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Limited Impact of Intermittent Preventive Treatment-Sulfadoxine-Pyrimethamine (IPTp-SP) and long-lasting insecticidal bed nets (LLINs) on Plasmodium falciparum Genetic Diversity and Population Structure among Pregnant Women in Cameroon

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Abstract

Background: Intermittent preventive treatment during pregnancy with Sulfadoxine-Pyrimethamine (IPTp-SP) together with long-lasting insecticidal bed nets (LLINs) are the recommended preventive interventions against malaria in pregnancy. However, transmission intensity and drug resistance in the parasite threatens the efficacy of IPTp-SP and may compromise naturally acquired pregnancy-specific immunity. This study determined the impact of IPTp-SP and LLIN use on the genetic diversity, infection complexity and population structure of *Plasmodium falciparum* in pregnant women across five geo-ecological zones of Cameroon.

Method: Pregnant women were enrolled through a cross-sectional survey conducted at antenatal clinics in selected health facilities across nine (9) regions of Cameroon between October 2019 and October 2022. Malaria was diagnosed with rapid diagnostic test (RDT) and quantitative real-time polymerase chain reaction (qPCR). The *P. falciparum* parasite was genotyped using neutral microsatellite and merozoite surface protein 2 markers. Genetic diversity, multiplicity of infection, and population structure were analysed using Geneious Prime (version 2019) and R (version 4.4.0) software, comparing infections across IPTp-SP and LLIN interventions and geo-ecological zones.

Results: Of 3,313 pregnant women enrolled, 58.8% (1947/3,313), were infected with *P. falciparum* while *P. malariae* and *P. ovale* were detected in 5.0% (165/3,313) and 3.4% (112/3,313) of the participants respectively. Overall, 36% (1191/3313) of participants received at least one dose of IPTp-SP and 30.2% (918/3044) reported concurrent use of IPTp-SP and LLINs. The overall prevalence of malaria in pregnancy was 26.9%, 35.7%, and 35.8 by mRDT, microscopy, qPCR respectively. IPTp-SP and bed net usage were associated with a significant reduction in polyclonal infections and heterozygosity (He), although the overall infection complexity and genetic diversity remain high. The genetic fingerprint of infections did not differ by use or type of interventions; however, low but significant genetic differentiation was observed between parasite infections from different geo-ecological regions.

Conclusions: Overall, these findings suggest sub-optimal protection from malaria infections by IPTp-SP and LLIN and limited influence on the overall genetic diversity and population structure of *P. falciparum* in pregnant women. Continuous molecular surveillance, SP efficacy studies and new approaches may be needed to strengthen malaria prevention in pregnant women across endemic settings.

Keywords: Malaria in pregnancy, *Plasmodium falciparum*, chemoprophylaxis, polyclonal infections, Genetic diversity

Background

Pregnancy-related malaria results in significant adverse maternal, foetal, and neonatal outcomes in malaria endemic areas. Malaria in pregnancy contributes to maternal anaemia, placental malaria, low birthweight, established risk factors for neonatal and childhood mortality [1,2]. The World Health Organization (WHO) recommends intermittent preventive treatment in pregnancy with sulfadoxine-pyrimethamine (IPTp-SP), consistent use of long-lasting insecticidal nets (LLINs), and prompt diagnosis and treatment of malaria infection to reduce these adverse outcomes [3]. The impact of IPTp-SP has been substantial estimated 551,000 (%) neonatal low-birthweight was averted in 2023 among women receiving IPTp-SP across malaria-endemic regions [2,4]. Despite the substantial benefit, malaria in pregnancy remains a significant public health challenge in many endemic countries.

In Cameroon, malaria among pregnant women doubled from approximately 13% in 2013 to 26% in 2021, before slightly dropping to an estimated 21% in 2023 [5]. Although uptake of IPTp-SP in Cameroon increased from 12% in 2011 to 32% in 2018 and 46% in 2022, coverage remains below national targets, with about 50% of pregnant women receiving the recommended three or more doses [5,6]. Late antenatal care attendance, limited access to health facilities, SP stock-outs, and inadequate follow-up systems continue to hinder optimal

coverage. Notably, IPTp-SP coverage declined markedly between the first and third doses, indicating substantial loss to antenatal follow-up [5]. Thus, maintaining a significant reservoir of sequestered malaria parasites in the population.

Malaria parasites infecting pregnant women represent a unique biological phenotype characterised by the expression of the placental adhesion ligand VAR2CSA, which mediates sequestration of infected erythrocytes within the placenta. This placental sequestration promotes chronic infections that contributes to severe maternal anaemia, poor fatal growth and adverse birth outcomes. These parasites are exposed to unique host immune pressures and repeated exposure to IPTp-SP, which may shape their genetic composition differently from the general parasite population in circulation. Understanding the population genetics of parasites infecting pregnant women is therefore important for elucidating mechanisms of parasite persistence, transmission, and adaptation within this vulnerable population.

Genetic diversity is a fundamental characteristic of *P. falciparum* populations and plays a critical role in parasite survival, transmission, and adaptation. High genetic diversity may facilitate immune evasion and reduced efficacy of chemopreventive and vaccine-based interventions in this vulnerable group by increasing antigenic and genomic variation [7,8]. Malaria control interventions, particularly IPTp-SP, can exert selective pressure on parasite populations, potentially influencing within-host infection complexity, allele frequencies, and population structure. Consequently, molecular surveillance of *P. falciparum* population infecting pregnant women provides an important framework for monitoring parasite diversity, assessing transmission dynamics, detecting emerging drug resistance, and evaluating the evolutionary impact of malaria intervention. Such molecular surveillance is equally relevant for other SP-based chemoprevention strategies, including Seasonal or Perennial Malaria chemoprevention (SMC or PMC) whose effectiveness may potentially be influenced by parasite population genetics [9,10].

