Submicroscopic *Falciparum* Malaria in Febrile Individuals in Urban and Rural Areas of Gabon

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Abstract. Characterization of the parasite reservoir is required to improve malaria control. Asymptomatic patients with subpatent parasitemia have been identified in Gabon, but the prevalence of such infections among febrile subjects is unclear. We assessed the prevalence of submicroscopic *Plasmodium falciparum* infections on an island (Port-Gentil), and in urban (Libreville), semiurban (Melen), and rural (Oyem) settings in Gabon. Blood samples ($N = 310$) from febrile patients were tested for malaria parasites by quantitative nucleic acid sequence–based amplification (QT-NASBA). Parasites were detected in 55.8% (173/310) of samples by microscopy and in 66.4% (206/310) of samples by 18S rRNA QT-NASBA. The proportion of submicroscopic infections differed considerably between sites. Gametocytes were found in 1% (3/310) of the individuals by microscopy and in 32% (99/310) by *Pfs25* mRNA QT-NASBA. Thus, submicroscopic parasitemia is frequent in febrile patients, and the detection of this condition is important, to improve disease control.

In the last decade, the malaria burden has considerably decreased in many countries.^{1,2} In areas presenting a recent decline in transmission intensity, submicroscopic parasitemia is particularly prevalent, as in areas of low malaria endemicity.^{3–5} These submicroscopic infections, commonly reported in asymptomatic individuals but underestimated among patients with symptoms, may contribute to the maintenance of disease transmission.^{6,7} In Gabon, the prevalence of malaria in febrile children decreased between 2000 and 2008, after the adoption of artemisinin-based combination therapies as the first- and second-line treatment of uncomplicated malaria. Insecticide-treated bed nets and sulfadoxine–pyrimethamine for intermittent preventive treatment were made freely available at all public health centers in Gabon.⁸ Nevertheless, a rebound of malaria morbidity was observed. Indeed, the risk of malaria increased in various areas of Gabon, concomitantly with a slowing of the prevention activities of the Malaria National Control Program.⁹ Few studies have investigated the extent of *Plasmodium falciparum* submicroscopic infection carriage in various settings of this country. Therefore, we performed a pilot study to assess the level of submicroscopic parasite carriage among symptomatic patients after the change in malaria policy in Gabon. The current study was part of a large cross-sectional survey conducted for the evaluation of malaria prevalence in four regional hospitals in Gabon, located in urban (Libreville), semiurban (Melen), and rural (Oyem) areas, and on an island (Port-Gentil). At each site, the prevalence of malaria was estimated by microscopy: 24.1% at Libreville, 31.3% at Melen, 44.2% at Oyem, and 6.5% at Port-Gentil. *Plasmodium falciparum* was the principal malaria-causing species identified.⁹ The study population, screened for malaria, consisted of patients who were either febrile or who had a recent history of fever. Informed consent for participation was obtained from the patients or their parents

or legal guardians. The following data were then collected: current fever or history of fever, age, and previous intake of antimalarial drugs. Fever was defined as an axillary temperature of at least 37.5°C. A blood sample was collected from each patient for thin and thick blood smears (15 μ L) and for blood spots (50 μ L) on Whatman standard filter paper (Whatman International Ltd., Maidstone, England) for molecular analysis. Thick smears were used for the detection of malaria parasites, as described by the method of Lambaré.¹⁰ We carefully spread 10 μ L of blood onto an area of 10 $\times$ 18 mm on a microscope slide and allowed it to dry. Slides were stained with 20% Giemsa and read under a light microscope ($\times$ 100) by two experienced technicians. Parasitemia was determined as the number of parasites per microliter of blood (p/ μ L). Matched thin blood smears were prepared for species identification. In cases of discordant results (presence or lack of asexual/sexual blood stages, mismatched species, or parasite density), the slides were reviewed by a third technician, who resolved any discrepancy. For parasite density determination, the mean of the two closest parasitemia values was used. Blood spots were dried and immediately placed in individual plastic bags at room temperature (20–25°C), in which they were stored for up to 1 year before processing. Nucleic acids were extracted with a semiautomated Mini-Mag analyzer (Biomérieux sa, Lyon, France), as previously described.¹¹ In brief, the blood spots on the filter paper were cut in four and each piece was placed in a sterile 1.5-mL tube at room temperature. Nuclisens lysis buffer (Biomérieux sa, Lyon, France) was heated in a water bath at 37°C for 30 minutes and then added to the sample-containing tubes. We added 50 μ L of silica solution (Nuclisens Magnetic Extraction Kits, Biomérieux sa, Lyon, France) to each tube. The nucleic acids were released from the paper and they bound to silica particles, forming complexes, during centrifugation. The complexes were washed three times each, in two different wash buffers. The nucleic acids were then eluted and stored at –20°C. Quantitative nucleic acid sequence–based amplification (QT-NASBA) with 18S rRNA and *Pfs25* mRNA was used for parasite and gametocyte detection.^{11,12} All samples, including positive and negative controls (filter paper free of DNA/RNA),

