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Assessing the burden of submicroscopic *Plasmodium* infections in a pre-elimination malaria setting in sub-Saharan Africa, Guinea-Bissau

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Abstract

Background Submicroscopic *Plasmodium* infections can be a source of persistent malaria transmission. The aim of this study was to assess their frequency, distribution, morbidity and associated factors in a pre-elimination malaria setting in sub-Saharan Africa, Guinea-Bissau, where the *Plasmodium falciparum* is the predominant *Plasmodium* species.

Methods Dried fingerprick whole blood samples from 601 participants in the 2017 national, household-based, cross-sectional survey to estimate malaria prevalence were subjected to DNA extraction. The DNA was used in nested end-point PCR assays targeting *genus*- and *species*-specific regions of the *Plasmodium* 18S rRNA genes. Statistical analysis of socio-demographic, clinical and molecular data was carried out using the Statistical Package for the Social Sciences, version 29. Factors associated with submicroscopic *P. falciparum* infections and their magnitude were sought using Chi-square test and multiple logistic regression models, respectively. Statistically significant level was considered at *P*-value < 0.05.

Results Nested PCR assays detected submicroscopic *P. falciparum* infections in 20.3% (95% CI = 16.8–23.8) of individuals microscopically negative for *Plasmodium* species in the general population and in 21.4% (95% CI = 9.9–36.5) of microscopically negative pregnant women. Submicroscopic *Plasmodium malariae* infections were also detected as co-infections in 3.0% individuals who were microscopically positive only for *P. falciparum*. Infections with other *Plasmodium* species were not detected. Submicroscopic *P. falciparum* infections were not associated with age, sex, or the presence of fever. A logistic regression model adjusted for ethnicity and health region showed that individuals from the Balanta and Bijagos ethnic groups, most of whom live in the low malaria-transmission areas of Quinara and Bissau, and the Bijagos archipelago, respectively, were less likely to have submicroscopic *P. falciparum* infections than individuals from the large Fula ethnic group, most of whom live in the high malaria-transmission area of Gabu. Submicroscopic *P. falciparum* infections were not associated with anaemia in children under 5 years of age.

Conclusion The results obtained highlight the contribution of asymptomatic and submicroscopic *P. falciparum* infections to malaria transmission in high malaria-transmission areas and the need for molecular-based tools to detect submicroscopic *Plasmodium* species.

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Background

Malaria remains a serious public health problem and the leading cause of morbidity and mortality in several endemic countries, despite the efforts made in recent decades to reduce its impact. Continued disruptions to national malaria control programmes due to socio-political-economic instability, the emergence of artemisinin-resistant *Plasmodium falciparum* parasites, histidine-rich protein 2 deleted *P. falciparum* parasites, pyrethroid-resistant *Anopheles* mosquitoes and climate change, have contributed to halting the global decline in malaria cases since 2015 [1–3].

To get back on track towards malaria control and elimination, in addition to overcoming the barriers mentioned above, it is essential to ensure that all cases of malaria are properly diagnosed and treated. These include asymptomatic malaria cases, which do not lead to seeking medical care, and submicroscopic *Plasmodium* infections, e.g., *Plasmodium* infections with levels of parasitaemia below the detection limit of routine diagnostic methods, such as optical microscopy or rapid diagnostic tests [4]. Without diagnosis and treatment, asymptomatic and submicroscopic *Plasmodium* infections are thought to sustain malaria transmission and contribute to residual malaria transmission and outbreaks [5] by acting as parasite reservoirs [4, 6] and providing female *Anopheles* mosquitoes with gametocyte forms of the parasite during a blood meal. Asymptomatic and submicroscopic *Plasmodium* infections have been reported in several malaria endemic countries [7–9] including Guinea-Bissau on the west coast of sub-Saharan Africa [10–12].

Malaria in Guinea-Bissau is mostly caused by *P. falciparum*, which causes life-threatening forms of the disease and remains endemic throughout the country with seasonal and regional variations [13]. The global trend in malaria incidence has also been observed in Guinea-Bissau over the past two decades. A huge decrease in estimated malaria cases from almost 500 000 to just over 100 000 was observed in the period 2000–2006, followed by a new increase in the subsequent years, culminating in 208 000 estimated malaria cases in 2011, and a new marked decrease in the subsequent period with 120 000 estimated malaria cases in 2017 [3]. A new period of progressive increase in malaria incidence has been observed since 2017, with 225 000 estimated malaria cases in 2022 [3]. Whether asymptomatic malaria cases and submicroscopic *Plasmodium* infections have contributed to the stagnation of malaria control and successive fluctuations in malaria incidence in Guinea-Bissau is unknown.

The aim of this study was to shed light on malaria epidemiology in Guinea-Bissau by assessing the frequency, distribution, morbidity and associated factors of submicroscopic *Plasmodium* infections, and characterizing the epidemiological profile of the infected individuals.

Methods

The 2017 Malaria Indicator Survey

This study was conducted using socio-demographic and clinical data and dried fingerprick whole blood samples from the Malaria Indicator Survey (MIS) carried out in Guinea-Bissau in 2017. The 2017 MIS was a household two-stage cluster survey, stratified by the 11 health regions of Guinea-Bissau, carried out towards the end of the rainy season (September to October), using the standardized Roll Back Malaria (RBM) protocol with a few local adaptations [14]. After informed consent was obtained, a structured questionnaire was applied collecting, among others, data on the occurrence of fever in the previous 14 days. Thick and thin fingerprick whole blood smears were collected from all individuals enrolled in the malaria prevalence survey and subjected to optical microscopy (OM) analysis by experienced microscopists at the laboratory of the National Institute of Health of Guinea-Bissau.

In parallel, drops of fingerprick whole blood, from each participant in the malaria prevalence survey, were collected onto filter paper and stored at room temperature with silica gel with colour indicator. Children aged from six to 59 months were screened for anaemia by measuring haemoglobin levels using the HemoCue® haemoglobinometer. Anaemia was considered in children with an haemoglobin (Hb) < 11.0 g/dL of whole blood according to WHO criteria for anaemia in children at this age range [15]. Those with history of fever on the day of visit were screened for *P. falciparum* parasites using rapid diagnostic tests (RDTs) based on the detection of the histidine rich protein 2 of the *P. falciparum* parasite. Positive cases were treated with the combination of artemether and lumefantrine (COARTEM®), the standard malaria treatment to treat *P. falciparum* infections adopted by the Guinea-Bissau Ministry of Health [16], or referred to a nearby health centre. Data were collected on Android devices using the Open Data Kit (ODK) data collection platform version GeoODK, with the added value

of capturing and visualizing Global Positioning System (GPS) coordinates of each household (HH). The 2017 MIS protocol was approved by the National Committee for Ethics in Health of Guinea-Bissau.