In Cameroon, *P. falciparum* is responsible for most malaria morbidity and mortality and exhibits, high genetic diversity, consistent with high transmission populations across West and Central Africa [11,12]. The widespread implementation of malaria control intervention, including IPTp-SP, exerts selective pressure on parasite population that may alter within-host infection complexity and favour the emergence, persistent or expansion of drug-resistant strains. Complete and partial resistance to SP is well established across African countries, including Cameroon, where molecular markers associated with SP resistance are reported to be highly prevalent [13]. Nevertheless IPTp-SP continues to provide substantial clinical benefits and remains a cornerstone of malaria prevention during pregnancy [9] Understanding

how intervention influence parasite population genetics is therefore essential for evaluating the long-term sustainability and effectiveness of malaria chemoprevention programmes.

Cameroon exhibits substantial spatial heterogeneity in malaria transmission intensity, mosquito vector species distribution and parasite population dynamics due to its geo-ecological diversity [14-16]. Its geo-ecological zones include; Sahelian, humid savanna, equatorial forest, coastal and highland, presenting one of the richest biodiversity in Africa [17]. Consequently, malaria transmission is broadly categorized into Sahelian (North) with seasonal, epidemic-prone risk; Humid Savanna (Central/Plateau) with moderate, seasonal transmission; Equatorial Forest (South) with high, perennial transmission; and Highlands/Coastal areas, all experiencing different vector dynamics and transmission intensities, creating varied control challenges [14,18,19]

These contrasting transmission settings influence parasite recombination rates, multiplicity of infection, genetic diversity and gene flow, which may probably affect the epidemiology of malaria in pregnancy and the effectiveness of control interventions. An understanding of parasite population dynamics across diverse transmission settings is important for targeted malaria control strategies that maximise intervention effectiveness while limiting the evolution and spread of antimalarial resistance.

While previous studies have assessed *P. falciparum genetic* diversity and population structure in Cameroon using a range of molecular tools, including MSP1/2 genotyping, microsatellites markers, and targeted and whole genome sequencing approaches [29, 56,57]. Study by Atuh et al. [51] showed high parasite diversity and significant evidence sub-structure between highland and coastal equatorial forest zones in the southwestern region [51]. However, most previous investigations have focused on the general population, with limited attention given to pregnant women, despite their heightened vulnerability to malaria and repeated exposure to IPTp-SP. Hence, little is known about the extent to which IPTp-SP and LLIN use influence parasite infection complexity, genetic diversity, and population structure among pregnant women across the diverse transmission settings of Cameroon. This study, therefore, assessed the prevalence of *Plasmodium* species infection, and the effect of IPTp-SP and LLIN usage on parasite infection, genetic diversity, and population structure of isolates from pregnant women in Cameroon; to contribute evidence that support the optimisation of interventions strategies against pregnancy malaria.

Materials and Methods

Study design, population and sampling

Participants for this study were recruited from cross-sectional surveys of pregnant women in selected towns/health districts across 9 out of 10 regions of Cameroon, conducted between October 2019 and October 2022. Each participating pregnant woman was recruited voluntarily from antenatal care (ANC) clinics following a signed informed consent. A total of twenty-seven (27) health facilities surveyed, selected to cut across the five diverse geo-ecological zones of Cameroon from North to South, with a mix of urban and rural health districts. These included the following regions and sites - Southwest (Mamfe and Mutengene), Littoral (Douala), South (Kribi), Adamawa (Meiganga), East (Bertoua), West (Bafoussam, Bafang and Dschang), Northwest (Bamenda Town, Nkwen, Azire, Santa and Ndop), North (Garoua) and Far North (Maroua) (Fig. 1). These sites cut across a geo-ecological and malaria transmission intensity gradient, characterised by differences in vector population dynamics and parasite species [20]. At recruitment the demographic and clinical information of each consented pregnant woman was document; maternal age, gravidity, parity, gestational age, history of fever during gestation as well as IPTp-SP usage, IPTp-SP dosage and LLIN use. LLIN use was defined as self-reported sleeping under a treated bed net the previous night proceeding enrolment. About two (2) ml of blood was then collected from each participant by venipuncture into EDTA tubes. Approximately 50µl of whole blood was also spotted on labelled Whatman No. 3 filter paper and allowed to air-dry overnight at ambient temperature to obtain a dried blood spot (DBS). Each DBS was stored in a sealed plastic bag containing desiccant sachets, protected from light, moisture and stored in a cold room until laboratory analysis following WHO recommendations [21].

