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RESEARCH ARTICLE

Submicroscopic infection from uncomplicated *Plasmodium falciparum* malaria of Franceville, southeastern Gabon

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Abstract

Background: In Gabon malaria is hyperendemic with perennial transmission. PCR provides a powerful tool to detect submicroscopic infection (SMI) allowing improving disease control strategies with regard to its eradication policy. The objective was to determine the prevalence of both patent and SMI of *P. falciparum* in symptomatic patients. **Materials and methods:** Two cross-sectional studies were carried out in two local hospitals of Franceville, from May to July 2011 and from February to May 2012. A total of 595 symptomatic patients were enrolled, 250 in the first and 345 in the second study. A clinical examination and parasitological diagnosis by microscopy and PCR were carried out in all patients. **Results:** Of the 250 patients enrolled in the first study, the prevalence of *P. falciparum* was 24.00% (60/250) and 9.60% (24/250) for patent and SMI respectively with an overall prevalence of 33.60% (84/250). In the second study, a prevalence of 21.45% (74/345) for patent infections and 9.57% (33/345) for SMI was obtained with a global prevalence of 31.01% (107/345). Overall, 66.40% and 69.00% of patients were free of *P. falciparum* infection respectively for study 1 and 2. The SMI occurs in symptomatic patients of Franceville in an average of 9.00% of cases with a relative high prevalence at the beginning and the end of the rainy seasons. **Conclusion:** These results should be used not only to develop the management of the disease but also to improve control strategies such as intermittent preventive treatment across the target population.

Introduction

In developing countries malaria still remains a major public health concern. The infection by *Plasmodium falciparum*, the most virulent species can rapidly progress to the severe disease such as anemia, cerebral malaria, respiratory distress, hypoglycemia etc. which are associated with a high mortality. Indeed, the detection of *P. falciparum* infection and proper management of symptomatic cases remains essential for disease control. The Roll Back Malaria initiative was launched jointly by WHO, the World Bank, UNICEF and UNDP in 1998 has been adopted by the endemic countries. This initiative has permitted to decrease significantly malaria morbidity and mortality according to the global distribution of cases and deaths published by the world malaria report 2009 [21]. Practically in all the endemic countries, a significant decline of disease rates has been demonstrated and more probably due to the large-scale bed net program and the improvement of case management such as enhanced diagnostic tests and implementation of highly effective anti-malarial drugs. However, to keep steadily declining prevalence until disease eradication we need to strengthen and improve the National Control Programs. This

necessarily requires the development and ownership of efficient diagnostic tools that allow targeted treatment of infected individuals and efficient measurement of epidemiological indicators [4, 19].

Many developing countries still experienced difficulty of obtaining timely and reliable epidemiological data and setting up monitoring and evaluation of their programs [4]. This was also the case for Gabon where the National Control Program of malaria tried since 2008 to improve data collection following the WHO's call for scaled-up control efforts [22]. In April 2008, five sentinel sites malaria surveillance were established in the country including one at Franceville to improve disease indicator surveys notably in pediatric wards.

In disease management, only patients with blood smears and/or rapid diagnostic test (RDT) *Plasmodium* positive are recommended to be treated [22, 1]. The treatment of patient negative (by microscopy and/or RDT) depends on clinical signs and appreciation of the physicians who do not always trust blood smear results [2, 14, 5]. To date, microscopy remains a gold standard technique to diagnose malaria [20, 12]. However, this method is insensitive to detect infected individuals harbouring a low parasite density (termed submicroscopic infections = SMI) [12]. It has been demonstrated that PCR provides a powerful tool to detect the SMI which are encountered in symptomatic as well as in asymptomatic individuals. It has been also documented that SMI are more frequent in endemic areas and that would have consequences on the accurate management of disease [18, 12, 3, 9].

The main objective of this study was to determine the true prevalence of *P. falciparum* infection (patent and sub-patent) in uncomplicated malaria during two periods of rainy season addressing therefore the issue of management of these infections with regard to the disease control and eradication policy.