The malaria prevalence survey enrolled 4011 children aged 6–59 months and 4796 individuals aged 5–99 years old. One hundred and twenty-one individuals were infected exclusively with *P. falciparum* parasites and four exclusively with *Plasmodium malariae* by OM analysis of blood smears. Malaria prevalence was estimated at 0.7% in children younger than 5, 2.4% in children aged 5–14 years, 1.9% in individuals aged 15–24 years, 0.5% in individuals aged 25–44 years and 0.3% in individuals aged 45 years or older. In a logistic regression model adjusted for health region, area of residence, age, sex and use of long-lasting insecticide-treated nets, the factors independently associated with higher malaria prevalence were the health regions of Bafata, Bijagos, Gabu and Tombali, the age group 5–14 years and the male sex. A detailed description of the 2017 MIS results was reported in the HC-NFM3 Malaria Project Document (PRODOC), which is publicly available online on the United Nations Development website's Transparency Portal (https://open.undp.org/projects/00130819) as a downloadable Portable Document Format (PDF) file.

Setting

Guinea-Bissau is located on the west coast of sub-Saharan Africa, 11 8037° N and 15 1804° W, and is composed by a continental and an island group with more than 80 islands. It borders Senegal to the north, Guinea to the east and south and the Atlantic Ocean to the west. It is divided in 11 health regions, namely Bafata, Bijagos, Biombo, Bolama, Cacheu, Farim, Gabu, Oio, Quinara, the Autonomous Sector of Bissau (SAB, the capital of Guinea-Bissau), and Tombali. The country lies in the malaria belt and present a perennial transmission pattern with a peak in the pre- and post-rainy season (May to mid-November).

Selection of dried fingerprick whole blood samples

The number of dried fingerprick whole blood samples (DBSs) to be analysed was calculated using the formula for the finite population correction [17] [$n = N^*X/(X+N-1)$], where $X = (Z_{\alpha/2})^{2*}p^*(1-p)/MOE^2$, $Z_{\alpha/2}$ is the critical value of the Normal distribution at α/2, MOE is the margin of error, p is the sample proportion, and N is the population size of individuals negative for *Plasmodium* species by OM according to the 2017 malaria prevalence survey, stratified by the age groups defined in the 2017 malaria prevalence survey data analysis. Values for α of 0.05, a sample proportion of 50.0% and an MOE of 2.0% were considered.

Dried fingerprick whole blood samples (DBSs) from individuals negative for *Plasmodium* species by OM (OM-negative individuals) across the country were selected to search for submicroscopic *Plasmodium* infections. For that, MIS identification codes present in the 2017 MIS anonymized database were sorted using the random number generator function followed by the truncate random numbers function of the Statistical Package for the Social Sciences (IBM® SPSS® Statistics) version 29. Three hundred eighty-one (381) DBSs from OM-negative individuals across the country were selected, and 122 DBSs from OM-negative individuals sharing HH or village with 98 individuals positive for *P. falciparum* by OM (OM-positive individuals) identified in 2017 MIS were also included. Then, a total of 503 DBSs from OM-negative individuals were selected, 11.0% of the calculated number to be selected. *Plasmodium* infections by different *Plasmodium* spp., including *P. malariae*, *Plasmodium ovale wallikeri*, *P. ovale curtisi*, and *Plasmodium vivax* were also searched among the 503 DBSs from OM-negative individuals and among the 98 DBSs from the 98 positive individuals for *P. falciparum* by OM. Then, a total of 601 DBSs were analysed in this study.

Laboratory investigation

DNA extraction

The 601 DBSs on filter paper were subjected to DNA extraction using the previously described saponin-Chelex® (Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany) method [18, 19]. Three 6 mm-diameter circles from each DBS were used. DNA was eluted in sterile MilliQ® water, quantified by using a spectrophotometer (NanoDrop™ One–Thermo Fisher Scientific, Waltham, MA, USA) and stored at − 20 °C until utilization and at − 80 °C afterwards. All DNA samples presented A260/A280 ratio > 1.8 and a concentration of 30 ng/μL on average.

Polymerase chain reactions and primer pairs targeting 18S rRNA genes of Plasmodium parasites

Samples were screened for *Plasmodium* parasites by two subsequent polymerase chain reactions [nested PCR assays (nPCR)] targeting the gene encoding the 18S rRNA, according to previously described [20–22]. The primers used in each reaction are shown in Additional file 1: Table S1. They were synthesized by STABVIDA Lda. (Caparica, Portugal).

A strategy targeting the five variants of the *P. falciparum* 18S rRNA gene was adopted to test individually the 503 DBSs from OM-negative individuals for *Plasmodium* spp. by OM. The alignment of the five complete sequences of 18S rRNA variants found in the 3D7 *P. falciparum* reference strain, (PF3D7_0112300,

PF3D7_0531600, PF3D7_0725600, PF3D7_1148600, PF3D7_1371000), respectively located on chromosomes (chrs) 01, 05, 07, 11 and 13, and available at GenBank https://www.ncbi.nlm.nih.gov/genbank/, using Clustal Omega (1.2.4) Multiple Sequence Alignment software, available on EBML-EBI website (https://www.ebi.ac.uk/Tools/services), is shown in the Additional file 1: Fig. S1. The hybridization loci of the primer pairs used in each reaction are shown in the same Additional file 1: Fig. S1. The 18S rRNA variants on chrs 05 and 07 share 100.0% identity, and the 18S rRNA variants on chrs 01, 11 and 13 share more than 99.0% identity. The 18S rRNA variants on chrs 05 and 07 share around 85% identity with the later three 18S rRNA variants on chrs 01, 11 and 13 [23]. Their unique feature is the stage-specific expression of the rRNAs during the parasite life cycle [24].

