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Short Communication

Dynamics of *Plasmodium* species and genotype diversity in adults with asymptomatic infection in Gabon

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ABSTRACT

Objectives: We investigated the diversity and dynamics of *Plasmodium* infection in serially collected samples from asymptomatic participants of a clinical trial assessing the efficacy and safety of ivermectin in Gabon. We checked whether the baseline sample reflected the *P. falciparum* genotype and *Plasmodium* species diversity seen over 7 days of follow-up.
Methods: Blood samples were collected at inclusion, every 8 hours until hour 72, daily until day 7, and on day 14. *Plasmodium* species was determined by qPCR and *pfmsp1* length polymorphism was assessed for *P. falciparum* genotyping.
Results: In 17/48 (35%) individuals, all *pfmsp1* genotypes identified during the assessed period were detected at baseline; in 31/48 (65%), new genotypes were found during follow-up. Additional sampling at hour 24 allowed the identification of all genotypes seen over 7 days in 50% of the individuals. Ivermectin did not impact the genotype dynamics. Mixed *Plasmodium* spp. infections were detected in 28/49 (57%) individuals at baseline, and detection of non-*falciparum* infections during follow-up varied.
Conclusions: Our results reveal complex intra-host dynamics of *P. falciparum* genotypes and *Plasmodium* species and underscore the importance of serial sampling in clinical trials for antimalarial drugs with asymptomatically *P. falciparum*-infected individuals. This might allow a more accurate identification of genotypes in multiple infections, impacting the assessment of drug efficacy.

Introduction

With 249 million estimated cases and 608,000 deaths worldwide in 2022 [1], malaria remains a public health concern. In endemic areas, individuals can be infected by different *Plasmodium* species and distinct genotypes of the same species. In serially collected samples, disappearance and reappearance of alleles over time was described in asymptomatic infections, raising the question of whether a single blood sample is sufficient to characterize parasite diversity within-host [2].

Clinical trials with new drugs or regimens for *Plasmodium falciparum* treatment usually rely on the polymerase chain reaction-adjusted parasitological cure rates (PCR-APCRs) for assessment of efficacy [3], distinguishing reinfection, resulting from a new exposure, from recrudescence, which happens when the initial treatment fails to eliminate the infection, a sign of insufficient drug action. The current recommendation is genotyping of *P. falciparum* merozoite surface protein-1 (*msp1*) and -2 (*msp2*) genes and a microsatellite (Poly-α, Pfpk2 or TA1) [3] in samples collected before treatment and upon recurrence.

This study examines the parasite population dynamics in adult Gabonese with asymptomatic *P. falciparum* infection who participated in a clinical drug trial [4]. We analyzed whether the baseline

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sample reflected *Plasmodium* species and genotype diversity of the infection seen during 7 days of follow-up.

Methods

Blood samples were collected in EDTA tubes from adults (≥18 years) living in Lambaréné, Gabon, and surrounding villages participating in a clinical trial for assessment of efficacy and safety of ivermectin for the treatment of asymptomatic *P. falciparum* infections from June 2019 to October 2020 [4]. The clinical trial consisted of a first, open-label, dose-escalation stage with 3 groups and a second double-blind, randomized, placebo-controlled stage [4]. Sampling was done prior to intervention (ivermectin or placebo), every 8 hours until hour 72, daily until day 7, and on day 14. Five hundred microliters of whole blood were mixed with 1300 µl of RNA*later* (Thermo Fisher Scientific) and stored at −20°C. Standard antimalarial treatment was given on day 7 for those who had not received rescue treatment before. The entomological infection rates reported near the study area were 73.2 infective bites per person (ib/p)/4 months in the dry season and 170.1 ib/p/8 months in the rainy season [5].

Total nucleic acids were purified from 750 µl of blood + RNA*later* mixture using QIAamp® Blood Mini Kit (Qiagen) or sbeadex Blood DNA Purification Kit (LGC). Parasite density was determined by reverse transcription-quantitative PCR targeting a conserved region of the 18s rRNA among *Plasmodium* [6]. *Plasmodium* species was assessed by qPCR in single-plex assays with specific primers and probes for the 18s rRNA of *P. falciparum* (Pf), *P. malariae* (Pm), *P. ovalecurtisi* (Poc), *P. ovalewallikeri* (Pow), and *P. vivax* (Pv) [6].