Materials and Methods:

Study area and Subjects: The study was performed at the "Centre International de Recherches Médicales de Franceville". Patients from 1 to 75 years old were enrolled at the two local Gabonese hospitals (Centre Hospitalier Régional Amissa Bongo and Hôpital de l'Amitié Sino-Gabonaise de Franceville, Haut Ogooué Province in southeastern Gabon). *P. falciparum* malaria is highly endemic with a perennial mode of transmission and some seasonal fluctuation [7]. Informed consent was obtained from 595 individuals or from their parents. The study was submitted and approved by the Research National Ethic Committee (CNER) of Gabon. It was also approved the Governor of the Haut Ogooué Province, and performed in accordance with the guidelines for human experimentation in clinical research of the Ministry of Public Health and Population of Gabon.

Blood sample collection: A total of 595 symptomatic patients were enrolled, 250 in May to July 2011 and 345 in February to May 2012. Blood was collected by venipuncture from all febrile patients using EDTA Vacutainers® (Becton Dickinson,Meylan, France). All the patients were screened for *P. falciparum* infection.

***P. falciparum* Diagnosis:** The thick and thin blood films were stained with Giemsa and examined by two experienced microscopists. The parasite load was expressed as the number of *P. falciparum* asexual forms per microliter of blood using Lambaréné method [13].

DNA preparation: DNA Templates were extracted using DNeasy Blood & Tissue kit (Qiagen, Germany) and collected into a clean tube and used for PCR immediately or stored at -20°C until use.

STEVOR gene amplification: Two point five microliters of the DNA template were amplified using a Perkin Elmer thermal cycler in a 25 µl reaction containing 1x PCR buffer as supplied by the manufacturer (200 mM Tris-HCl, pH 8.7, 100 mM KCl, 100 mM $(NH_4)_2$ SO_4, 20 mM $MgSO_4$, 1% Triton-X 100, 1mg/ml of bovine serum albumin), 17µM each of dATP, dCTP, dGTP and dTTP (Invitrogen, Cergy Pantoise, France), 0.75pM of each primer P5, P18, P19 and P20, and 0.625 unit of Taq DNA polymerase (Invitrogen, Cergy Pantoise, France). The reaction mix for the nested PCR was the same as that of the PCR, however, 0.75 pM of primers P17 and P24, and 1µl of the first PCR product were added. PCR and nested PCR were performed in the following conditions: 93°C for 3 min and 35 cycles of 93°C for 30sec, 55°C for 50sec and 72°C for 30sec. After amplification, 10 µl of each nested PCR product was mixed with 5 µl of loading dye EZ-Vision THREE DNA Dye as loading Buffer 1X (ref: N313, Interchim, France), and analyzed by electrophoresis on a 1.2% agarose gel and the DNA was visualized and photographed under ultraviolet light (Quantum ST4, 1100/26M).

Statistical analysis: Data were analyzed with Statview® and EPI INFO 6 software® (Center for Diseases Control – CDC- ; Organisation Mondiale de la Santé ; version française : Epicentre et Ecole Nationale de Santé Publique). Proportions were analyzed with the $\chi 2$ test. Significance was assumed at $p < 0.05$.

Results

Description of the sample:

Study 1: Two-hundred-fifty (250) patients were enrolled in the first study period with an average age of 33 (±18) including 189 women (75.60%) and 61 men (24.40%). Children between 1-5 years old were 30 (12.00% of patients enrolled).

Study 2: Three hundred forty five (345) subjects were recruited during the second period of data collection. The average age of patients enrolled was 31 (± 19) years. The number of men and women were 127 (36.80%) and 218 (63.20%) respectively. The number of children under 5 years was 47 (13.60%).

Patent infection:

Study 1: The prevalence of patent infection (*Plasmodium* microscopy positive = ME+) was 24.00% (60/250) (Figure 1).

Study 2: Seventy four (74) blood smears were *P. falciparum* positive by microscopy from the 345 samples examined with an estimated prevalence of 21.45% [$CI_{95\%}$, 17.12-25.78]. The median parasitemia was 2013 parasites/µL blood. The monthly distribution of the number of *P. falciparum* infection detected by microscopic examination is shown in the figure 2. Among these 74 ME+ individuals, twelve (12) were children under 5 years.

Submicroscopic infection:

Study 1: Twenty four samples (24) of 250 were positive by PCR assay with a prevalence of 9.60% (Figure 1).

Study 2: Of 345 blood specimen examined, 271 (78.50%) were negative by microscopy. Among these 271 blood smears, thirty three (33) submicroscopic infections were detected by PCR with a prevalence of 9.57%. The prevalence of submicroscopic infection significantly decreases from 18.18% to 8.20% in February until May, while that of patent infection increases from 15.91% to 26.23% in the same period (Figure 2).