To target the *P. falciparum* 18S rRNA variants on chr 05 and 07, the primer pair rPLU1/rPLU5 [37–1675 base pair (bp)], which targets a 1.6 kilobase (kb) conservative fragment common for *Plasmodium* genus, was used in the first reaction, followed by the primer pair FAL1ass/FAL2ass (666–854 bp), which targets a 188 bp fragment specific for *P. falciparum*, in the second reaction. To target the *P. falciparum* 18 S rRNA variants on chr 01, 11 and 13, the primer pair rPLU6/rPLU5 (609–1675 bp), which targets a 1.1 kb conservative fragment common for *Plasmodium* genus, was used in the first reaction, followed by the primer pair rFAL1/rFAL2 (665–870 bp), which targets a 205 bp fragment specific for *P. falciparum*, in the second reaction (Additional file 1: Table S1).

The presence of *Plasmodium* infections with different *Plasmodium* spp. was also investigated. For that, three primer pairs were used in parallel in the first *genus*-specific reaction, the primers rPLU1/rPLU5 and rPLU6/rPLU5 mentioned above, and the primer pair rPLU6/rPLU2, which targets a shorter sequence than the others. The previously described primer pairs for *P. malariae* and *P. vivax* [20], and *P. ovale wallikeri* and *P. ovale curtisi*, the same primer is used to detect both *P. ovale* spp. [25] (Additional file 1: Table S1), were used in the second *species*-specific reaction. For the sequences amplified with the primer pair rPLU6/rPLU2, the *species*-specific reaction to identify *P. ovale* spp. was carried out with primers rPLU6/Powc1. The 503 DBSs from OM-negative individuals and 98 DBSs from 98 OM-positive individuals exclusively for *P. falciparum* were analysed. Samples were tested in pools of five containing 30 ng/μL of extracted DNA from each DBS. The pools with positive results were analysed individually for the identification of the positive DBS.

PCR procedures and conditions

First and second PCR assays were made in a final volume of 20μL, containing 1×Colorless GoTaq® Flexi buffer (Promega Biotech Iberica SL, Madrid, Spain), 2 mM de $MgCl_2$ (Promega Biotech Iberica SL, Madrid, Spain), 250 μM of each dNTP (Promega Biotech Iberica SL, Madrid, Spain), 125 nM of forward and reverse oligonucleotide primers, 0.02U/μL of GoTaq® G2 Flexi DNA polymerase (Promega Biotech Iberica SL, Madrid, Spain) and 30 ng of extracted DNA in the first reaction and 1μL of the amplified product in the second reaction. The PCR assays were run in a Biometra T1 thermocycler (Alfagene®, Thermo Fisher Scientific, Waltham, MA, USA). DNA extracted from the 3D7 *P. falciparum* reference strain, which is maintained in culture in the laboratory according to standardized conditions previously described [26, 27] was used as positive control and the reaction mix without DNA was used as negative control in all PCRs.

The previously described PCR cycling parameters [20–22] were used with slight modifications regarding the number of cycles from 30 to 35 cycles in the first and second reactions, according to previous in-house optimizations comparing the sensitivity and specificity of different PCR techniques using *Plasmodium* spp. DNA as template. The product of the second reaction was visualized in 2% agarose gel stained with GreenSafe ® (NZYTech Corporation, Lisbon, Portugal) under ultra-violet (UV) light.

Geo-mapping of *P. falciparum* infections

The GPS coordinates of each HH were plotted on a Leaflet interactive map, generated using *Python* version 3.10.12, in *Google Colab*, with the package *Folium* version 0.14.0. The composed image presented was produced from prints of the interactive map and edited in *Photoshop CS5*. The location of individuals was displayed on the map distributed in three groups: (i) positive for optical microscopy (OM^+); (ii) negative for optical microscopy and positive for PCR ($OM^-/nPCR^+$); (iii) negative for both diagnostic methods ($OM^-/nPCR^-$). As the goal was geo-mapping these three types of individuals, we did not represent as an independent group the OM-positive individuals negative for nPCR ($OM^+/nPCR^-$) and then, they were included in the first group (OM^+). The HHs were coded on the map according to the different types of individuals living in them. The HHs inhabited by OM-positive individuals and individuals with submicroscopic *P. falciparum* infections detected only by nPCR were represented by black circles (OM^+ / $OM^-/nPCR^+$), the HHs inhabited by OM-positive individuals without submicroscopically-infected individuals were represented by red circles (OM^+), the HHs inhabited by individuals

with submicroscopic *P. falciparum* infections without microscopically-infected individuals were represented by green circles ($OM^-/nPCR^+$) and the HHs inhabited only by non-infected individuals were represented by orange circles ($OM^-/nPCR^-$).

Statistical analysis

Raw data from the 601 individuals included in this study were extracted from the 2017 MIS anonymized database. Statistical analysis of socio-demographic, clinical and molecular data was performed in the Statistical Package for the Social Sciences version 29 (IBM® SPSS® Statistics 29). Absolute and relative frequencies (number and percentage of cases) were calculated by each categorical variable. The distribution of values of the continuous variables age and parasitaemia levels estimated by OM was tested for normality by the Shapiro–Wilk test and the results were displayed in Additional file 1: Table S2. Then, the distribution of values of age and parasitaemia levels was described as median, interquartile range (IQR) and minimum (min) and maximum (max) values, the distribution of parasitaemia levels were displayed in boxplot graphics, and group comparisons for independent variables were performed using Mann–Whitney U-test or Kruskal–Wallis H-test, as appropriate. Agresti-Coull and Wald 95% confidence intervals were calculated to estimate the frequency of submicroscopic *P. falciparum* infections among OM-negative individuals. Associations between categorical variables were tested using Pearson Chi-square test (X^2). In case of failure of assumptions of the Pearson Chi-square test, *P* values were calculated by Fisher's exact test. To adjust for confounding, the magnitude of the associations was estimated by the odds ratios obtained by multiple logistic regression models considering the binary dependent variables: submicroscopic *P. falciparum* infections (yes/no) and microscopic *P. falciparum* infections (yes/no), using the entry method, and excluding those independent variables without significance to the model or responsible for outliers with a standardized residues > 3, until to reach the final model. The independent variables to be considered in the models were tested for absence of multicollinearity, e.g., Collinearity Coefficients Tolerance > 0.1 and Variance Inflation Factor (VIF) < 10 in a linear regression model, and the categories of the variable ethnicity with 10 or less cases (Mansonca, Nalu, Saracole, Wolof and those from unknown ethnicity, Additional file 1: Table S3) were grouped to be considered in the model with submicroscopic *P. falciparum* infections. The same procedure was not carried out for the model regarding microscopic *P. falciparum* infections to not biased the results, as most infections were related to two ethnicities Fula and Balanta. The models comprising the independent variables were compared to the model without predictors and with the model in the previous step using the Omnibus test. The power of the model, e.g., the percentage of cases that are explained by the model was verified by the Nagelkerke R-square ($R^2_{Nagelkerke} = |0–1|$). The Wald test was used to verify the statistically significant coefficients of each variable in the model and to show the odds that each statistically significant variable offered of having or not having submicroscopic or microscopic *P. falciparum* infections compared to the large and reference categories. Significance levels $P < 0.05$ were considered for all comparisons.