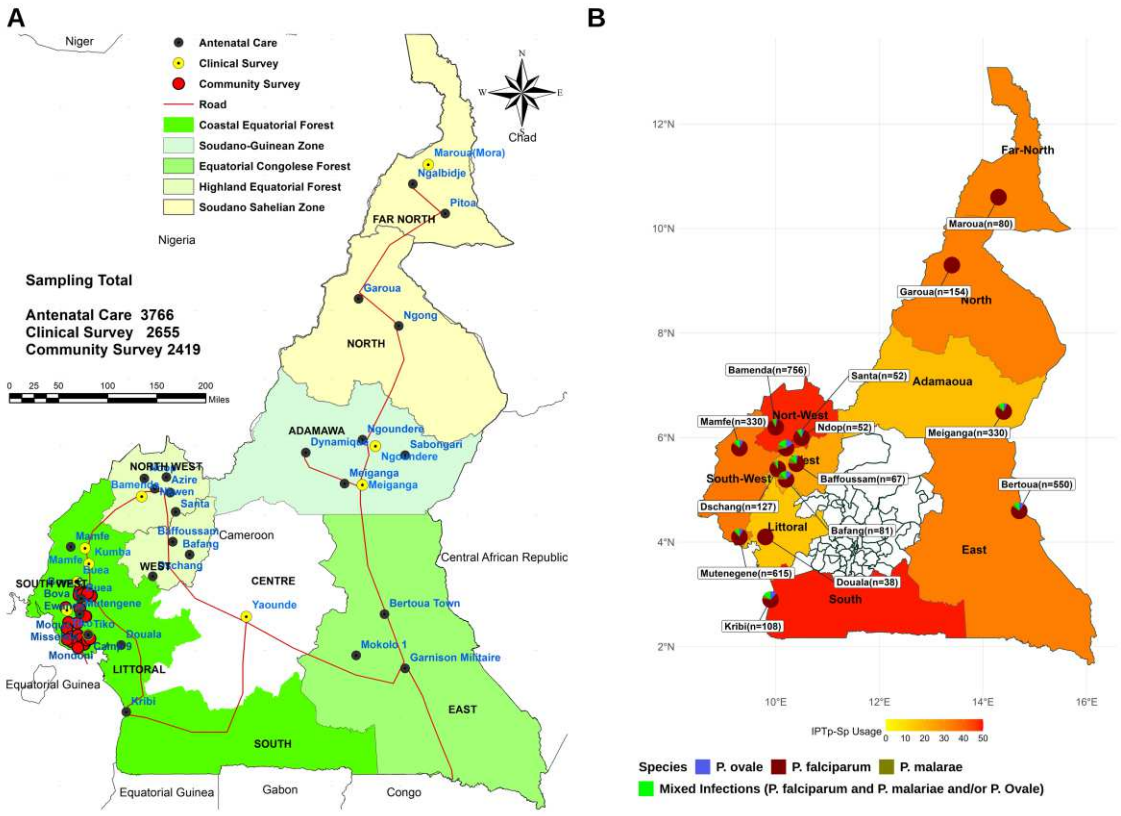


Figure 1. Map of Cameroon, showing sampling sites in (A), with each region numbered and colour-coded by designated ecological zones, and sites shown as red points in the regions. The central region (8) was not included in the study. (B) Distribution of samples and prevalence of *Plasmodium species* shown as pie charts. The heat scale per region depicts the coverage of IPTp-SP usage across (https://www.diva-jis.org).

Diagnosis of malaria and parasitaemia determination

At recruitment, all participants were tested for malaria using RDT. Parasite densities were determined by light microscopy of thick blood films stained with 10% Giemsa (Sigma, Sino-American) following standard WHO protocols [22,23]). For molecular diagnosis, DNA was extracted from DBS using the Chelex®-Tween 20 method. *P. falciparum* infection was detected using an ultrasensitive quantitative real-time polymerase chain reaction (qPCR) assay targeting the acidic terminal sequence of the var gene (varATS≈59 copies/genome) [Table S1] [24]. A quantitative real-time semi-nested PCR speciation assay, involving pre-amplification by conventional PCR followed by species specific qPCR was used to detect P. *ovale* and *P. malariae* [Table S1] [25], while infection with *P. vivax* infections were tested using the protocol reported Hofmann et al. [24].

Plasmodium falciparum merozoite surface protein-2 (MSP-2) allelic genotyping

The merozoite surface protein 2 (msp2) gene was successfully genotyped in 476/576 *P. falciparum* qPCR and microscopy positive samples. MSP2 repeat size polymorphism was determined by nested PCR involving primary amplification of the entire polymorphic gene segment of block 3, followed by a nested reaction targeting the allelic type-specific regions using specific primers [Table S1**]** [27,28]. The size of PCR products was determined following capillary electrophoresis on QIAxcel Advanced System with QIAxcel DNA High Resolution Kit, the 15 bp/1 kb QX Alignment Marker (Qiagen cat# 929521) and the 15-3000 bp QX size marker (Qiagen cat# 929556) according to the manufacturer's instructions. Alleles in each family were considered the same if the fragment sizes were within a 20 bp interval. Infections with a single msp2 allele were considered as monoclonal, whereas those with two or more alleles were classified a polyclonal [30].

Microsatellite genotyping and allele scoring

Plasmodium falciparum isolates from 552/576 infections were successfully genotyped using nine neutral polymorphic microsatellite markers (ARA2, Pfg377, PfPK2, TA87, TA81, TA42 2490, Polya and TA60) distributed across five parasite chromosomes [31]. Each locus was amplified following a hemi-nested 2-step PCR with fluorescent end-labelled sequence primer pairs as previously described [Table S2] [28,31]. Electropherograms or peaks generated for each isolate were visually inspected, and allele sizes were determined from the predominant peaks before being binned based on the repeat motif length for each locus using Geneious Prime software (Applied Biosystems version 2019). For samples with multiple amplicons, only peaks with height > 200 relative fluorescent units (RFU) were retained for subsequent analysis. Secondary peaks at heights<30% of the height of the predominant allele peaks were considered as stutters and artefacts and excluded. Samples with more one peak at any of the loci were considered as polyclonal infections.

Genetic diversity analysis

Initial infection genetic diversity was assessed based on the MSP2 allotype, classifying infections as either IC3D7 or FC27 allelic family. Further diversity was assessed across the nine microsatellite loci. Only the dominant alleles were used to assemble infection genotypes for population genetic diversity analysis, as previously described [32]. Population-level genetic diversity was assessed by estimating expected heterozygosity (He), observed heterozygosity (Ho) and number of alleles per locus (allelic richness (A). Expected heterozygosity was computed as $He=1-\sum pi^2$, where pi is the allele frequency at locus i [33]. Observed heterozygosity (Ho) was calculated as the proportion of heterozygous genotypes

observed. Mean He and Ho were compared among intervention groups (No IPTp-SP, IPTp-SP, IPT-SP + bed net).

Multiplicity of infection

Within-host parasite diversity or multiplicity of infection (MOI) was defined as the maximum number of genetically distinct parasite clones determined from multiplicity of distinct alleles (fragment peak) within or across MSP2 families, or microsatellite per infection. Infections with MOI=1 was considered as monoclonal, whereas those with MOI > 1 were classified as polyclonal [34]. Further assessment was done using a modified version of the within-host fixation index or the variant of inbreeding coefficient/within-host diversity (*F_{WS}) derived from microsatellites [35,36]. The *F_{WS} index expresses the probability that any random pair of infections carry different alleles at a specific locus and was calculated for each sample using the equation *F_{WS} =1–Hw/Hs, where Hw=allele frequency of each unique allele at a locus for every individual and Hs=heterozygosity of the parasite population. A low *F_{WS} indicates high within-host diversity relative to the population, while samples with *F_{WS} > 0.95 were considered clonal, those with *$F_{WS} \leq 0.70$ were classified as high complexity [37,38].

