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High Prevalence of Asymptomatic *Plasmodium falciparum* Infection in Gabonese Adults

Matthias P. Dal-Bianco,* Kai B. Köster, Ulrich D. Kombila, Jürgen F. J. Kun, Martin P. Grobusch, Ghyslain Mombo Ngoma, Pierre B. Matsiegui, Christian Supan, Carmen L. Ospina Salazar, Michel A. Missinou, Saadou Issifou, Bertrand Lell, and Peter Kremsner

Medical Research Unit, Albert Schweitzer Hospital, Lambaréné, Gabon; Department of Parasitology, Institute of Tropical Medicine University of Tübingen, Germany; Infectious Diseases Unit, Division of Clinical Microbiology and Infectious Diseases, National Health Laboratory Service and Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

Abstract. *Plasmodium falciparum*, the most common malarial parasite in sub-Saharan Africa, accounts for a high number of deaths in children less than five years of age. In malaria-endemic countries with stable transmission, semi-immunity is usually acquired after childhood. For adults, severe malaria is rare. Infected adults have either uncomplicated malaria or asymptomatic parasitemia. During a period of one year, we screened 497 afebrile males to investigate the prevalence of asymptomatic *P. falciparum* parasitemia in villages near Lambaréné, Gabon by use of three different methods. A total of 52% of the individuals had parasites detected by a subtelomeric variable open reading frame polymerase chain reaction (*stevor*-PCR), 27% of the rapid diagnostic test results were positive, and 12% of the thick blood smears with low parasitemias had *P. falciparum*. Most positive cases were only detected by the *stevor*-PCR. Asymptomatic *P. falciparum* parasitemia in adults living in a malaria-endemic country is frequent.

INTRODUCTION

Plasmodium falciparum accounts for most malaria-related morbidity and mortality in sub-Saharan Africa. Incidence of malaria in these countries can be perennial or epidemic and caused by stable or unstable transmission. In an area of stable malaria transmission, infections with this parasite species can occur in different ways. Infants up to an age of three months rarely have parasites or malaria.[1] Children less than five years of age have greater morbidity due to severe malaria.[2,3] Asymptomatic parasitemia is uncommon in this age group.[4] Older individuals are more likely to have uncomplicated malaria or asymptomatic parasitemia.[5] A difference in the type of disease is related to age. Over time, each disease episode leads to acquisition of immunity. In an area highly endemic for malaria, protection from severe malaria is acquired early in childhood, although it takes longer to develop protection from less severe disease.[6] Although immunoprotective mechanisms clear a large proportion of infected erythrocytes, a certain amount can persist in the circulation, which leads to asymptomatic parasitemia. Besides frequent reinfections, immune evasion by antigenic variation explains the mechanism, which leads to persistent infection even at a submicroscopic level.[7]

Detection of low parasitemia with *P. falciparum* is a challenge. Although diagnostic methods have improved greatly over the past decade, for malaria diagnosis the classic thick blood smear method still remains the standard in clinical malaria diagnosis. Rapid diagnostic tests (RDTs) are a fast and easy-to-perform alternative method of diagnosis, but they show decreased sensitivity at lower parasitemia.[8] Most of these tests are based on detection of several proteins, including histidine-rich protein 2 (HRP-2), which is the most sensitive antigen marker for *P. falciparum*.[9] Because of its long half-life, detection of this protein does not always correlate with the presence of viable parasites, thus leading to false-positive results and lower specificity.[10] Parasite-specific aldolase (panmalarial antigen) is used as a complementary antigen in second-generation HRP-2 tests being produced for use with the four *Plasmodium species* that infect humans.[9] Polymerase chain reaction (PCR) techniques detect parasites at densities less than one parasite per microliter. The subtelomeric variable open reading frame (*stevor*) gene sequences are unique to *P. falciparum* and can be used for highly sensitive PCR assays. With this method, 10 parasites can be detected in 1 mL of blood.[11]

In this study, using three different methods for parasite detection, we report the prevalence of asymptomatic *P. falciparum* infection in adults living near Lambaréné, Gabon.