Results

Socio-demographic profile of participants

The distribution of the 601 individuals, whose DBSs were included in this analysis, according to health region, age group, and sex is shown in Table 1. The age groups defined in the data analysis of the 2017 malaria prevalence survey were maintained in this study. The distribution of individuals by age group and sex is similar in each of the 11 health regions (Chi-square test, $X^2 = 49.1$, $df = 40$, $P = 0.15$, and $X^2 = 9.5$, $df = 10$, $P = 0.49$, respectively). Age ranged from 6 months to 98 years old, most were children of 6 months to 4 years old (age group 0–4 years, 35.0%), 75.0% lived in the rural zones located on mainland or island regions, and most were female (60.0%). Females were older than males (females age median = 15.0 years old, interquartile range (IQR) = 3.5–27.0 years old, min = 6 months, max = 98 years old *versus* males age median = 7.0 years old, IQR = 2.0–23.0 years old, min = 6 months, max = 95 years old, Mann–Whitney U Test, $U = 48\,442.5$, $Z = 2.841$, $P = 0.004$). Of 361 females included in this analysis, 144 (40.0%) aged 15–44 years old and 40 (28.0%) were pregnant.

More than 60.0% of the 601 participants belonged to the ethnic groups Fula (n = 224) (who live mainly in the health regions Bafata and Gabu), Balanta (n = 98) (in the health regions Quinara and the Autonomous Sector of Bissau, SAB, the capital of Guinea-Bissau) and Bijagos (n = 68) (in the health regions Bijagos and Bolama). The Fulas are present in several countries in the Sahel and West Africa and are the largest ethnic group in Senegal and Guinea, while the Balanta are the largest ethnic group in Guinea-Bissau and are also present in Senegal, Guinea, The Gambia, and Cape Verde [28, 29]. The frequency of all ethnic groups in each of the 11 health regions is shown in Additional file 1: Table S3.

Nested PCR assays identified *Plasmodium* infections missed by OM

The 503 DBSs from individuals negative for *Plasmodium* spp. by OM were individually tested for the

Table 1 Distribution of the 601 DBS's donors analysed in this study by health region, age group and sex

Health region	Total N (%)	Age group (years) n (%)					Sex n (%)		Statistical analysis
		0–4	5–14	15–24	25–44	≥45	Male	Female	
Bafata[a]	89 (15.0)	28 (32.0)	24 (27.0)	14 (16.0)	13 (15.0)	10 (11.0)	37 (43.0)	49 (57.0)	$X^2=49.1$, $df=40$, $P=0.15$[b]
Bijagos	20 (3.0)	11 (55.0)	1 (5.0)	2 (10.0)	3 (15.0)	3 (15.0)	7 (35.0)	13 (65.0)	$X^2=9.5$, $df=10$, $P=0.49$[c]
Biombo[a]	37 (6.0)	9 (24.0)	7 (19.0)	9 (24.0)	5 (14.0)	7 (19.0)	10 (28.0)	26 (72.0)	
Bolama	22 (4.0)	9 (41.0)	7 (32.0)	1 (4.5)	4 (18.0)	1 (4.5)	10 (46.0)	12 (54.0)	
Cacheu	37 (6.0)	10 (27.0)	6 (16.0)	6 (16.0)	7 (19.0)	8 (22.0)	14 (38.0)	23 (62.0)	
Farim	15 (2.0)	4 (26.7)	5 (33.3)	3 (20.0)	3 (20.0)	0 (0.0)	7 (47.0)	8 (53.0)	
Gabu	142 (24.0)	55 (39.0)	26 (18.0)	31 (22.0)	19 (13.0)	11 (8.0)	57 (40.0)	85 (60.0)	
Oio	48 (8.0)	13 (27.0)	8 (16.0)	9 (19.0)	9 (19.0)	9 (19.0)	15 (31.0)	33 (69.0)	
Quinara	82 (14.0)	39 (48.0)	15 (18.0)	11 (13.0)	12 (15.0)	5 (6.0)	40 (49.0)	42 (51.0)	
SAB	82 (14.0)	26 (32.0)	20 (24.0)	20 (24.0)	9 (11.0)	7 (9.0)	27 (33.0)	55 (67.0)	
Tombali	27 (4.0)	9 (33.0)	2 (7.0)	5 (19.0)	7 (26.0)	4 (15.0)	12 (44.4)	15 (55.6)	
Total (N)	601 (100.0)	213 (35.0)	121 (20.0)	111 (19.0)	91 (15.0)	65 (11.0)	236 (40.0)	361 (60.0)	

[a] Missing sex information for three individuals in Bafata and one individual in Biombo. The total number of males and females is 597

[b,c] Chi-square test (X^2) was used to test for association between the distribution of individuals by age group and sex in each health region, respectively. *df* = degrees of freedom

presence of submicroscopic *P. falciparum* infections using the PCR strategies described in the Methods section. Of the 503 DBSs, 102 were positive for *P. falciparum* infections: four DBSs were positive for *P. falciparum* exclusively using the strategy targeting the 18S rRNA variants on chrs 05 and 07, 82 DBSs were positive for *P. falciparum* exclusively using the strategy targeting the 18S rRNA variants on chrs 1, 11, and 13, and 16 DBSs were positive for both strategies. The same strategies confirmed *P. falciparum* infections in 82 of the 98 DBSs from the 98 individuals positive for *P. falciparum* by OM.

Of the 601 DBSs screened for *Plasmodium* infections by different species using nPCR, three DBSs from the 98 individuals exclusively positive for *P. falciparum* by OM were also positive for *P. malariae*. No single or mixed infections with *P. ovale wallikeri*, *P. ovale curtisi*, or *P. vivax* were detected among the 601 DBSs analysed.

