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Plasmodium falciparum infection in febrile Congolese children: prevalence of clinical malaria 10 years after introduction of artemisinin-combination therapies

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Abstract

OBJECTIVES To investigate the proportion of malaria infection in febrile children consulting a paediatric hospital in Brazzaville, to determine the prevalence of submicroscopic malaria infection, to characterise *Plasmodium falciparum* infection and compare the prevalence of uncomplicated *P. falciparum* malaria according to haemoglobin profiles.

METHODS Blood samples were collected from children aged <10 years with an axillary temperature ≥37.5 °C consulting the paediatric ward of Marien Ngouabi Hospital in Brazzaville. Parasite density was determined and all samples were screened for *P. falciparum* by nested polymerase chain reaction (PCR) using the *P. falciparum* msp-2 marker to detect submicroscopic infections and characterise *P. falciparum* infection. Sickle cell trait was screened by PCR.

RESULTS A total of 229 children with fever were recruited, of whom 10% were diagnosed with uncomplicated malaria and 21% with submicroscopic infection. The mean parasite density in children with uncomplicated malaria was 42 824 parasites/μl of blood. The multiplicity of infection (MOI) was 1.59 in children with uncomplicated malaria and 1.69 in children with submicroscopic infection. The mean haemoglobin level was 10.1 ± 1.7 for children with uncomplicated malaria and 12.0 ± 8.6 for children with submicroscopic infection. About 13% of the children harboured the sickle cell trait (HbAS); the rest had normal haemoglobin (HbAA). No difference in prevalence of uncomplicated malaria and submicroscopic infection, parasite density, haemoglobin level, MOI and *P. falciparum* genetic diversity was observed according to haemoglobin type.

CONCLUSION The low prevalence of uncomplicated malaria in febrile Congolese children indicates the necessity to investigate carefully other causes of fever.

keywords fever, children, *Plasmodium falciparum*, uncomplicated malaria and submicroscopic infection, sickle cell trait, Republic of Congo

Introduction

Malaria remains a public health problem of overwhelming importance. In 2015, the malaria global burden was estimated at 214 million cases resulting in 438 000 deaths [1]. Children under five and women in their first pregnancy are at particular risk of complications and severe disease [2]. The transmission and incidence rates of *Plasmodium falciparum* malaria are higher in sub-Saharan Africa [2]. Eighty eight percent of the malaria cases in 2015 occurred in the WHO African Region [1]. In the past decade, massive interventions including long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), artemisinin-based-combination treatment (ACTs) and intermittent preventive treatment (IPT) of infants, children and pregnant women [3] led to a significant reduction of malaria burden [4] particularly in Ethiopia and Zambia [5, 6] and to near-elimination or elimination in Botswana, Namibia, Cabo Verde, Algeria and Morocco. [1, 7]. The malaria mortality rate is estimated to

have dropped by 60% globally between 2000 and 2015; therefore, substantial progress has been made towards the World Health Assembly target of reducing the malaria burden by 75% by 2015, and the Roll Back Malaria (RBM) Partnership target of reducing deaths to near-zero [1]. As the incidence of malaria diminishes, a better understanding of non-malarial fever is important for effective management of illness in children [8]. WHO guidelines recommend malaria parasitological confirmation for all patients suspected of malaria before treatment [1, 9].

An interesting feature of the epidemiology of malaria is some degree of protection against infection and severity of disease, provided by the concomitant presence of haemoglobinopathies, such as sickle cell trait (HbAS) and other red cell abnormalities [10]. Sickle cell haemoglobin (HbS) is a mutant allele of β-globin and one of the well-known balanced polymorphisms providing heterozygotes (HbAS) with 60% – 90% protection against high-density *P. falciparum* parasitaemia, acute uncomplicated malaria, severe malaria and malaria mortality [11]. This advantage has provided a strong selection pressure in large parts of sub-Saharan Africa with moderate-to-intense malaria transmission, resulting in high population frequencies of this mutation [11].

Although the fact that HbAS confers protection against the severe form of malaria, the related mechanisms are still not fully understood [12]. Some studies reported similar or fewer malaria parasite prevalence and/or density in HbAS than HbAA children during *P. falciparum* infection [13, 14], suggesting a better control of the development of infection [15, 16]. By contrast, a recent study in Gabon found *P. falciparum* malaria to be strongly associated with sickle cell trait prevalence [17].