SUBJECTS AND METHODS

Study area and population. The study was conducted in the Albert Schweitzer Hospital in Lambaréné, Gabon, which is located within the African rain forest belt. Precipitation in this region is highest in March and November and lowest from July to August. The entomologic inoculation rate has been estimated to average 50 infective bites per person per year. Malaria is hyperendemic and perennial with moderate seasonal variation.[12,13]

During an epidemiologic survey, we enrolled 497 male 18–51-year-old individuals (mean ± SD age = 30 ± 8 years) in the study. All participants were healthy volunteers without clinical signs and symptoms of malaria from villages located ≤ 50 km from Lambaréné. Written informed consent was obtained before screening for *P. falciparum* parasitemia. Demographic data and dates of sampling were documented. For each individual only one finger prick was performed. We immediately prepared a thick blood smear for an RDT and dotted blood on Whatman (Brentford, United Kingdom) filter paper for analysis by a *stevor*-PCR. Samples were collected by a field-worker team from March 2005 to April 2006. In *Plasmodium spp.*–positive cases diagnosed from blood smear or by RDT, subjects were examined by a physician in our clinical trial unit. If symptoms of malaria developed, individuals were treated orally with artesunate (Arsumax®, 50 mg; Sanofi-Aventis, Gentilly, France) for five days. Ethical clearance was

* Address correspondence to Matthias P. Dal-Bianco, Medical Research Unit, Albert Schweitzer Hospital, BP 118 Lambaréné, Gabon and Department of Parasitology, Institute of Tropical Medicine University of Tübingen, Wilhelmstrasse 27, 72074, Tübingen, Germany. E-mail:matthiasdalbianco@yahoo.com

TABLE 1
Results for each test method*

	Thick blood smear	Rapid diagnostic test	*stevor* PCR
No.	493	496	470
Plasmodium falciparum	57 (12%)	132 (27%)	244 (52%)
Negative	436 (88%)	364 (73%)	226 (48%)

* *stevor* PCR = subtelomeric variable open reading frame polymerase chain reaction.

obtained from the ethics committee of the International Foundation Albert Schweitzer Hospital.

Malaria blood smear. In this study, the Lambaréné method for thick film preparation and reading was applied as described elsewhere.[14] Blood smears were stained with a 20% Giemsa solution. Two experienced technicians individually examined the microscopic slides. The main objective was to find *P. falciparum*. The technicians read at least 100 microscopic high-power fields with oil-immersion (CH30 microscope; Olympus, Tokyo, Japan). Parasitemia was calculated as parasites per microliter (counted parasites/no. of microscopic fields) × 600.[14] The final result was obtained by calculating the arithmetic mean from parasite count of the first and second readings. If different results for parasitemia were obtained, a third experienced technician read the slides to obtain a final result. A third reading was also conducted if the ratio of results between higher and lower parasitemias was > 1.5 or if both results for parasitemia were less than 300 parasites/μL and had a difference of more than 100 parasites/μL.

Rapid diagnostic test *Immunochromatographic antigen detection.* We used an RDT (NOW® ICT Malaria Test; Binax, Inc., Portland, ME), which detects markers for all four human pathogenic *Plasmodium* species. For each individual, 15 μL of EDTA-whole blood was applied to the test strip. The reagent, which contained buffer solution and detecting antibody against HRP-2 or parasite-specific aldolase labeled with colloidal gold, was then applied. All of tests were performed in accordance with the manufacturer's instructions and showed a well-visible control line. Tests were performed within five minutes after finger prick blood sampling. Interpretation of results was done after 15 minutes. Results were noted and the used test cards were archived.

PCR technique (stevor genes amplification). Thirty microliters of capillary blood of each individual, collected from the same finger prick, was applied onto filter paper (Whatman). The filter papers were dried and stored individually in sealed plastic bags. The DNA from dried blood spots was purified using a purification kit (Qiamp DNA mini kit; Qiagen, Valencia, CA).