In summary, the nPCR assays detected 102 submicroscopic *P. falciparum* infections among the 503 DBSs from negative individuals for *Plasmodium* spp. by OM. This represents 20.3% submicroscopic *P. falciparum* infections among OM-negative individuals in Guinea-Bissau (Wald 95% CI = 16.8–23.8, $Z=0.100$, $P=0.92$ for 20.0% submicroscopic *P. falciparum* infections among OM-negative individuals in Guinea-Bissau). The nPCR assays also detected three co-infections with *P. malariae* (3.0%) that were missed by OM among the 98 individuals exclusively positive for *P. falciparum* by OM. These results were incorporated in the box-plot graphic with the distribution of the parasitaemia levels of the 98 OM-positive individuals in Additional file 1: Fig. S2.

Geo-mapping of *P. falciparum* infections

The 601 individuals whose corresponding DBSs were included in this analysis were distributed across 305 HHs, as shown on the map of Guinea-Bissau in Fig. 1. The average number of members per HH was nine (min–max: 1–39) and the most common number of members per HH was six.

The 20 HHs represented by the black circles had a total of 23 OM-positive individuals and 28 individuals with submicroscopic *P. falciparum* infections. All HHs, excluding one, were in the Fula territory, most near the north border with Senegal. The 68 HHs represented by the red circles had a total of 75 OM-positive individuals. They were spread over 10 health regions, excluding Farim, with 63.0% in the Bafata and Gabu health regions, e.g., in the Fula territory. The 64 HHs represented by the green circles had a total of 74 individuals with submicroscopic *P. falciparum* infections. They were spread over 10 health regions, excluding Bijagos, with 76.0% in the health regions of Biombo, Gabu, Oio, SAB and Tombali. The remaining 153 HHs represented by the orange circles had a total of 247 non-infected individuals.

Factors associated with submicroscopic *P. falciparum* infections

The frequencies (number and percentages of cases) of the 102 infected individuals with submicroscopic *P. falciparum* infections among the 503 OM-negative individuals by health region, age, sex, ethnicity, pregnancy and presence of fever are shown in Table 2. Associations between the socio-demographic and clinical data and the presence of submicroscopic *P. falciparum* infections were tested

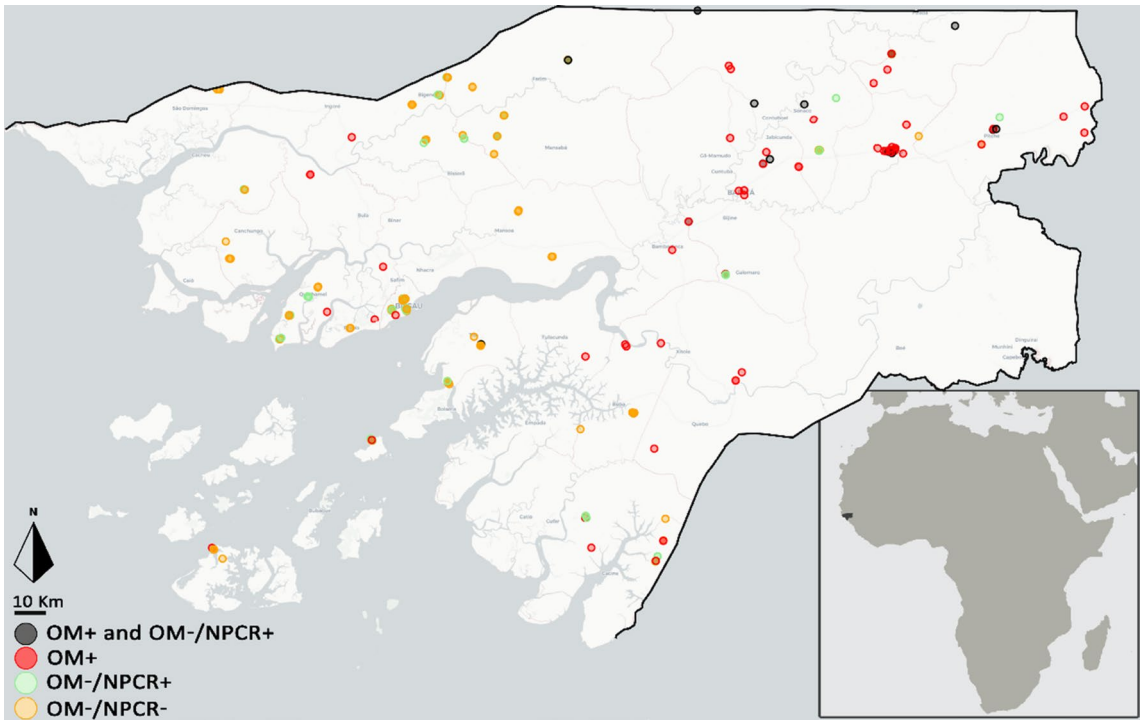

Fig. 1 Spatial distribution of the 305 HHs by GPS coordinates. Guinea-Bissau is located on the west coast of sub-Saharan Africa, as shown on the map of the African continent at the bottom right. Of the 305 HHs plotted on the map by the corresponding GPS coordinates, 20 (black circles) were inhabited by OM-positive individuals and individuals with submicroscopic *P. falciparum* infections, 68 (red circles) were inhabited by OM-positive individuals, 64 (green circles) were inhabited by individuals with submicroscopic *P. falciparum* infections, and 153 (orange circles) were inhabited by non-infected individuals. Circles are opaque to better visualize overlapping HHs. OM-positive individuals negative for nPCR were included into one of the groups with OM-positive individuals (black or red circles), accordingly. The Leaflet interactive map was generated using *Python* version 3.10.12, in *Google Colab*, with the package *Folium* version 0.14.0. The composed image presented was produced from prints of the interactive map and edited in *Photoshop CS5*

using Chi-square test and multiple logistic regression models. Similar data related to the 98 individuals with microscopic *P. falciparum* infections are shown in Additional file 1: Table S4.