Prevalence of *P. falciparum*: The true prevalence of *P. falciparum* infection were 33.60% (84/250) and 31.01% (107/345) for study 1 and 2 respectively. Overall, 66.40% (study 1) and 69.00% (study 2) of the samples assessed by microscopy and PCR were negative for *P. falciparum* infection.

Clinical status: Overall, 592 (99.50%) were classified as uncomplicated malaria. Only 3 children with severe malaria were observed including 2 hyperparasitemia and 1 severe anemia.

Co-infection: One co-infection *P. falciparum* and *P. malariae* and 2 cases of mono-infection by *P. malariae* were obtained.

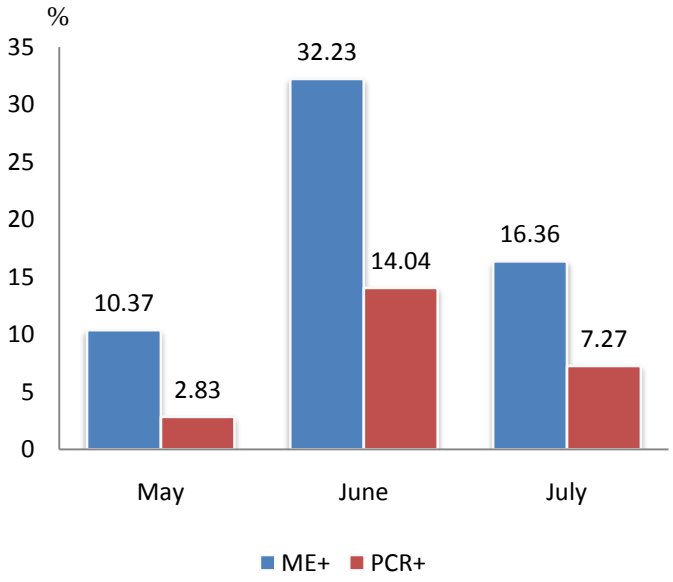

Figure 1: Study 1. Distribution of *P. falciparum* infections.

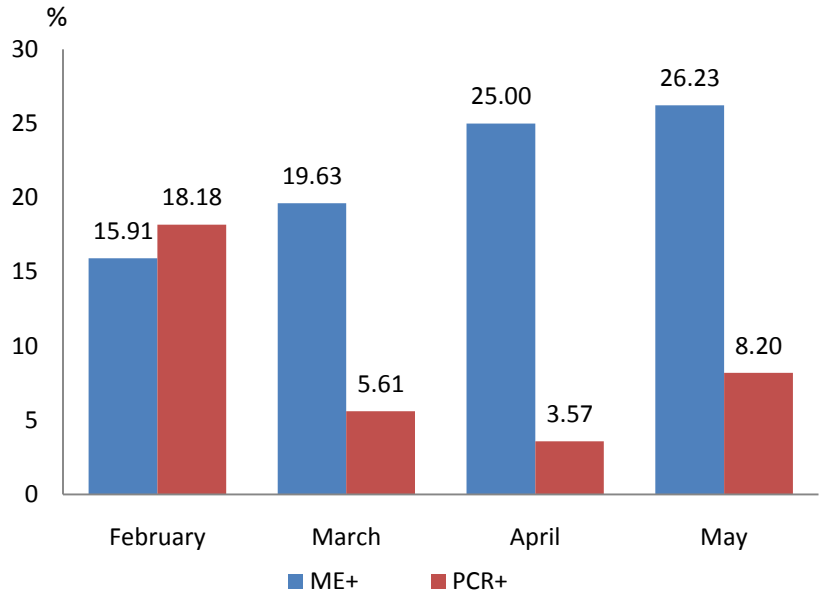

Figure 2: Study 2. Distribution of *P. falciparum* infections.