For *P. falciparum* genotyping, the polymorphic region of the *pfmsp1* block 2 was amplified by nested PCR [7,8]. Amplicon sizing was performed in the automated capillary gel electrophoresis QIAxcel Advanced System (Qiagen) using a protocol with 3-5 base pairs (bp) resolution. We refer to "genotype pattern" as the set of all *pfmsp1* genotypes detected in one sample at a given time point. The PCRs are described in Table S1 and primers, probes, and cycling conditions in Table S2. Figures were generated with R (version 4.3.0).

Results

Description of participants

In total, 49 participants were included. Five participants were included in each group in the dose-escalation stage and 17 in each group in the second trial stage. Table S3 describes their baseline data.

Decline in parasitemia observed during the follow-up

At baseline, parasitemia ranged from 4 to 17,029 parasites (p)/µl (median = 1,635 p/µl). Regardless of intervention, participants presented a decline in parasitemia during follow-up [4], ranging from 0.1 to 11,203 p/µl (median = 183 p/µl) on day 7 (Figure S1).

P. falciparum *genotype diversity and dynamics overtime*

Analysis of parasite diversity within-host revealed a variation in genotype patterns detected throughout follow-up in 35/48 (73%) individuals, with some genotypes detected at certain time points and undetectable in others (Figure S2). Of note, 2 individuals presented unique genotype patterns at each time point; one individual had 17 different genotypes detected in total, and the other had 11 different genotypes. Among the participants treated with standard antimalarial before day 7, only one showed a decrease in the number of genotypes over time.

Ivermectin had no impact on the parasite genetic diversity. The proportion of genotypes detected at baseline that disappeared on day 7 was similar between the ivermectin (22%, 11/52) and placebo (23%, 5/36) groups (Figure S2).

The baseline sample often does not capture the whole genotype diversity

In 17/48 (35%) individuals the whole set of genotypes identified during 7 days of follow-up was already detected at baseline, whereas in 31/48 (65%), a total of 85 new genotypes were identified in the subsequent samples (Figure S2). Therefore, we evaluated which timepoint during follow-up would be most suitable to detect new genotypes and have the complexity of infection characterized (Table 1). One additional sampling 8 hours after inclusion increased the probability of detecting all genotypes present in the infection to 46% (22/48), whereas one additional sampling after 24 hours increased the probability to 50% (24/48) (Figure 1a). No other collection timepoint of our samples increased the probability further.

Plasmodium *spp. diversity and fluctuation over time*

Baseline samples showed a high species diversity: Pf/Pm/Poc corresponded to 24% (12/49), Pf/Pm 22% (11/49), Pf/Pow 6% (3/49), and Pf/Poc and Pf/Pm/Poc/Pow 2% (1/49) each. One participant was identified with *P. malariae* mono-infection and excluded from *pfmsp1* genotyping and subsequent analysis. *P. vivax* was not found. Among the follow-up samples, a fluctuation in the detection of non-*falciparum* species was observed, with 15/28 individuals detected with Pf + non-*falciparum* at baseline presenting negative qPCR for a non-*falciparum* species in at least one follow-up timepoint (Figure 1b).

Discussion

Testing antimalarials in asymptomatically infected individuals allows one to obtain drug safety data in a relevant population and concomitantly gather first activity data. Studies should consider the high complexity of *Plasmodium* species and genotypes occurring in these populations. In this study, the baseline sample reflected the diversity observed during 7 days of follow-up in only 35% of participants. This finding might have an important implication: clinical trials for new antimalarial drugs relying on PCR-APCRs [3] may misclassify a recrudescence as reinfection. As shown here, new genotypes can be detected within short sampling intervals. Additional sampling within 24 hours gives a 50% probability of identifying all genotypes, compared to 35% at baseline, according to our data. One cannot rule out that the genotypes identified subsequently to the baseline represent recent infections that were still in the liver at baseline and were newly emerging in blood when they were first detected. However, the fluctuating nature of appearance and disappearance of the genotypes during the follow-up time indicates that for most genotypes rather the limit of detection is the reason for upcoming genotypes and not a new infection.

Moreover, the identification of *Plasmodium* species in baseline samples revealed that 57% had mixed infections, and fluctuation in the detection of non-*falciparum* species in follow-up samples was observed in 15/28 participants, indicating that *Plasmodium* species diversity can also be missed in a single sampling.

The complex intra-host dynamics of *Plasmodium* species and *pfmsp1* genotypes observed in this study align with previous research emphasizing that a single sampling cannot comprehensively characterize the diversity of infections with multiple genotypes [2].