Health region, ethnicity, age, sex, and pregnancy

The 102 individuals with submicroscopic *P. falciparum* infections were identified in 10 of the 11 health regions, namely Bafata (n = 17), Biombo (n = 11), Bolama (n = 3), Cacheu (n = 5), Farim (n = 2), Gabu (n = 30), Oio (n = 10), Quinara (n = 3), SAB (n = 13), and Tombali (n = 8). They belonged to 10 of the 15 ethnicities included in this study, namely Balanta (n = 3), Balanta Mané (n = 3), Beafada (n = 8), Bijagos (n = 6), Fula (n = 48), Mancanha (n = 1), Mandinga (n = 12), Mandjaco (n = 4), Mista (n = 7), and Pepel (n = 10). They aged 6 months to 74 years old (median = 20.0 years old, IQR = 3.75—34.0 years old) and 60.0% were women. Of the 40 pregnant women included in this study, 27 were negative for *Plasmodium* spp. by OM in the 2017 MIS, and six of them had submicroscopic *P. falciparum* infections detected in this study by nPCR. Thirteen pregnant women were infected with *P. falciparum* by OM in the 2017 MIS (Additional file 1: Table S4). These data showed 46.1% (6/13) increase in the number of pregnant women infected with *P. falciparum* parasites, and a proportion of pregnant women with submicroscopic *P. falciparum* infections among OM-negative pregnant women of 21.4% (Agresti-Coull, 95% CI = 9.9–36.5, $Z = -0.19$, $P = 0.83$ for 25.0% submicroscopic *P. falciparum* infections among pregnant women with OM-negative results in Guinea-Bissau). Statistically significant associations were observed between submicroscopic *P. falciparum* infections and the variables health region ($X^2 = 34.23$, $df = 10$, $P < 0.001$) and ethnicity ($X^2 = 45.60$, $df = 10$, $P < 0.001$) (Table 2). No associations were observed regarding age, sex, and pregnancy. On the opposite, the microscopic *P. falciparum* infections were associated with health region ($X^2 = 58.76$, $df = 10$, $P < 0.001$), age ($X^2 = 41.90$, $df = 4$, $P < 0.001$), sex ($X^2 = 5.31$, $df = 1$, $P = 0.021$), and pregnancy ($X^2 = 53.46$, $df = 14$, $P < 0.001$) (Additional file 1: Table S4).

Table 2 Associations of socio-demographic and clinical data with submicroscopic *P. falciparum* infections using Chi-square tests and multiple logistic regression models

Socio-demographic/clinical	Number of individuals n (%)	Submicroscopic. *P. falciparum* infections n (%)	[a]Statistical analysis	[b]Statistical analysis					
				Wald	*df*	*P*-value	OR	95% CI for OR	
Health region									
Gabu	142 (24.0)	30 (30.0)	$X^2 = 34.23$, $df = 10$, $P < 0.001$*	23.520	10	0.009*			
Bijagos	20 (3.0)	0 (0.0)		0.000	1	0.998	0.000	0.000	
Biombo	37 (6.0)	11 (33.0)		0.154	1	0.695	1.183	0.511	2.742
Bolama	22 (4.0)	3 (16.0)		1.489	1	0.222	0.444	0.120	1.636
Cacheu	37 (6.0)	5 (15.0)		2.850	1	0.091	0.408	0.144	1.155
Farim	15 (2.0)	2 (14.0)		1.372	1	0.241	0.394	0.083	1.871
Bafata	89 (15.0)	28 (32.0)		0.062	1	0.803	0.914	0.452	1.848
Oio	48 (8.0)	10 (21.0)		1.145	1	0.285	0.640	0.282	1.450
Quinara	82 (14.0)	3 (4.0)		13.598	1	0.000*	0.099	0.029	0.338
SAB	82 (14.0)	13 (16.0)		4.349	1	0.037*	0.459	0.221	0.954
Tombali	27 (4.0)	8 (38.0)		0.567	1	0.451	1.456	0.547	3.875
Age group									
0–4	213 (35.0)	28 (15.0)	$X^2 = 8.81$, $df = 4$, $P = 0.06$	8.623	4	0.071			
5–14	121 (20.0)	13 (13.0)		0.053	1	0.818	1.088	0.531	2.229
15–24	111 (19.0)	22 (24.0)		3.308	1	0.069	1.788	0.956	3.344
25–44	91 (15.0)	21 (24.0)		3.360	1	0.067	1.812	0.960	3.420
≥ 45	65 (11.0)	18 (30.0)		6.022	1	0.014	2.347	1.187	4.,640
Sex									
Female	361 (60.0)	61 (20.0)	$X^2 = 0.29$, $df = 1$, $P = 0.59$	0.427	1	0.513	1.160	0.743	1.811
Male	236 (40.0)	41 (22.0)							
Fever									
No	478 (80.0)	78 (19.0)	$X^2 = 2.11$, $df = 1$, $P = 0.15$	2.527	1	0.112	1.534	0.905	2.599
Yes	123 (20.0)	24 (26.0)							
Ethnicity									
Fula	224 (37.0)	48 (30.0)	$X^2 = 45.60$, $df = 10$, $P < 0.001$*	33.189	10	0.000*			
Balanta Mané	21 (4.0)	3 (15.0)		1.829	1	0.176	0.415	0.116	1.484
Beafada	20 (3.0)	8 (44.0)		1.574	1	0.210	1.883	0.700	5.064
Bijagos	68 (11.0)	6 (9.0)		9.099	1	0.003*	0.248	0.100	0.613
Balanta	98 (16.0)	3 (4.0)		16.023	1	0.000*	0.086	0.026	0.286
Mancanha	14 (2.0)	1 (7.0)		2.639	1	0.104	0.181	0.023	1.423
Mandinga	51 (9.0)	12 (25.0)		0.417	1	0.518	0.785	0.376	1.637
Mandjaco	31 (5.0)	4 (13.0)		3.242	1	0.072	0.362	0.120	1.094
Mista	21 (4.0)	7 (33.0)		0.109	1	0.741	1.177	0.447	3.099
Pepel	37 (6.0)	10 (31.0)		0.026	1	0.871	1.070	0.471	2.430
[c]Grouped	16 (3.0)	0 (0.0)		0.000	1	0.999	0.000	0.000	
Pregnancy									
Pregnant	40 (28.0)	6 (22.0)	$X^2 = 0.00$, $df = 1$, $P = 1.0$						
Non-pregnant	104 (72.0)	20 (21.0)							

[a] Chi-square tests between submicroscopic *P. falciparum* infections and the socio-demographic and clinical characteristics

[b] Logistic regression data for each independent variable separated

[c] Grouped ethnicity comprises the ethnicities Mansonca, Nalu, Saracole, Wolof, and unknown ethnicity

df = degrees of freedom

OR = odds ratio

*Statistically significant

Fever

One hundred twenty-three individuals of the 601 analysed in this study reported fever in the 14 days prior to blood sampling and 76 of them also reported fever on the day of blood collection. Among the 123 individuals who reported fever, 26.0% were infected with submicroscopic *P. falciparum* parasites and among the individuals without fever, 19.0% were infected with submicroscopic *P. falciparum* parasites. No association was observed between fever or submicroscopic *P. falciparum* infections (Table 2). Regarding the individuals with microscopic *P. falciparum* infections, 14.0% of whom reported fever were infected versus 26.0% of whom without fever. A statistically significant association between fever and microscopic *P. falciparum* infection showed that most of them was asymptomatic ($X^2=9.81$, $df=1$, $P=0.002$) (Additional file 1: Table S4).