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were analyzed in duplicate. Samples were considered positive when the fluorescence of the target amplicons exceeded the mean fluorescence of three negative controls plus 20 standard deviations. Cultured gametocytes were used to generate a trend line, which was subjected to serial 10-fold dilutions from 10^6 to 10 gametocytes per milliliter, as a control for the identification of positive results and to assess amplification efficiency.

Data were analyzed with Statview 5.0 (SAS Institute, Cary, NC). The proportions of study participants were compared in Pearson's chi-square tests and continuous variables were analyzed in Mann-Whitney or Kruskal-Wallis tests. We considered P values below 0.05 to be significant.

In total, 310 patients, from 1 to 86 years of age, were included in this study; 15.8% (49/310) were adults (Table 1). Participants from Libreville were the oldest.

The proportion of study participants with microscopic asexual parasitemia was 55.8% (173/310), but gametocytes were detected in only 1% (3/310) of patients. All the gametocyte carriers were patients from Libreville, the urban area (2.8%; 3/106). Parasite prevalence by microscopy was 32% (21/66) on the island, 62.2% (66/106) in the urban area, 60.5% (46/76) in the semiurban area, and 64.5% (40/62) in the rural area, and the difference between settings was significant ($P < 0.01$) (Table 1).

Malaria infection was detected by 18S rRNA amplification in 66.4% (206/310) of the patients, including those diagnosed by microscopy ($N = 173$). The QT-NASBA method detected malaria parasites in 35% (23/66) of the patients from the island, 75.5% (80/106) of those from the urban area, 79% (54/76) of those from the semiurban area, and 71% (49/62) of those from the rural area ($P < 0.01$) (Figure 1A). *Pfs25* mRNA amplification detected gametocytes in 32% (99/310) of the samples, including the three for which gametocytes were observed by microscopy.

The frequency of gametocytes, detected by *Pfs25* mRNA amplification, was similar in semiurban (42%; 31/76) and rural (40.8%; 26/62) areas ($P = 0.08$) (Figure 1A), but this frequency tended to be higher in the urban area than in the

island: 29.2% (31/106) versus 16.6% (11/66), respectively ($P = 0.06$).

Submicroscopic infections were detected by molecular methods in 24.1% (33/137) of the 137 samples classified as negative on the basis of microscopy results. The rate of submicroscopic infections differed between sites, and was significantly lower on the island (4.4%; 2/45) than in the urban (35%; 14/40), semiurban (26.7%; 8/30), and rural (40.9%; 9/22) areas (Figure 1A) ($P < 0.01$). The proportion of study participants with submicroscopic infections was lower (15%; 3/20) among patients over the age of 11 years than among those 5 to 10 years of age (32.4%; 12/37) (Figure 1B). The carriage of submicroscopic gametocytes did not differ significantly between age groups: 29.5% (46/156) for 0- to 4-year-olds, 37.5% (33/88) for 5- to 10-year-olds, and 30.1% (19/63) for children over the age of 11 years (Figure 1B).

Submicroscopic malaria infections, including subgametocytemia, have been identified as a relevant source of human-to-mosquito transmission^{6,13} In this study, we assessed the frequency of submicroscopic *P. falciparum* infections in febrile patients from urban and rural areas of Gabon. We found that 24.1% of participants with no malaria parasite detection by microscopy presented subpatent parasitemia. This proportion differed between sites and was lower on the island, where parasite detection rates by microscopy is also the lowest.