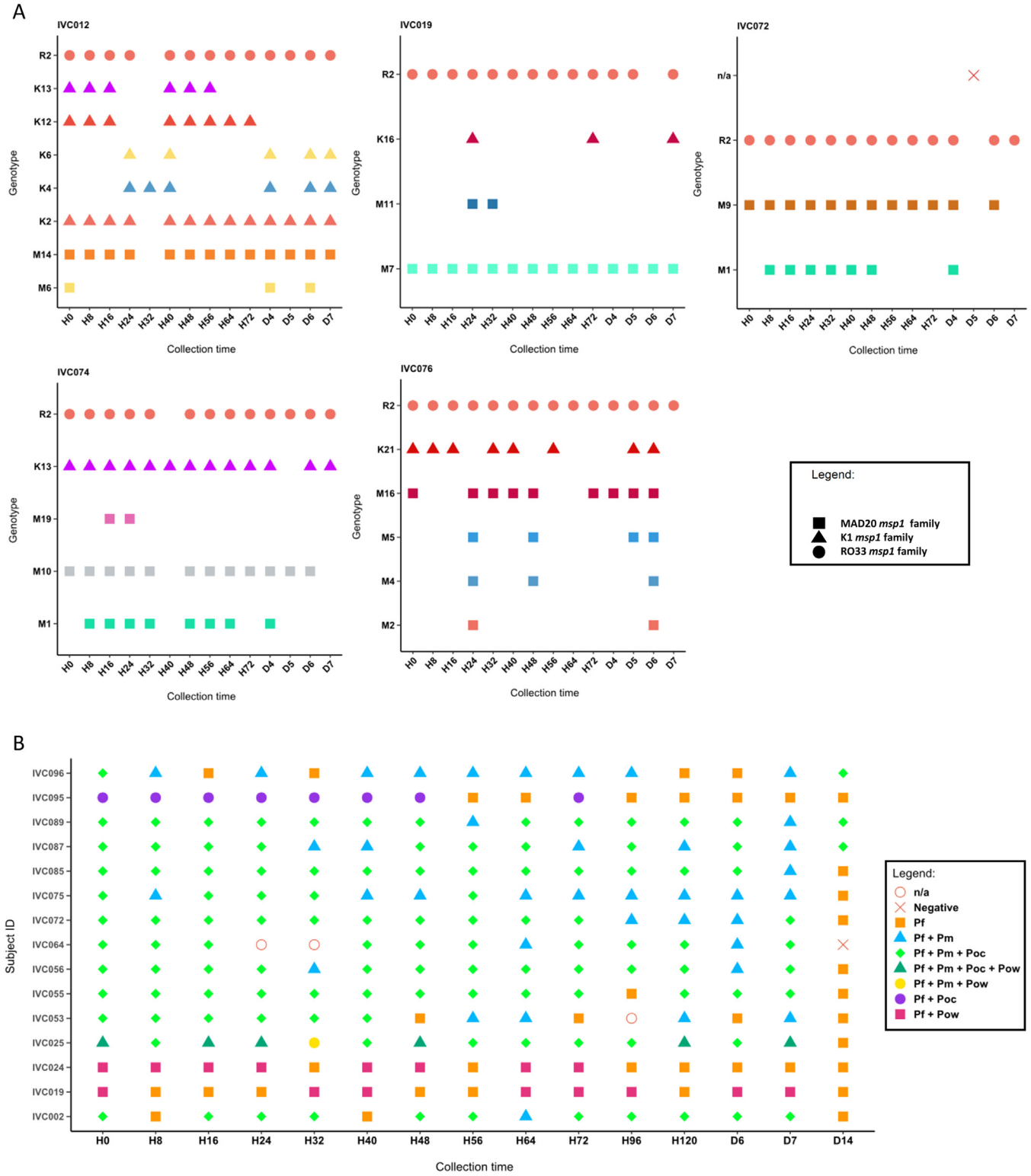

Figure 1. *Plasmodium falciparum* genotype and *Plasmodium* species diversity in asymptomatic Gabonese adults followed for 14 days. (a) Dynamics of *pfmsp1* genotypes in 5 participants depicting the contribution of one additional sampling at hour 24 to cover all genotypes present in the multiple infection. The dynamics in all participants presenting new genotypes in relation to the baseline sample during the follow-up period are shown in Figure S2. The decodes of the genotypes presented on the y axis are found in Table S2. n/a: samples without genotyping information. (b) Variation in the detection of non-*falciparum* species during follow-up observed in 15 individuals who were identified with mixed infections at baseline (H0). n/a: not available sample, Pf: *Plasmodium falciparum*, Pm: *P. malariae*, Poc: *P. ovalecurtisi*, Pow: *P. ovalewallikeri*.