Multiple logistic regression models

After assuring the absence of multicollinearity between the independent variables, binary logistic regression models were carried out to verify whether health region and ethnicity were predictors of submicroscopic *P. falciparum* infection (yes/no). The models carried out separately for each variable were statistically significant (health region, Omnibus tests, $X^2=40.97$, $df=10$, $P<0.001$, $R^2_{Nagelkerke}=0.123$; ethnicity, Omnibus tests, $X^2=52.62$, $df=10$, $P<0.001$, $R^2_{Nagelkerke}=0.156$). Hierarchic binary logistic regression models comprising both variables were built with the order of entry alternating between the two variables. The model with health region at the first step followed by ethnicity at the second step was statistically significant (Omnibus test, $X^2=64.36$, $df=20$, $P<0.001$, $R^2_{Nagelkerke}=0.189$) and the entry of the ethnicity still contributed to the model (Omnibus test, $X^2=23.40$, $df=10$, $P=0.009$), however both variables were not significant in the model. On the opposite, the model with ethnicity at the first step followed by health region at the second step was statistically significant (Omnibus test, $X^2=64.36$, $df=20$, $P<0.001$, $R^2_{Nagelkerke}=0.189$), but the entry of the health region did not contribute to the model (Omnibus test, $X^2=11.74$, $df=10$, $P=0.30$) and both variables were not significant in the model. Then, the final model comprises only the ethnicity as a predictor of submicroscopic *P. falciparum* infections (Table 2). Individuals from the ethnicities Balanta (4.0%) and Bijagos (9.0%) were less infected with submicroscopic *P. falciparum* infections than individuals from the other ethnicities (Table 2) and were less likely to have submicroscopic *P. falciparum* infections than individuals in the large ethnic group and reference category Fula (Balanta, OR = 0.086, 95% CI = 0.03–0.29; Bijagos, OR = 0.248, 95% CI = 0.10–0.61). Balanta and Bijagos people live in the health regions Quinara, SAB, Bijagos and Bolama, where individuals were less likely to have submicroscopic *P. falciparum* infections than individuals living in the more numerous health region and reference category Gabu, most occupied by Fula ethnicity (Quinara, OR = 0.099, 95% CI = 0.03–0.34; SAB, OR = 0.459, 95% CI = 0.22–0.95).

A hierarchic binary logistic regression model was built comprising all variables associated with microscopic *P. falciparum* infections according to the Chi-square tests showed in Additional file 1: Table S4, to search for predictors of microscopic *P. falciparum* infections among the 601 individuals included in this study. The entry of ethnicity was not significant to the model and was removed. The final model comprising age, health region, fever and sex, in this order, was statistically significant (Omnibus test, $X^2=116.52$, $df=16$, $P<0.001$, $R^2_{Nagelkerke}=0.301$) and the four variables were statistically significant in the model (Additional file 1: Table S4). The results showed that individuals aged 5–14 years old were more likely to have microscopic *P. falciparum* infections than children under 5 years old (OR = 4.352, 95% CI = 2.33–8.11); individuals living in the health regions Biombo, Cacheu, Farim, Oio, Quinara and SAB were less likely to present microscopic *P. falciparum* infections than individuals living in Gabu and individuals with fever were more likely to have microscopic *P. falciparum* infections than individuals without fever (OR = 2.140, 95% CI = 1.21–3.79). The results showing that males were more likely to have microscopic *P. falciparum* infections than females were not considered, as the inferior limit of the 95% CI includes the value 1.00 (OR = 1.685, 95% CI = 1.00–2.82).

Haemoglobin levels in submicroscopic and microscopic *P. falciparum* infections

Of the 213 children aged 6–59 months included in this analysis, 178 participated in the anaemia prevalence survey, and 83 had anaemia. Of the 83 anaemic children, 68 had mild anaemia (11.0 g/dL > Hb ≥ 9.0 g/dL), 14 had moderate anaemia (9.0 g/dL > Hb ≥ 7.0 g/dL), and one child had severe anaemia (Hb < 7.0 g/dL). Data are shown in Table 3. Of the 68 children who had mild anaemia, five were positive for *P. falciparum* by OM (OM^+), 10 were infected with submicroscopic *P. falciparum* infections ($OM^-/nPCR^+$) and 53 were not infected with *Plasmodium* parasites ($OM^-/nPCR^-$), whereas of the 14 children with moderate anaemia, three were positive for *P. falciparum* by OM, one had submicroscopic *P. falciparum* infection, and 10 were not infected with *Plasmodium* parasites. The single child with severe anaemia (Hb = 4.8 g/dL) was positive for *P. falciparum* by OM (OM^+).

Table 3 Frequency of anaemic children aged 6–59 months infected with microscopic and submicroscopic *P. falciparum* infections

Variable	Number of individuals n (%)	Microscopic n (%)	Submicroscopic n (%)	Statistical analysis	
Anaemia					
Mild	68 (38.0)	5 (7.0)	10 (16.0)	$X^2 = 7.54$, $df = 3$, $P = 0.048$*# [a]	$X^2 = 0.32$, $df = 2$, $P = 0.89$# [b]
Moderate	14 (8.0)	3 (21.0)	1 (9.0)		
Severe	1 (1.0%)	1 (100.0)	0 (0.0)		
No	95 (53.0)	10 (11.0)	15 (18.0)		
Anaemia					
Yes	83 (47.0)	9 (11.0)	11 (15.0)	$X^2 = 0.00$, $df = 1$, $P = 1.0$[a]	$X^2 = 0.07$, $df = 1$, $P = 0.80$[b]
No	95 (53.0)	10 (11.0)	15 (18.0)		

[a,b] Chi-square tests related to comparisons between anaemia and microscopic *P. falciparum* infections or submicroscopic *P. falciparum* infections, respectively.
df = degrees of freedom

P-values according to Fisher's exact test

* Statistically significant

Anaemia was not associated with submicroscopic or microscopic *P. falciparum* infections, the degree of anaemia was not associated with submicroscopic *P. falciparum* infections, but it was associated with microscopic *P. falciparum* infections (Table 3).