Since the introduction of ACTs in Republic of Congo (RoC) in 2006, a reduction of malaria clinical cases from 45% to 16% was observed [18–20]. The prevalence of clinical malaria in children varies between 8% and 29% in Brazzaville and Pointe-Noire, the two main cities in RoC [21]. The prevalence of HbAS in RoC is about 19% [19], and to our knowledge, no specific investigation has yet been conducted to assess the proportion of malarial fevers and the influence of symptomatic uncomplicated malaria in the HbAS population.

This study was conducted in a paediatric unit of Marien Ngouabi Hospital (MNG Hospital) in Brazzaville during the transmission season, to (i) investigate the proportion of febrile paediatric cases attributed to malaria; (ii) determine the prevalence of a submicroscopic malaria infection reservoir in Congolese children under 10 years old; (iii) characterise *P. falciparum* infection by determining mean parasite density, multiplicity of infection (MOI), genetic diversity and haemoglobin level; and (iv) to compare the prevalence of uncomplicated malaria in HbAA and HbAS carriers.

Materials and methods

Study area

The RoC is divided into 12 administrative departments subdivided into 94 districts and 70 townships. The department of Brazzaville has nine districts, of which Talangaï is one. Our investigation was conducted in our study site [21] at MNG Hospital in Talangaï in the northern part of Brazzaville, along the Congo and the Tseme rivers. In this area, malaria transmission is perennial with *P. falciparum* being the predominant species and *Anopheles gambiae* the main mosquito vector [22].

The health policy in RoC is organised around 'socio-sanitary circumscriptions', namely *circonscriptions socio-sanitaire* (CSS) or *district sanitaire*, which are allocated according to the number of inhabitants. The CSS is subdivided into health areas, where integrated health centres are established [23]. MNG Hospital is situated in the CSS of Talangaï. Due to an explosion in March 2012 in the centre of Brazzaville that led to the closing-down of many health centres, MNG Hospital was the only paediatric referral hospital in the area at the time of the study.

Procedures

From September 2014 to February 2015, a cross-sectional study was conducted at the paediatric ward of MNG Hospital. Children aged from one to 10 years presenting with fever (axillary temperature ≥37.5 °C) on the day of consultation and those with a history of fever 48 h before consultation at hospital were enrolled if informed consent from parents or guardians was obtained. Complete clinical and demographic data were recorded, including axillary temperature on the day of consultation, age, sex, bed net use and prior self-medication with antimalarial drug. Febrile children whom the clinician diagnosed with other pathologies such as severe diarrhoea, pulmonary infection or HIV were not included.

From each enrolled patient, 4 ml of whole blood was collected in EDTA for thick and thin smear preparation, parasite and human DNA extraction and haemoglobin concentration measurement.

Extraction of parasite and human DNA

Genomic DNA was extracted from 200 µl of whole blood samples collected in EDTA using QiaAmp DNA

kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The extracted DNA was stored at −20 °C until use.

Genotyping of β-globin

β-globin HbAA, HbAS and HbSS genotypes were determined using allele-specific PCR method as described by Wu *et al.* [24]. Individuals with HbSS were excluded from the study.

Malaria diagnosis

Thick and thin blood smears of each patient were stained with 20% Giemsa for 15 min. Slides were examined independently by two qualified laboratory technicians and were considered negative after the observation of at least 100 fields. Asexual blood-stage parasites were counted against 200 leucocytes and the parasite density was calculated and expressed as the number of parasites per microlitre of blood (p/μl), assuming a leucocyte count of 8000/μl of blood. Thin blood smears were examined for the identification of malaria species.

Plasmodium falciparum submicroscopic infection, multiplicity of infection and genetic diversity

Overall, isolates were screened for *P. falciparum* infection using msp-2 marker, and then, positive samples were further analysed to determine MOI and *P. falciparum* genetic diversity [25, 26]. Positive controls and DNA-free negative controls were included in each set of reaction. Individual alleles were identified by fragment length and by corresponding allele-specific primers used. PCR fragment sizes were estimated using a 100-base pair (bp) DNA ladder marker (BioLabs, New England). Submicroscopic infection was identified as microscopy-negative and PCR-positive. Multiplicity of infection (MOI) was calculated as the mean number of *P. falciparum* genotypes per infected individual [27].

Case definitions

Febrile patients were classified into three groups according to the parasitological results: (i) uncomplicated malaria infection patients who were febrile (axillary temperature ≥37.5 °C) without any sign of another disease (respiratory distress, known HIV patient, etc.) who had a malaria parasite-positive blood smear with <250 000 parasites/microlitres of blood (parasite density above this threshold was considered as a severe); (ii) febrile patients with a submicroscopic infection who carried *P. falciparum* infections detected only by PCR [28–31]; and (iii) *P. falciparum* -uninfected febrile patients who presented negative thick blood smears and negative PCR. Haemoglobin (Hb) levels were measured using CYANHemato cy006. Anaemia was defined as haemoglobin concentrations <11 g/dl [32, 33].