Population differentiation and structure

Genetic differentiation among parasite subpopulations was assessed using Wright's population fixation index (F_{ST}) implemented in the hierfstat and adegenet R packages [39-40]. It measures differences in the distribution of within- and between-population allele frequencies. For multiallelic microsatellite loci and assuming Hardy–Weinberg proportions, F_{ST} between ecozones, and between clinical groups, was estimated as: F_{ST}= HT-HS/HT =1-(HS/HT), where HT is the total heterozygosity and HS is the average subpopulation heterozygosity. Pairwise values were calculated among intervention groups and geo-ecological zones. Values close to zero or negative values resulting from sample variance were interpreted as lack of genetic differentiation.

The degree of allelic correlation was determined by linkage disequilibrium (LD), D, among microsatellite alleles was calculated as: $D=pAB-pA\cdot pB$. Where: - pAB is the frequency of the haplotype containing both alleles A and B. - pA is the frequency of allele A. - pB is the frequency of allele B. The normalized linkage disequilibrium (D') was determined as: $D'=DD$max, where Dmax is the maximum possible value of D, calculated as: Dmax=min($pA\cdot(1-pB)$,$(1-pA)\cdot pB$). This normalization allows D' to range between -1 and 1, indicating the strength and direction of the association. LD between two loci was further determined by the squared correlation coefficient ($r2$) calculated as: $r2=D2pA\cdot(1-pA)\cdot pB\cdot(1-pB)$. Population genetic structure was explored using Discriminant

Analysis of Principal Components (DAPC) and visualised with scatter plots using the adegenet and factoextra packages in R. For this, allele frequencies for each population group were transformed into a genetic variance–covariance matrix, and the first two principal components were used to visualize clustering of isolates by intervention or geographic groups.

Statistical analysis

Participant demographic and clinical data were entered into Microsoft Excel and exported to SPSS version 21 for descriptive analysis. Binned microsatellite genotypes generated in Geneious Prime (version 2019) software (Applied Biosystems) were exported as text files for population genetic analyses R (v4.4.0). Genotyped data was cleaned and filtered using the dplyr package by removing incomplete or non-numeric entries before downstream analysis. Allele frequency distributions, MOI, heterozygosity estimates, pairwise, and linkage disequilibrium statistics were visualized using scatterplots and heatmaps to depict patterns of genetic diversity, relatedness, and population structure. The significance of isolation by distance was evaluated using the Mantel statistic based on Pearson's product-moment correlation between genetic and geographic distance, at $\alpha = 0.05$.

Results

Demographic and obstetric characteristics of the study population

A total of 3,313 pregnant women aged 14–40 years were surveyed in the study during the rainy and dry seasons across nine (9) regions in Cameroon. The study population included: multigravida (40.2%), second semester of gestation (53.7%), non-anemic (63.0%), greater than 25 years of age (49.5%), on IPTp-SP was (62.2%) and those who had taken at least one IPTp-SP dose (47.5%). In addition, most participants came from High Equatorial Forest (34.26%), were of the Semi-Bantu ethnic group (54.56%), most were not using bed nets (74.0%), mostly in the second trimester of gestation (53.7%), and had 1-3 children (53.3%) at enrolment (Table 1).

Table 1: Baseline Socio-demographic characteristics of parturient mothers

Parameter	**mean ± (SD)**	**[Range]Unit**
Age	25.9 (±5.9)	[14-40] years
Gestational age	24.4 (±7.1)	[10-45] weeks
Hb level	11.9 (±1.9)	[6.1-19.5] g/dl
Gravidity	2.4 (±1.5)	[1-9] pregnancies
Parity	1.3 (±1.4)	[0-8] children
Weight	68.7 (±13.8)	[20-180] kg
IPTp-SP dosage	1.7 (±1.0)	[1-4] doses
Auxiliary temperature	36.6 (±1.0)	[26-43] ^{O}C

Parameter	Sub-class	Total No	% (n)
Age group (years)	≤ 20	3115	21.0 (655)
	21 - 25		29.5 (918)
	> 25		49.5 (1542)
Gravidity	Primigravidae	2720	38.4 (1045)
	Secundigravidae		21.4 (583)
	Multigravidae		40.2 (1092)
Parity	0	2656	38.7 (1029)
	1 - 3		53.3 (1415)
	≥ 4		8.0 (212)
Trimester of gestation	First	2855	6.4 (184)
	Second		53.7 (1532)
	Third		39.9 (1139)
IPTp-SP status	Non-Use	3154	62.2 (1963)
	Use		37.8 (1191)
IPTp-SP Dosage	Not specified	1191	12.7 (151)
	1		47.5 (566)
	2		20.3 (242)
	≥ 3		19.5 (232)
Bed net status	Bed net usage	3109	26.0 (810)
	Bed net non-usage		74.0 (2299)
Bed net & IPTp-SP Use	Enrolment	2950	67.8 (2001)
Anaemic Status	Anaemic	1988	37.0 (736)
	Non-Anaemic		63.0 (1252)

Prevalence of malaria parasite infection

The prevalence of malaria parasite infection was 17.3% (514/2,973), 14.6% (402/2,762) and 60.3% (1984/3,313) by blood mRDT, microscopy and qPCR respectively, with 58.8% (1947/3,313), 5.0% (165/3,313) and 3.4% (112/3,313) harbouring *P. falciparum, P. malariae* and *P. ovale* respectively (Fig. 1A, 2A, BC). Most of the microscopy diagnosed infections had low parasite densities, with a geometric mean parasite density (GMPD) of 260 parasites/µl of blood, ranging from 78 – 36,178 (Table 1). *Plasmodium falciparum* was the most prevalent species, while no *P. vivax* infection was detected at all study sites (Fig. 1B). Two hundred and seventy-seven participants (8.4%) had mixed infections with either *P. falciparum* and/or *P. malariae* and/or *P. ovale*, indicating ongoing but limited transmission of non-*falciparum* species.