A nested PCR was conducted according to the modified protocol of Cheng and others for detection of plasmodial DNA.[11] The PCR was conducted in a thermocycler (PTC-200; MJ Research, Watertown, MA). For the first-round PCR, we used four primers: P5, 5′-gggaattcTTTATTTGATGAAGATG-3′ (lowercase bases are not part of the *stevor* sequence); P18, 5′-TTTCA(C)TCACCAAACATTTCTT-′3; P19, 5′-AATCCACATTATCACAATGA-3′; and P20, 5′-CCGATTTTAACATAATATGA-3′. Ten microliters (5.0–25 ng) of DNA was added to 3 μL of P5, P18, P19, and P20 (10 μM), 10 μL of 10× buffer, 20 μL of $MgCl_2$ (25 mM), 0.8 μL of dNTP (25 mM), 0.5 μL of *Taq* polymerase (5 U/μL), and 46.7 μL of distilled water. A total of 22 cycles (93°C for 30 seconds, 50°C for 50 seconds, and 72°C for 30 seconds) were performed. For the second-round PCR, primers P17, 5′-ACATTATCATAATGAC(T)CCAGAACT-3′ and P24, 5′-GTTTCCAATAATTCTTTTTCTATC-3′ were used. Two microliters of first-round PCR products were added to 2 μL of P17 and P24 (10 μM), 5 μL of 10× buffer, 5 μL of $MgCl_2$ (25 mM), 0.8 μL of dNTP (25 mM), 0.25 μL of *Taq* polymerase (5 U/μL), and 33 μL of distilled water and 35 cycles (93°C for 30 seconds, 55°C for 50 seconds, and 72°C for 30 seconds) were performed. Five microliters of this PCR product was added to 1 μL of Sybr Green (Biozym Scientific, Hessisch Oldendorf, Germany), 2 μL of 5× buffer, and 2 μL of distilled water and analyzed by electrophoresis on a 2% agarose gel (Peqlab, Erlangen, Germany). Positive and negative controls were run in parallel with each test sample. The presence of DNA fragments was interpreted as a positive test result.

Statistical analysis. Results of 497 individuals were used for the assessment of data quality on the basis of descriptive statistics, including means and standard deviations, medians and interquartile ranges, and proportions. Data of 466 individuals with results for each test method were analyzed for stratification of parasitemia and comparison of the diagnostic methods by calculating the coefficient of agreement. Sample size calculation was not done. Data analyses were performed using SAS JMP version 5 (SAS Institute, Inc., Cary, NC) and Excel® 2002 Microsoft (Redmond, WA).

RESULTS

Parasitemias in the thick blood smears of asymptomatic carriers. A total of 57 (12%) (95% confidence interval [CI] = 9–15%) of 493 individuals showed *P. falciparum* parasites

TABLE 2
Stratification by parasite density in thick blood smear and correlation with rapid diagnostic test (RDT) and stevor PCR*

	Parasite densities				
	0	1–100	101–1,000	> 1,000	> 0
No.	412	28	19	7	54
Mean age (years) [SD]	30 [8]†	25 [6]	31 [9]	27 [8]	27 [8]
Parasite count/μL‡	0	33 (6–94)	317 (110–990)	4,197 (1,200–16,650)	136 (6–16,650)
RDT positive	75	25	19	7	51
RDT negative	337	3	0	0	3
stevor PCR positive	188	28	19	7	54
stevor PCR negative	224	0	0	0	0

* *stevor* PCR = subtelomeric variable open reading frame polymerase chain reaction. Total number = 466.
† Missing data for six individuals.
‡ Parasite count/μL = geometric mean (range).

during microscopic examination. The range of parasitemia was 6 to 16,650/μL and the geometric mean was 136/μL (Table 1).

Of 466 individuals for whom all three diagnostic tests were performed, 28 had a low parasitemia (<100/μL), 19 of 466 individuals had a parasitemia between 100 and 1,000/μL, and 7 of 466 individuals had a parasitemia greater than 1,000/μL (Table 2).

Three individuals had *P. malariae* infections, which were reported as negative for *P. falciparum*, and one individual, in whom the other tests were not done, had a mixed infection of *P. falciparum* and *P. ovale*.

Results of RDT in the absence of clinical symptoms. Histidine-rich protein 2 was detected in 132 (27%) (95% CI = 23–31%) of 496 RDTs (Table 1). In 17 of these tests with positive results, there was also a line for the parasite specific aldolase, which indicated a *P. falciparum* or mixed infection.

Screening for *P. falciparum* by PCR. Overall, 244 (52%) (95% CI = 47–56%) of 470 samples were positive in the *stevor* PCR (Table 1).

Comparison of diagnostic test results. A total of 466 samples for which results of all three tests were available were analyzed (Tables 2–4). Although 114 of 466 tests results were positive in the PCR and in either one or both of the other tests, 128 of 466 test results were positive only in the PCR. The coefficients of agreement (Cohen's kappa) were 0.22 for thick blood smear and *stevor* PCR, 0.30 for RDT and *stevor* PCR, and 0.81 for RDT and thick blood smear. When we used the PCR as the gold standard, the sensitivity and specificity were 22% and 100% for the blood smear and 46% and 93% for the RDT. The positive predictive values and negative predictive values were 100% and 54% for the blood smear and 88% and 62% for the RDT.