Data management and analysis

All data were entered using Epi-data (version 3.1, 'The EpiData Association', Odense, Denmark), cleared and analysed with the STATA software package (version 11, StataCorp, Texas, USA). Quantitative variables were described with mean ± standard deviation (SD). Qualitative variables were described with frequency (*n*) and percentage. The comparison of the malaria prevalence in the HbAA and HbAS groups was made using the Mann–Whitney *U*-test. The Student's *t*-test was used to compare the MOI and the mean haemoglobin level between uncomplicated and submicroscopic malaria infection. Odds ratios (OR) permitted to evaluate the association between haemoglobin profile, age, axillary temperature and haemoglobin concentration. For disperse parasite density values with high parasitaemia, parasite densities were expressed as the geometric mean. The significant correlation test was used to verify the correlation between age and *P. falciparum* infection. All tests were considered statistically significant when P was <0.05.

Ethics

The study was approved by the Institutional Ethics Committee of the Fondation Congolaise pour la Recherche Médicale. Written informed consent from the parent or guardian was required prior to inclusion in the study.

Results

Characteristics of febrile Congolese patients recruited in Brazzaville

A total of 229 children aged from one to 10 years were enrolled in the study. We found 13% heterozygous (HbAS) and 87% normal (HbAA) carriers, respectively. The patient's main characteristics are summarised in Table 1. The children's mean age was 3.1 ± 2.5 years; 46% were female. About 44% reportedly slept under insecticide-treated bed nets. Antimalarial self-treatment was administrated to 31% of the children before coming to hospital, and anaemia was found in 42%.

The proportion of uncomplicated malaria was 10% and that of submicroscopic *P. falciparum* infection 21%.

The prevalence of uncomplicated malaria was not significantly different according to haemoglobin type (9% *vs.* 13%; $P = 0.277$). We found that 69% were *P. falciparum* -uninfected febrile patients.

Plasmodium falciparum infections in Congolese children

Plasmodium falciparum was the only species found in blood smears. The mean parasite density was 42 824 parasites/μl. Parasitaemia increased with age ($P = 0.007$). The multiplicity of infection (MOI) was 1.59 in patients with uncomplicated malaria and 1.69 in patients with submicroscopic infection; we observed no statistical difference in the MOI between both groups ($P = 0.653$). The mean haemoglobin level was 10.1 ± 1.7 in children with uncomplicated malaria and 12.0 ± 8.6 in those with submicroscopic infection. The type of infection did not influence the haemoglobin level ($P = 0.398$) (Table 2).

Msp-2 genotyping revealed that 42% (30/71) of all *P. falciparum* -infected patients (uncomplicated and submicroscopic malaria infection) harboured multiple infections. The FC27 and 3D7 allelic families were highly diverse with 14 and 11 different alleles, respectively (Figures 1 and 2). The FC27 allelic family was the predominant with 53%.

The association between MOI and the log parasite density was examined, and no association was found. Regarding haemoglobin type, although the HbAA group had an apparently larger number of infected children, multiplicity of infection and parasite densities, it did not differ significantly from the HbAS group ($P = 0.514$).

Discussion

The objective of this study was to determine the proportion of fever attributed to malaria cases and the prevalence of submicroscopic malaria infection in febrile Congolese children <10 years consulting at a paediatric hospital in Brazzaville. To the best of our knowledge, this investigation is the first to report the proportion of febrile cases attributed to malaria after the introduction of ACTs in the country.

In RoC and many other African countries, fever is often considered to be a symptom of clinical malaria