There was spatial variation in IPTp-SP uptake, with the highest coverage (40–50%) observed in the Northwest and South regions, and lower coverage (10–30%) across the Adamawa, East, North, and Far North regions (Fig. 1B). From participant reports, about 36% (1191/3313) confirmed taking at least one dose of IPTp-SP, while only 19.5% reported taking ≥ 3 doses. Of

those using IPTp-SP, 918 were also using bed nets. IPTp-SP and IPTp-SP + bed net users had significantly lower (p=0.0001) malaria parasite infections than non-IPTp-SP users.

The prevalence of malaria parasite infections in IPTp-SP + bed net users, IPTp-SP users and IPTp-SP non-users were (18.2% vs 26.9% vs 73.1%, p=0.0001) by mRDT, (24.9% vs 35.7% vs 64.3%, p<0.0001) by light microscopy and (27.3% vs 35.8% vs 64.2%, p<0.0001) by real-time PCR (Fig. 2B). The prevalence of sub-microscopic *P. falciparum* was significantly higher (65.4%) in women who were not on chemoprevention, compared to 28.4% and 34.6% for those using IPTp-SP + bed or IPTp-SP alone, respectively (Fig. 2C). Overall malaria prevalence decreased with increasing IPTp-SP dosage when assessed by RDT and microscopy. RDT positivity declined from 17.9% among women who received no IPTp-SP dose to 8.5% among those who received ≥3 doses, while microscopy positivity decreased from 12.3% to 7.7% across the same groups. These findings suggest that IPTp-SP use was associated with reduced detectable malaria infection during pregnancy.

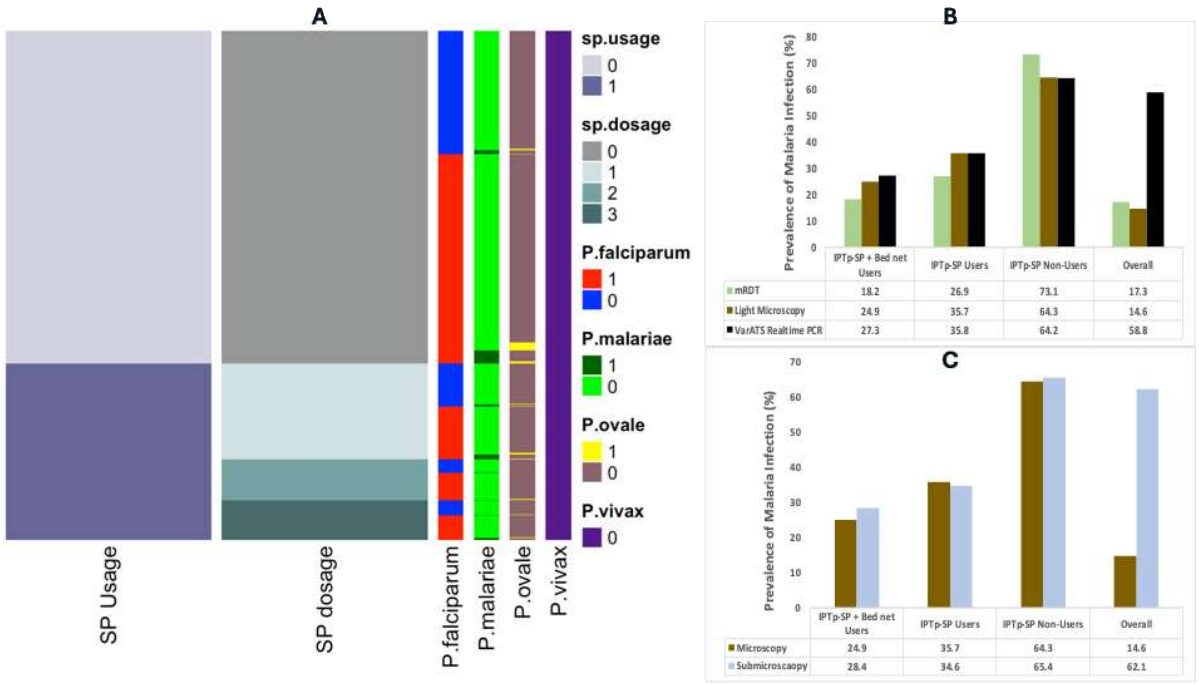


Figure 2. IPTp-SP use and *Plasmodium* species infections in Cameroon. A) Distribution of *Plasmodium spp* infection. Colour codes represent groups for SP users or diagnosis for each *Plasmodium species*, where 1 depicts infection and 0 for no infected detected, B) Barplot of parasite prevalence determined by light microscopy, RDT and qPCR in IPTp-SP users and non-users, and C) Prevalence of microscopic and sub-microscopic malaria parasite infection between IPTp-SP users and non-users.

Infection Complexity of *Plasmodium falciparum*

A combine analysis on MSP2 IC3D7_MSP2 FC27 allelic families across the IPTp-SP or IPTp-SP+bed net users and non-users showed a significant difference (p=0.0041) in polyclonal infections between groups. A wide distribution of multiplicity of infection (MOI) was estimated, ranging from 1 to 7 clones in infections across all groups (Fig. 3A). The prevalence of polyclonal infections significantly decreases (p=0.0038) from 50% in non-IPTp-SP to 34.9% in IPTp-SP user groups. Infections estimated to have more than 5 clones were only observed in the group of women who were not on IPTp-SP. Women on IPTp-SP or in combination with bed nets had a narrower distribution of MOI with an estimated maximum of 5 clones detected, although this was not significant different from other groups (p=0.17). However, the fraction (¼) of infections with 2 or more clones was significantly lower in groups that were on IPTp-SP chemoprevention. Increasing SP dosage was associated with lower malaria parasite complexity. Polyclonal infections declined from 26.0% among women with no SP exposure to 14.8% among those receiving ≥3 doses, while mean MOI decreased from 1.36 to 1.15. Despite these declining trends, differences in polyclonal infection and MOI were not statistically significant ($p > 0.05$). The MOI varied across the geo-ecological zones, with the Soudano Guinean Zone showing the highest and most variable MOI with up 7 clones. However, the Western Highlands estimated the lowest and least variable MOI with most infections clustered around one to two clones, indicating lower parasite diversity and transmission intensity setting. The overall mean inbreeding coefficient, determined by *Fws, was low across all groups, an indication of high within-host diversity, high polyclonal infections and high genetic diversity (Fig. S1A). The median *Fws value was not significantly different across SP user groups.