Alternatively, genotyping methods using next-generation sequencing can be considered, providing insights into minor clones, especially in highly endemic areas [9,10]. However, strict quality criteria must be applied, and standardized guidelines should be established to obtain reliable information [11]. Our findings underscore the importance of considering serial sampling in clinical trials for antimalarial drugs in populations with asymptomatic infections in highly endemic areas, as this could provide more accurate identification of species and genotypes present in the infection. They also contribute to the interpretation of data collected at a single time

Table 1
Number of new *pfmsp1* genotypes detected per timepoint of sampling in 31 Gabonese adults with asymptomatic infection who did not have the whole set of genotypes identified during follow-up detected at baseline

Subject	Number of genotypes		Number of new genotypes												
	Total until D7	Baseline	D1			D2			D3			D4	D5	D6	D7
			H8	H16	H24	H32	H40	H48	H56	H64	H72				
IVC083	17	9	-	2	1	-	1	-	-	-	-	3	1	-	-
IVC096	14	10	-	-	-	1	-	1	1	-	1	-	-	-	-
IVC097	11	5	1	1	-	2	-	1	-	-	1	-	-	-	-
IVC005	10	2	1	3	-	-	-	-	3	-	-	-	-	-	1
IVC043	9	4	4	-	-	-	-	-	-	-	-	1	-	-	-
IVC085	9	2	-	-	1	-	3	1	-	2	-	-	-	-	-
IVC012	8	6	-	-	2	-	-	-	-	-	-	-	-	-	-
IVC046	8	5	2	-	-	-	-	-	-	-	-	1	-	-	-
IVC087	7	2	-	-	-	-	-	1	-	-	1	1	-	2	-
IVC095	8	6	-	1	-	-	1	-	-	n/a	-	-	n/a	-	n/a
IVC084	7	4	2	-	-	-	-	-	-	-	1	n/a	n/a	-	-
IVC025	6	2	1	-	-	1	-	-	-	-	-	-	-	1	1
IVC076	6	3	-	-	3	-	-	-	-	-	-	-	-	-	-
IVC068	5	2	1	1	1	-	-	-	-	-	-	n/a	n/a	n/a	-
IVC074	5	3	1	1	-	-	-	-	-	-	-	-	-	-	-
IVC002	4	3	-	-	1	-	-	-	-	-	-	-	-	-	-
IVC019	4	2	-	-	2	-	-	-	-	-	-	-	-	-	-
IVC023	4	2	-	-	-	1	-	-	-	-	-	1	-	-	-
IVC024	4	3	-	-	-	-	-	1	-	-	-	-	-	-	-
IVC044	4	2	-	-	-	-	-	-	-	-	-	-	1	-	1
IVC089	4	2	-	-	-	1	-	-	-	-	1	-	-	-	-
IVC004	3	2	-	-	-	-	-	-	-	-	-	-	1	n/a	-
IVC041	3	2	1	-	-	-	-	-	-	-	-	-	-	-	-
IVC063	3	2	-	-	n/a	n/a	1	-	-	n/a	-	-	-	-	-
IVC065	3	2	1	n/a	n/a	n/a	-	-	-	-	-	-	-	-	n/a
IVC072	3	2	1	-	-	-	-	-	-	-	-	-	n/a	-	-
IVC029	2	1	-	-	-	-	-	-	-	-	-	1	-	-	-
IVC036	2	1	-	-	-	-	-	1	-	-	-	-	n/a	-	n/a
IVC053	2	1	1	-	-	-	-	-	-	-	-	n/a	-	-	n/a
IVC055	2	1	1	-	-	n/a	-	-	-	-	n/a	-	n/a	-	-
IVC077	2	1	-	-	-	-	-	-	-	-	-	-	-	1	-

D: day; H: hour; n/a: genotyping information not available; -: no new genotype.

point, concurrently recognizing their limitations in capturing the complex nature of infection dynamics.

Declarations of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical approval

The clinical trial was approved by the Institutional Ethics Committee of the Centre de Recherches Médicales de Lambaréné (CERMEL) under reference number CEI/CERMEL 006/2019 on 16 May 2019. It is registered with the Pan African Clinical Trials Registry (PACTR201908520097051). The study was conducted according to the ICH-GCP and the declaration of Helsinki as well as all applicable national laws and guidelines.

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Author contributions

JH and BM conceived the study. DEM and RZM collected samples and organized the clinical trial data. JI and AG performed lab experiments. JI analyzed the data. MRo contributed to genotyping data analysis. JI wrote the first draft of the manuscript with input from JH and MRo. GMN, AAA, MRa, and PGK provided resources. All authors reviewed and approved the final version of the manuscript.

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