Table 1: Monthly prevalence of *P. falciparum*

		Prevalence (%)						p
		February	March	April	May	June	July	
Study 1								
	ME+	-	-	-	10.37	32.23	16.36	0.001
	PCR+	-	-	-	2.83	14.04	7.27	0.001
Study 2								
	ME+	15.91	19.63	25.00	26.23	-	-	0.30
	PCR +	18.18	5.61	3.27	8.20	-	-	0.02

Discussion

P. falciparum malaria, a leading life threatening disease, still remains a major public health concern in developing countries, especially in sub-Saharan Africa. In endemic areas, all the febrile individuals with demonstrated parasitemia should be treated. The identification of parasitemia in these febrile patients is therefore essential to progress from disease control to elimination (The maIERA, 2011). However, as malaria transmission is decreasing in many endemic settings we are facing a new challenge of diagnosing parasite low densities termed submicroscopic infections (SMI). The improvement of available diagnostic tools for correct case management will be therefore essential. In attempt to this goal PCR assays have been performed and used as an alternative to microscopy in detecting the four main *Plasmodium* species [16].

In this work, *P. falciparum* infections were detected by combined microscopy and PCR from uncomplicated but symptomatic individuals. PCR assay targeting a conserved fragment of STEVOR gene was used. The assay has a sensitivity of 100% and the target gene is expressed only by *P. falciparum* [6]. Only blood smears *P. falciparum* negative by microscopy were tested by PCR.

The prevalence of patently infected individuals have been estimated to 24.00% and 21.40% respectively for the first and second study confirming therefore previous epidemiological investigation in this area [23].

The peak prevalence of both patent and SMI was observed only in June compared to May and July in the first study (Figure 1). This may be related to the abundance of rainfall and its distribution over the period. The month of June corresponds to the end of the rainy season and is consistent with the fact that malaria transmission rate increases at the end of the rainy season in this area [7].

In the second study, there is no significant difference between the prevalence of patent infection during the four months from February (at the beginning of the great rainy season) to May (Figure 2). This result could be explained by the fact that rainfall did not significantly varied between the four months and consequently the entomological

inoculation rate was relatively the same. Of interest, it has been previously reported that the peak of entomological inoculation rate in Benguia (periphery of Franceville) is generally obtained in June [8]. Taking into account this previous result and considering that the great rainy season ends in June, the prevalence of patently infected individuals could further increase beyond May.

Thirty tree (33) samples of 345 (9.60%) were detected by PCR. No major difficulty was encountered during amplification steps. A specific band of 250 base pairs DNA is obtained after the electrophoretic migration in all positive samples PCR.

As shown in figure 2, the prevalence of *P. falciparum* SMI is higher in February than that of the tree other months (Mach, April and May). The peak of SMI in February corresponds to the end of the short dry season and the beginning of the great rainy season. This result may show that *P. falciparum* infection even at low level of parasitemia continues during the dry season, thus confirming therefore the perennial transmission of disease. Elissa et al. have previously demonstrated that malaria transmission in Franceville is perennial with fluctuations depending on the rainy seasons. The SMI could be associated with low transmission intensity during dry season. Thus, the introduction of PCR assay has allowed the identification of early increase of *P. falciparum* infection at the beginning of rainy season. These results could be used to develop strategies such as the choice of the best period for intermittent preventive treatment across the target population.

Overall, our data show that around 9.00% of *P. falciparum* infected individuals namely SMI fail to be detected by conventional microscopy and this would have a negative impact on disease control programmes. A recent study in the field has shown that there is a limited clinical benefits to treat all SMI (PCR positive) namely in an immune population from area with high malaria transmission intensity [9]. Nevertheless, in endemic areas, the SMI can be associated with severe disease [15, 11, 10] and reduced birth weight in primagravidae [3]. Therefore, it is understandable that physicians do not always consider blood smears results for anti-malarial treatment. On the other hand, PCR may be a useful tool for malaria epidemiological indicators assessment. To estimate the parasite rate (PR) and the annual parasite incidence (API) malariologists always use only the rate of blood smears positive (slide positivity rate SPR among symptomatic individuals suspected as cases) the PCR is not taken into account. Unfortunately, this method of data calculation underestimates the API which plays an important role in the global malaria eradication programme.

Conclusion: Submicroscopic infection of *P. falciparum* occurs in symptomatic patients in an average of 9% of cases with a relative high prevalence at the beginning and the end of the rainy season. These results could be used not only to improve the management of disease but also to develop strategies that can prevent malaria. For example, such study could guide the choice of the period to administer anti-malarial drug in an intermittent preventive treatment across the target population.

As perspectives this work should be extended over a period of year in urban as well as in rural areas. It would be also of interest to investigate the etiologic agents associated with symptoms from individuals free of malaria.

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