for semi-quantitation of parasitemia and allowed us to demonstrate that individuals who were microscopy positive had higher levels of parasitemia than those who were microscopy negative. This again supports the idea that this method is particularly well suited to detection of lower level parasitemia. The multiplex LDR-FMA system also allowed testing for all four human *Plasmodium* species from a single sample and confirmed that malaria in this area is almost exclusively caused by *P. falciparum*.

The present study brings to light important challenges confronting clinicians when evaluating and treating patients with malaria symptoms in areas like the highlands of Kenya, where transmission is low. Current Kenyan Ministry of Health guidelines direct that artemether-lumefantrine be given only to those who are microscopy positive for *P. falciparum*.[16] This directive is appropriately intended to prevent overuse of artemether-lumefantrine and decrease the development of drug resistance. The present data demonstrate that the majority of individuals with symptoms of malaria in this highland area were not infected with *P. falciparum*, even when tested by PCR, but if LDR-FMA detection of *P. falciparum* is clinically relevant, as many as 49% of clinical cases (83 of 169) attributable to malaria might have gone untreated. A key factor in assessment of the clinical relevance of this finding is whether the absence of recurrent illness or malaria complications in symptomatic individuals with infection by PCR reflects the effects of treatment obtained outside the health center system, a degree of natural immunity or some combination of these two factors.

The large increase in MAF among symptomatic individuals when MAF was assessed by nested PCR (28.2%) as compared with microscopy (14.6%) supports the idea that a substantial proportion of the additional cases detected by PCR are clinically relevant. Furthermore, approximately a third of those who were LDR-FMA positive, microscopy negative were either treated for malaria at the initial visit because of ill appearance and lack of another diagnosis or returned because of persistent symptoms. Conversely, approximately two thirds of those who were LDR-FMA positive, microscopy negative did not receive treatment or complain of persistent symptoms, and LDR-FMA-positive, microscopy-negative individuals had significantly lower frequencies of measured fever than LDR-FMA-positive, microscopy-positive individuals (28.6% versus 68.9%, $P < 0.0001$). Taken together, the findings suggest that detection of parasitemia by LDR-FMA is clinically relevant in a sub-set, likely a minority of individuals. Further studies in additional populations are required to better delineate risk of disease without treatment in PCR positive, microscopy negative symptomatic individuals in areas of low, unstable transmission.

The LDR-FMA method of *P. falciparum* detection has limitations, including the use of assigned cutoff levels that may result in false positives or false negatives if set too low or too high. Negative control samples were used to optimize the cutoff and, in this study, conservative values were chosen to avoid false positives, thus enhancing specificity. Even with the conservative cutoff level, LDR-FMA detected more infection than the other PCR methods, suggesting there was no lack of sensitivity for this assay. The high concordance of LDR-FMA and MSP-2 PCR also suggests that the LDR-FMA assay was specific. Although PCR, in general, remains impractical in clinical settings in developing countries, LDR-FMA may be a useful research tool for detection of *Plasmodium* species infection, particularly in areas where more than one species of *Plasmodium* causes human disease. It might also be effective in highland areas, where parasite densities are frequently low.

In summary, our study findings document that the malaria attributable fraction in symptomatic individuals or individuals with measured fever in a highland area is increased when assessed by nested PCR in comparison to microscopy. The precise clinical relevance of detection by nested PCR or LDR-FMA in microscopy-negative individuals in this area remains to be elucidated. The present data suggest that persistent infection requiring reevaluation occurs in a minority of these individuals, but further studies are required to definitively address this question. Among the PCR methods, LDR-FMA detected the highest frequency of *P. falciparum* infection and appears to be a sensitive and specific method for monitoring infection by *P. falciparum* and other human *Plasmodium* species in individuals with malaria symptoms in areas of low transmission.

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Table 1

Comparison of results obtained for different *P. falciparum* PCR tests as compared with microscopy in symptomatic individuals in a highland area

Test	Positive	Negative	% Positive	Microscopy negative, PCR positive	Microscopy positive, PCR negative
Microscopy	95	284	25.1	-	-
Nested PCR	139	240	36.7	60	16
MSP2 PCR	156	223	41.1	72	11
LDR-FMA	169	210	44.5	83	9

Table 2

Comparison of microscopy and standard PCR tests for *P. falciparum* detection to LDR-FMA*

Test	**Sensitivity**	**Specificity**	**PPV%**	**NPV%**
Microscopy	50.9 (43.4-58.4)	95.7 (93.0-98.5)	90.5 (84.6-96.4)	70.8 (65.5-76.1)
Nested PCR	75.1 (68.6-81.7)	94.3 (91.2-97.4)	91.4 (86.7-96.0)	82.5 (77.7-81.3)
MSP-2 PCR	85.8 (80.5-91.1)	94.8 (91.8-97.8)	92.9 (88.9-97.0)	89.2 (85.2-93.3)

*For this comparison, LDR-FMA results were considered “true positives.” PPV denotes positive predictive value and NPV denotes negative predictive value. The figures in parentheses show the 95% confidence intervals.

Table 3

Pairwise comparisons of concordant tests for *P. falciparum* detection between different test methods

	Nested PCR Kappa (95% CI)	MSP-2 PCR Kappa (95% CI)	LDR-FMA Kappa (95% CI)
Microscopy	0.54 (0.45-0.63)	0.52 (0.42-0.61)	0.49 (0.39-0.58)
Nested PCR		0.76 (0.75-0.87)	0.71 (0.63-0.78)
MSP-2 PCR			0.81 (0.75-0.87)