Table 1 Characteristics of Congolese febrile patients recruited in Brazzaville

Variables (%)	All ($n = 229$) 100	AA ($n = 199$) 87	AS ($n = 30$) 13
Age, years, mean ± SD [min–max]	3.1 ± 2.5 [1–10]	3.1 ± 2.5 [1–10]	2.9 ± 2.3 [1–8]
Sex ratio F/M (%)	105/124 46/54	88/111 44/56	17/13 57/43
Axillary temp. °C, mean ± SD [min–max]	37.9 ± 1.1 [34.6–40.8]	37.9 ± 1.1 [35.8–40.8]	37.8 ± 1.2 [34.6–40.0]
Haemoglobin, g/dl, mean ± SD [min–max]	11.3 ± 4.2 [4.9–69.4]	11.3 ± 4.4 [4.9–69.4]	11.2 ± 1.4 [7.4–13.9]
Bed net use, *n* (%)	219 (96)	189 (95)	30 (100)
Treated bed net use, *n* (%)	101 (44)	2 (46)	9 (30)
Previous antimalarial self-treatment, *n* (%)	71 (31)	60 (30)	11 (37)
Anaemia, *n* (%)	96 (42)	86 (43)	10 (33)
P. f. uncomplicated malaria, *n* (%)*	22 (10)	18 (9)	4 (13)
P. f. submicroscopic infection, *n* (%)	49 (21)	41 (21)	8 (27)

*Mann–Whitney $P = 0.277$

Table 2 *Plasmodium falciparum* infections in Congolese children

Type of infection	Number	Age* (mean ± SD)	Sex (F/M)	Axillary temp. (mean ± SD)	MOI**	Hb level*** (mean ± SD)
Uncomplicated malaria	22	3.8 ± 2.5	11/11	38.8 ± 1.2	1.59	10.1 ± 1.7
Submicroscopic infection	49	3.5 ± 2.9	23/26	37.7 ± 0.9	1.69	12.0 ± 8.6

Student's *t*-test with inequality variance (*P*-value: *0.662; **0.653; ***0.398). MOI, Multiplicity of infection.

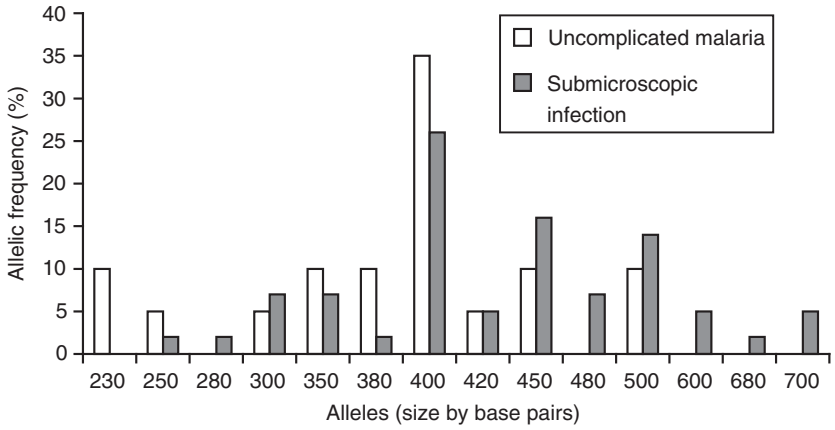

Figure 1 Allelic frequency of FC27 msp-2 gene alleles in *Plasmodium falciparum falciparum* isolates from Congolese children.

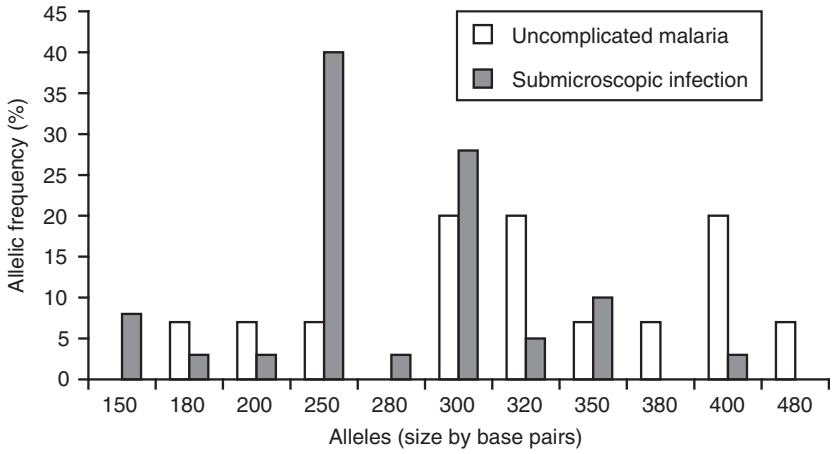

Figure 2 Allelic frequency of 3D7 msp-2 gene alleles in *Plasmodium falciparum* isolates from Congolese children.

infection [34] and treated accordingly. The prevalence of clinical malaria shifted from 36% before the introduction of ACTs [19] to 10% in our study. This important decrease could be credited to the local efforts led by the Malaria National Control Program [23] since 2006, which include free antimalarial treatment for pregnant women and under-fives in public health centres, massive distribution of long-lasting insecticide-treated nets (LLINs) and intermittent preventive treatment (IPT) for pregnant women [23]. Moreover in RoC, ACTs are easily accessible and affordable, as can be concluded from the fact that 31% of children presented to hospital after antimalarial self-medication. A similar (6–13%) drop in malaria prevalence occurred in other endemic sub-Saharan countries such as Gabon, Cameroon, Equatorial Guinea, Ghana, Burkina Faso, Kenya, Tanzania and Ethiopia [35–43]. Children suffering from uncomplicated *P. falciparum* malaria in the study were treated according to the national treatment policy of RoC, where the recommended first-line antimalarial is artemether–lumefantrine [19, 23].