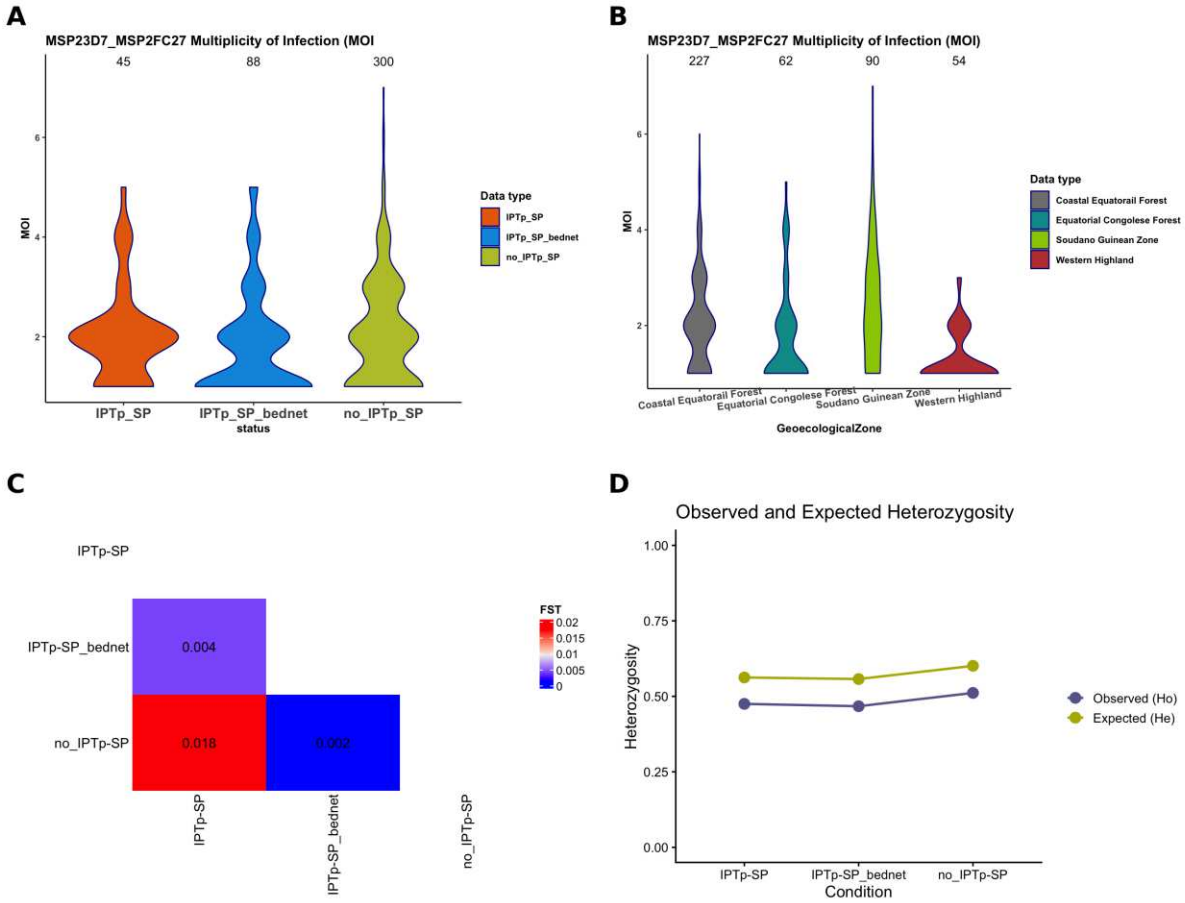


Figure 3. Infection Complexity of *Plasmodium falciparum* A) The violin plots show the distribution of polyclonal infections determined by MSP2 repeat polymorphism across study groups; IPTp-SP, IPTp-SP +bed net and Non-IPTp-SP Users. B) The inbreeding coefficient (*Fws) within study groups; SP, SP +bed net and Non-SP Users. The number above each plot indicate the sample size for each group. C) This heat map shows FST values between group pairs; non-IPTp-SP versus SP Users (red), SP + bed net versus non-IPTp-SP users (Purple) and SP versus IPTp-SP + Bed net (blue). The colour intensity represents the FST values on a scale ranging from 0 to 0.006 inside each cell predicting the level of genetic differentiation. D) Observed (H_o) and expected (H_e) heterozygosity of *Plasmodium falciparum* across intervention groups.

Genetic diversity and population structure of *Plasmodium falciparum*

The allelic richness (Na) per microsatellite locus ranged from 3 (low) for locus TA40 to 30 (high) for loci TA81 and 2490. All, except the PFG377 loci had allele sizes within the range of the 3D7 reference genome across all the study groups. There were an exceptionally high allele frequency (1.0) and significant difference in the TA40 locus (Fig. S1B). Observed and expected heterozygosity indicated a moderate reduction in parasite genetic diversity as determined by lower heterozygosity in populations using IPTp-SP and bed net interventions. Also, at the geo-

ecological level a slightly decreased heterozygosity was see in the western highlands and coastal equatorial forest (Fig. S1C). The mean observed heterozygosity declined slightly from non-IPTp-SP (H_o=0.601) to IPTp-SP (H_o=0.563) and SP + bed nets (H_o=0.557) user groups. Also, the expected heterozygosity showed a similar but smaller decline, dropping slightly IPTp-SP (H_e=0.511) to IPTp-SP + bed net (H_e=0.467) group (Fig. 3D). Generally, the expected heterozygosity declined with increasing SP dosage, from women without SP exposure to women receiving ≥3 doses.