DISCUSSION

In the present study, we found a high prevalence of asymptomatic carriers of *P. falciparum* in a young male adult population. Each of the methods used has its own advantage. Most positive cases were detected by the *stevor* PCR. The method has been reported to be at least 100-fold more sensitive than other PCR assays.[11] Assuming that DNA fragments remain in blood for only a short time, the PCR should only detect living parasites.[15] The thick blood smear and PCR results show the presence or absence of parasites at an exact time point. The RDT, due to the long half-life of its HRP-2 marker, provides evidence of the situation in the weeks before sampling. An increase in parasitemia, with a subsequent decrease below the microscopic detection limit, seems likely for those with a positive result in the RDT and PCR. Sequestered parasites can be detected by the RDT, which cannot be done with the blood smear or the most sensitive PCR method. In most cases, a positive RDT result should detect asexual stages because most of the HRP-2 is released during schizont rupture. Sequestered gametocytes do not contribute to production of this marker.[16] This finding indicates that methods determining circulating and sequestered parasite loads must be distinguished. In this context, another useful method is quantification of malaria pigment in leukocytes.[17] It seems reasonable that there is a higher sequestered parasite load in people with asymptomatic parasitemia from malaria-endemic countries than in travelers returning from the tropics.

Although sensitivity for the thick blood smear was not high, the advantages of this procedure were high specificity, quantification of parasitemia, and easy handling. Not sufficiently screening blood transfusions that contain low parasite densities and administered to non-immune individuals, such as children, can be a concern because of transfusion-malaria. Detection of parasites depends on the amount of blood processed. Even the most sensitive PCR method may not be successful because only a small amount of blood is usually examined.

During the last century, efforts were made to control vectors of malaria. Attempts were made either to eradicate mosquitoes or to protect individuals from mosquito bites. However, less attention was paid to the human reservoir of *P. falciparum*. A study in Brazil underlines the importance of asymptomatic carriers with low-level parasitemias for malaria transmission. Persons with PCR-detectable parasitemia may serve as a reservoir for transmissible parasites.[18] Therefore, treatment may be useful for malaria control. However, persistent low-level parasitemia seems to be a prerequisite for enduring semi-immunity in African adults. Thus, asymptomatic *P. falciparum* parasitemia in adults living in a malaria-endemic country seems to be common.

TABLE 3

Positive results obtained in each of the three tests*

stevor PCR	Thick blood smear	Rapid diagnostic test	No.
+	+	+	51
+	+	−	3
+	−	+	60
+	−	−	128
−	−	+	15
−	−	−	209

* *stevor* PCR = subtelomeric variable open reading polymerase chain reaction. Total number = 466.

TABLE 4

Sensitivity specificity, positive predictive value (PPV), and negative predictive value (NPV)*

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Thick blood smear	22	100	100	54
Rapid diagnostic test	46	93	88	62

* Total number = 466. The subtelomeric variable open reading frame polymerase chain reaction was used as the gold standard.

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Authors' addresses: Matthias P. Dal-Bianco, Pierre B. Matsiegui, Michel A. Missinou, Saadou Issifou, Bertrand Lell, and Peter Kremsner, Medical Research Unit, Albert Schweitzer Hospital, BP 118 Lambaréné, Gabon and Department of Parasitology, Institute of Tropical Medicine University of Tübingen, Wilhelmstrasse 27, 72074, Tübingen, Germany. Kai B. Köster, Ulrich D. Kombila, Ghyslain Mombo Ngoma, Christian Supan, and Carmen L. Ospina Salazar, Medical Research Unit, Albert Schweitzer Hospital, BP 118 Lam-

baréné, Gabon. Jürgen F. J. Kun, Department of Parasitology, Institute of Tropical Medicine University of Tübingen, Wilhelmstrasse 27, 72074, Tübingen, Germany. Martin P. Grobusch, Infectious Diseases Unit, Division of Clinical Microbiology and Infectious Diseases, National Health Laboratory Service and School of Pathology, Faculty of Health Sciences University of the Witwatersrand, 7 York Road, Parktown 2196, Johannesburg, South Africa and Medical Research Unit, Albert Schweitzer Hospital, BP 118 Lambaréné, Gabon.

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