Submicroscopic malaria is commonly reported in asymptomatic individuals, including adults from Gabon, but the data presented herein highlight the nonnegligible frequency of submicroscopic infections among febrile patients.⁷ There is still debate whether all cases of fever with any level of parasitemia should be classified as malaria. However, in the absence of other causes, submicroscopic malaria may have been responsible for the cases of observed or reported fever in this study. Thus, the detection of submicroscopic infections remains important to ensure appropriate treatment of the patient and for determination of the size of the reservoir. Undetected and untreated submicroscopic malaria

TABLE 1
Baseline characteristics of the study population

	All (N = 310)	Libreville (N = 106)	Melen (N = 76)	Oyem (N = 62)	POG (N = 66)
% Females (n/N)	50 (155/310)	58.5 (62/106)	55.2 (42/76)	45.1 (28/62)	34.8 (24/66)
Mean temperature, °C (±SD)	38.6 (0.4)	39.0 (0.6)	38.5 (1.1)	38.1 (1)	38.8 (0.7)
Febrile,* % (n)	273 (88)	106 (100)	62 (81)	40 (65)	65 (99)
Median age, (IQR) months	48 (24–108)	120 (36–348)	40 (24–82)	42 (24–66)	48 (24–96)
Age group					
0–4 years, % (n/N)	50.6 (157/310)	31.1 (33/106)	63.1 (48/76)	64.5 (40/62)	54.5 (36/66)
5–10 years, % (n/N)	28.7 (89/310)	20.8 (22/106)	33.0 (25/76)	35.5 (22/62)	30.3 (20/66)
≥ 11 years, % (n/N)	20.6 (64/310)	48.1 (51/106)	4.0 (3/76)	—	15.2 (10/66)
Malaria infection, assessed by microscopy					
Median asexual parasite density (IQR), p/μL	9,520 (1,400–36,925)	3,220 (448–29,400)	24,125 (8,400–70,000)	8,400 (2,100–59,850)	5,600 (2,100–14,725)
Asexual parasite prevalence, % (n/N)	55.8 (173/310)	62.2 (66/106)	60.5 (46/76)	64.5 (40/62)	32.0 (21/66)
Asexual parasite prevalence by age group,					
0–4 years, % (n/N)	25 (77/310)	14 (15/106)	38 (29/76)	40.3 (25/62)	12.1 (8/66)
5–10 years, % (n/N)	17 (52/310)	11.3 (12/106)	21.0 (16/76)	24.2 (15/62)	15.1 (10/66)
≥ 11 years, % (n/N)	14 (44/310)	37.7 (40/106)	1.3 (01/76)	—	4.5 (03/66)
Gametocyte prevalence, n/N	3/310	3/106	0	0	0

IQR = interquartile range; POG = Port-Gentil; SD = standard deviation.

*Fever was defined as axillary temperature ≥ 37.5°C.

FIGURE 1. (A) *Plasmodium falciparum* carriage, as assessed by microscopy and quantitative nucleic acid sequence–based amplification (QT-NASBA), by site. Submicroscopic infections are defined as 18S RNA-NASBA positive and microscopy negative. Among the individuals from Libreville, 40 were microscopically negative, and among them, 14 have been found malaria infected by polymerase chain reaction (PCR)—35% (14/40) of submicroscopic infections. Among those from Port-Gentil, 45 were microscopically negative, and among them, two have been found malaria infected by PCR—4.4% (2/45) of submicroscopic infections. Among those from Melen, 30 were microscopically negative, and among them, eight have been found malaria infected by PCR—26.7% (8/30) of submicroscopic infections. Among those from Oyem, 22 were microscopically negative, and among them, nine have been found malaria infected by PCR—41% (9/22) of submicroscopic infections. (B) *Plasmodium falciparum* carriage, as assessed by microscopy and QT-NASBA, by age. Submicroscopic infections are defined as 18S RNA-NASBA positive and microscopy negative.

infections should be considered as a major contributor to the reservoir of infectious parasites, because the spread of the parasite from these individuals to competent mosquito vectors could increase malaria transmission. Moreover, there may be clinical benefits to treating chronic (submicroscopic) infections even though further analysis of the relationship between submicroscopic infection and symptoms is required.¹⁴

The frequency of submicroscopic gametocyte carriers has been reported to be high in Gabon. In 2008, 49% of children from rural areas and 32.6% of those from urban areas carried submicroscopic gametocytemia.¹¹ In 2011, in a study performed in Libreville, submicroscopic gametocytes were found in 34.6% of pregnant women.¹⁵ A similar proportion (32%) was found in this study. However, other studies have reported very different submicroscopic gametocyte frequencies.^{4,16,17} Prevalence rates of submicroscopic malaria (24.1%) are consistent with those reported for previous studies in Tanzania.^{4,16,18} The frequencies esti-

mated here were obtained from a population of symptomatic patients attending health-care centers; this may limit the characterization of the human reservoir. Our findings show that malaria prevalence is underestimated in febrile patients and that the distribution of submicroscopic malaria infections is highly heterogeneous in Gabon. These findings also highlight the need for assessments of submicroscopic infection rates to improve malaria control strategies.

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