There was no genetic differentiation in the *P. falciparum* interventions populations, with very low microsatellite fixation Index (F_{ST}) values (0 to 0.005) observed between the study groups (Fig 3C), irrespective of intervention. This was equally the case with geo-ecological zones (Fig S1D). Discriminant Analysis of Principal Components (DAPC) scatter plot did not separate the isolates by intervention groups (Fig. 4A). However, partial clustering by eco-epidemiological zones, indicating several distinct clusters was observed. Infections from coastal equatorial forest region were distributed across all 3 clusters. Isolates from the Western Highland (typical from the West and Northwest highlands) mostly clustered together, whereas those from other ecological zones were distributed across multiple clusters (Fig. 4B). Partial sub-clustering of infections from the western highlands was evident from clustered heatmap of genetic distances (Fig. 4C). Overall, pairwise genetic distances were predominantly low to moderate, consistent with limited isolation by distance and extensive mixing of infections across the various geo-ecological strata. However, a weak but statistically significant correlation was observed between parasite genetics and geographic distance and ($r = 0.026$, $p = 0.007$) (Fig. 4D).

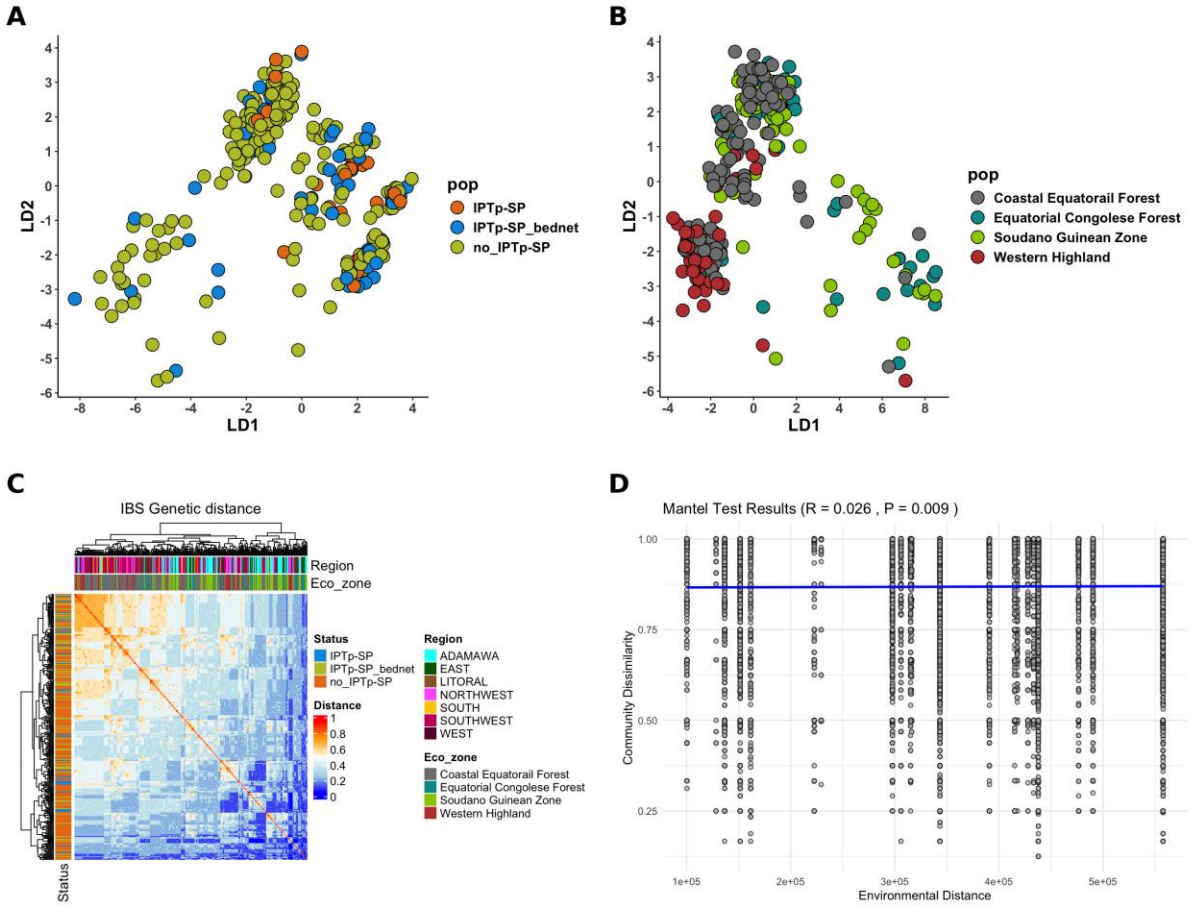


Figure 4. Genetic diversity and population structure of *P. falciparum* A) Discriminant Analysis of Principal Components (DAPC) scatter plot for Genetic population clustering of *Plasmodium falciparum* showing no distinct clusters within the three study groups. The spread in the points/isolates and lack of discrete clusters shows similarities in the *P. falciparum* population. B) Genetic population clustering of *Plasmodium falciparum* by geo-ecological zone. Partial separation of Western Highland isolates and substantial overlap among forest and savannah zones. C) Pairwise genetic distance heatmap. Widespread admixture with no clear grouping by region, eco-zone, or intervention status. D) Environmental distance versus community dissimilarity. Weak positive relationship with significant association within geo-ecological zones.