Our observation implies that 90% of fevers could be related to other conditions such as upper or lower respiratory tract infections, gastroenteritis, helminth or other infections [44]; this fully justifies the importance of confirming the presence of malaria parasites before treatment [9]. Interestingly, if WHO Integrated Management of Childhood Illness [45] had been applied, all enrolled children would have received unnecessarily antimalarials while although only 10% needed it. However, malaria diagnosis by microscopy or rapid diagnostic tests necessitates training. A recent study in RoC pointed out the unavailability of rapid diagnostic tests in the laboratories and the need for sustained microscopy training for better performance [21].

We did not investigate non-malarial causes of fever. However, the hospital records seem to indicate that gastrointestinal and respiratory diseases were common etiologies.

We found a prevalence of 21% of submicroscopic malaria infection only detected by PCR. This is high, and these children form a malaria infection reservoir that possibly contributes 20% – 50% of human-to-mosquito transmissions [46]. This proportion can reach 80% in areas where the community parasite prevalence detected by microscopy is <10% [47]. In Burkina Faso, for example, a study using quantitative nucleic acid sequence-based amplification (QT-NASBA) showed that children are a more important *P. falciparum* infectious reservoir than adults and submicroscopic infections are an important source of onward malaria transmission [48]. Many studies reported the importance of using more sensitive methods for malaria diagnosis. In a reduced transmission area such as Senegal, enzyme-linked immunosorbent assay was used to evaluate the sero-epidemiological situation of *P. falciparum* malaria [49]. A study in Kenya of asymptomatic children [50] showed the common presence of gametocytaemia in children, especially in under-fives. Similarly in Gabon, submicroscopic gametocytaemia was common in febrile children [51]. With the falling malaria prevalence in many malaria endemic areas in sub-Saharan Africa [51], targeting gametocytes is important to maintain malaria control and elimination. Unfortunately, we did not investigate the presence of gametocytes in isolates collected from Congolese children.

The multiplicity of *P. falciparum* infection of about 1.5 resembles previous findings in Brazzaville before and just after the introduction of ACTs [19, 52, 53]. Genetic diversity of *P. falciparum,* which plays a major role in the natural acquisition of immunity to malaria infections, also corresponded to previous investigations in the area [19, 52, 53]. The similarity in MOI and genetic diversity might imply that, although both parasite density and malaria prevalence in the area diminished, the distribution and the type of *P. falciparum* parasite circulating in the area did not change. Control measures may have limited malaria expansion but not parasite diversity. Even though 96% of the enrolled children slept under mosquito nets, no impact on *P. falciparum* genetic diversity was detected. Similar findings were reported in Malawi and Kenya [54, 55].

Sickle cell trait prevalence in our Congolese cohort was 13%, reflecting previously reported ranges from 5% to 40% in Central Africa [56–58]. Sickle cell trait has long been associated with protection against severe forms of *P. falciparum* malaria [14, 59]. The HbS variant appears to reduce the risk of infection with *P. falciparum.* However, the epidemiological evidence supporting this conclusion remains incomplete [14, 59]. In our study, the proportion of children presenting with uncomplicated malaria was 9% for HbAA and 13% for HBAS. The prevalence here is similar to the proportion of *P. falciparum* infection in asymptomatic Congolese children [52], showing that sickle cell trait carriage seems to not influence malaria infection. However, this observation could be modulated by several factors. The sample size may have been too small to detect the impact of sickle cell trait carriage. The children's age may have played a role: perhaps younger children would have shown an influence on infection parameters. Lastly, self-medication should be carefully considered, as it is an important confounding factor.

In conclusion, in this population of Congolese children under 10 presenting with fever, 10% were diagnosed with uncomplicated malaria. No influence of sickle cell trait and age was observed on parasite density, multiplicity of infection and *P. falciparum* strain diversity. This could be due to the small sample size and/or the high efficacy of ACTs associated with case home management.

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