Discussion

We conducted an epidemiological survey of genetic diversity of *P. falciparum* among pregnant women who are using the current malaria interventions recommended against malaria in pregnancy by WHO intermittent preventive treatment (IPTp-SP) and long-lasting insecticide-treated bed nets. These strategies are widely implemented to mitigate the negative effects of malaria in pregnancy on mother and unborn child. Our findings revealed an overall low to

moderate adherence to both interventions, with a significant proportion of gravid women contracted infections during pregnancy. A larger proportion of these infections were detected by sensitive quantitative PCR, indicating that sub-microscopic infections that likely also go undetected by RDTs may be more prevalent in this group than appreciated. This high rate of sub-microscopic infections is consistent with previous reports on the large burden of malaria in pregnant women than evident from routine rapid RDT or microscopy diagnosis [9,42,43]. These infections were dominated by *P. falciparum*, but also non-falciparum species, whose impact on health of mother and unborn child need further evaluation. The presence of non-falciparum species *(P. malariae* and *P. ovale*) despite low frequences indicates an ongoing transmission of non-falciparum malaria. *P. malariae* can establish long-lasting blood-stage infections, while *P. ovale* can reactivate from dormant hepatic hypnozoites (relapse), allowing both species to persist in the human host for extended periods. Their presence highlights the need for species-inclusive surveillance for malaria control [4]. However, there was an overall significant reduction in infection rates and species complexity with the use of IPTp-SP and more so with the additional used of LLINs, affirming known protection of these interventions, consistent with reduced odds of infection among users as shown by other studies [44,45]. Overall, these results highlight that routine diagnostics tools substantially underestimate the true burden of malaria in pregnancy (MiP) and that sub-microscopic infections constitute a significant malaria reservoir, needing more effective interventions. IPTp-SP coverage remains suboptimal in Cameroon as previously shown [46]. Thus, approaches to enhance adherence to both chemoprevention and LLINs would be beneficial for pregnant women in Cameroon.

From genotyping polymorphic MSP and microsatellite loci, we observed that pregnant women who are not adhering to IPTp-SP had more complex infections, constituting several genetically distinct *P. falciparum* clones. However, persistent infections, some with multiple clones were seen, in users of this chemoprevention reflecting its limitations. Hence SP remains moderately effective in clearing and preventing infections. This could be partly due to SP resistance driven by mutations in *P. falciparum* dihydrofolate reductase and dihydropteroate synthetase, or the high force of infection (transmission intensity) across the country resulting in reinfections despite SP use. SP has been discontinued as first line therapy across Africa, but the footprint of drug resistance remains, with high rates of mutations associated with its resistance persisting as reported across Cameroon [13]. Reduced drug effectiveness or efficacy and the presence of resistant parasite strains has previously been reported in the same area of Cameroon [46,47]. Nevertheless, evidence indicates that IPTp-SP retains partial effectiveness, possibly through suppression of parasite density [48]. High prevalence of *Pfdhfr-Pfdhps* mutations, including

quintuple and sextuple mutants, has been documented, potentially compromising IPTp-SP efficacy [2,43,48,49], hence the need for additional interventions to prevent MiP. As most infections were not genetically distinct by use of non-use of interventions, these may not need specific user group customisation. However, infections in pregnant women from different parts of the country showed genetic sub-clustering based on region of sampling. The most distinct group were infections from the highland plateaus of North and North-West regions. These regions are highly characterised by very low malaria transmission intensity as evident in the reduced levels of infection complexity/MOI mostly dominated by monoclonal infections. This might limit the possibilities for superinfection and parasite recombination, resulting in less genetically diverse parasite population. Geo-ecological and vector differences can create localized transmission patterns that explain the partial geographic structuring observed in the Western Highland parasites despite overall population homogeneity [19,50]. This geographic divergence was corroborated by the small but significant isolation by distance, as shown before for population samples drawn from the same regions of the country [51]. Such geo-ecological clustering of infection genotypes is uncommon in most of Africa, where populations are thought to be freely mixing due to high transmission. Only large regional population structure had been shown in parasite populations across Africa, but the scale of spatial sampling within Cameroon and most of central Africa was low, probably missing this local sub-structuring [52]. Globally, genetic differentiation appears more strongly associated with eco-epidemiological conditions than with study group classification, indicating that geography outweighs treatment exposure in shaping parasite structure [53-55]. It is likely that that local conditions have allowed populations in this region to drift, further facilitated by lower connectivity and reduced geneflow with the southern and coastal regions of the country. More extensive genome wide genetic analysis could reveal insights into the wider nature of this divergence and its impact on malaria and interventions towards elimination.

This study is limited by the fact that information on IPTp-SP uptake and LLIN use was partly based on self-report and antenatal records which might result bias in reporting coverage. Although it provides insight into parasite genetic diversity and population structure (genetic epidemiology study) in MiP, it did not directly assess IPTp-SP therapeutic efficacy and assess genetic markers associated with SP efficacy. Finally, microsatellite markers and MSP2 antigenic marker, provide lower genomic resolution than contemporary genome sequencing approaches, which could further characterize parasite population structure and transmission dynamics.

Conclusion

Plasmodium falciparum infections persist among pregnant women in Cameroon, irrespective of IPTp-SP and bed net interventions with sub-microscopic infections constituting a substantial hidden reservoir of malaria transmission. The genetic diversity of infections does not differ due to IPTp-SP or bed net use, but these interventions reduce the within host complexity of infections. This coupled to the subtle eco-geographic sub-structure of infections calls for extended surveillance and incorporation of genetic data to refine elimination strategies.

Declarations

Ethical approval and consent

Ethical clearance (Ref. No. 2019/09/1188/CE/CNERSH/SP) was obtained from the Cameroon National Ethics Committee (CNERSH) while administrative authorization was obtained from the Regional Delegate of Public Health, District Medical Officer, and Chief Medical Officers as appropriate. After adequate sensitization about the study objectives, risks and possible benefits, only pregnant women who volunteered to participate by signing a written informed consent form were enrolled. Assent was equally obtained from the guardians of pregnant women below 16 years of age in line with national gynaecological guidelines.

Availability of data and materials

The dataset generated and analysed for this study are available on request and insert links to supplementary materials and data on the manuscript.

Clinical trial number

Not applicable

Competing interests

The authors declare that they no competing interests.

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