Discussion

The aim of this study was to assess the frequency, distribution, and morbidity of submicroscopic *Plasmodium* infections, as well the factors associated with them in a pre-elimination malaria setting, Guinea-Bissau. Guinea-Bissau is an endemic malaria country located on the west coast of sub-Saharan Africa, with seasonal and regional variations in malaria transmission, where the *P. falciparum* is the predominant *Plasmodium* species.

Submicroscopic *Plasmodium* infections, probably low-density infections with less than 100 parasites/μL of whole blood [30], can sustain malaria transmission in low and high malaria-transmission areas. The results obtained in this study showed a frequency of 20.0% submicroscopic *P. falciparum* infections in the general population and among pregnant women with a microscopically-negative diagnosis in Guinea-Bissau. They also showed that submicroscopic *P. falciparum* infections were widely distributed throughout the country, occurring in 10 of the 11 health regions, and in almost all ethnic groups, age groups, males, females and pregnant women. The factors associated with submicroscopic *P. falciparum* infections among the 503 microscopically-negative individuals were ethnicity and health region according to Chi-square tests. A logistic regression model comprising both variables showed that ethnicity was a predictor of submicroscopic *P. falciparum* infections, and individuals from the Balanta and Bijagos ethnic groups, most of whom live in the low malaria-transmission health regions of Quinara and SAB, and the Bijagos archipelago, respectively, were less likely to have submicroscopic *P. falciparum* infection than individuals from the larger Fula ethnic group, most of whom live in the high malaria-transmission health region of Gabu. This suggests that submicroscopic *P. falciparum* infections are mostly asymptomatic and may contribute to maintaining malaria transmission, especially in high malaria-transmission areas, acting as silent parasite reservoirs and a barrier to achieving malaria control and elimination in these areas. Further investigation using more sensitive molecular strategies that allow parasite quantification and stratification of different levels of parasitaemia may help to identify predictors of submicroscopic *P. falciparum* infections.

On the opposite, health region, ethnicity, age, sex, pregnancy and presence of fever were associated with microscopic *P. falciparum* infections using Chi-square tests. Despite the presence of fever being associated with microscopic *P. falciparum* infections, some infected individuals with microscopic *P. falciparum* infections did not report fever, even younger individuals. In addition to the development of an anti-parasitic acquired immune response, this suggests the development of immune tolerance in exposed individuals, allowing the occurrence of asymptomatic infections even at parasitaemia levels detected by OM [31].

A logistic regression model comprising age, health region, fever and sex showed that the first three factors were predictors of microscopic *P. falciparum* infections. Individuals aged 5–14 years old, and fever were more likely associated with microscopic *P. falciparum* infections than children under 5 years old, and the absence of fever. Possible reasons for the emergence of malaria in children aged 5–14 years [32–36] include successful intervention programmes targeted at very young

children, and various social, cultural, and economic behavioural factors that increase exposure of children older than 5 to infected mosquito bites and reduce the uptake and effectiveness of interventions in this target group [37]. For example, children under 5 are commonly prioritized for long-lasting insecticide nets use, whereas children older than 5 are not [38]. Higher levels of parasitaemia were also observed in pregnant women compared with non-pregnant women of the same age range, probably explained by pregnancy-associated immunosuppression [39], highlighting the vulnerability of this group to malaria [40]. Finally, individuals living in the health regions of Biombo, Cacheu, Farim, Oio, Quinara and SAB were less likely to have microscopic *P. falciparum* infections than individuals living in Gabu.

The results obtained also showed a frequency of 3.0% of mixed infections with *P. falciparum* and *P. malariae* in individuals infected only with *P. falciparum* by OM in the 2017 national malaria prevalence survey. The identification of mixed infections by OM is not easy, even for experienced microscopists, especially when one species is numerically predominant and the others are present at low levels [22]. In addition to the experience of the microscopist, the quality of the blood smear preparation [41] and the low-incidence of non-*falciparum* infections in certain areas contribute to the difficulty of detecting morphological differences between different *Plasmodium* spp. at the ring stage [42], which may hamper the detection of mixed *Plasmodium* infections. *Plasmodium malariae* is a neglected *Plasmodium* parasite that has been largely observed in sub-Saharan Africa as co-infection with *P. falciparum* as the dominant species [43]. The results obtained showed the necessity to consider *P. malariae* infections in the strategies focused on malaria control and elimination in Guinea-Bissau.

Absence of amplification of *P. falciparum* genetic fragments was observed in 16 DBSs samples from microscopically positive individuals for *P. falciparum*. In addition to reflecting possible damage to the samples due to prolonged storage, which could have reduced the quality and quantity of extracted parasite DNA, this may indicate, at a lower probability, possible single nucleotide polymorphisms in the *P. falciparum* 18S rRNA genes that deserve further investigation, especially considering the scarcity of *P. falciparum* sequences from Guinea-Bissau deposited in genetic databases.

Regarding the morbidity of malaria infections, the results obtained showed that submicroscopic *P. falciparum* infections were not associated with anaemia or degrees of anaemia, but microscopic *P. falciparum* infections were associated with severe anaemia.

Conclusions

The results obtained indicate the urgent need for molecular-based strategies, including PCR-based point-of-care devices, to screen for submicroscopic *P. falciparum* infections and mixed *Plasmodium* infections; that submicroscopic *P. falciparum* infections should not be ignored in surveillance strategies, especially in high malaria-transmission areas, and that malaria surveillance strategies can be designed to target the predictors of microscopic *P. falciparum* infections to achieve malaria control and elimination.

Abbreviations

CHR	Chromosome
DBS	Dried fingerprick whole blood sample
DF	Degrees of freedom
DNA	Desoxyribonucleic acid
EBML-EBI	European Molecular Biology Laboratory-European Bioinformatics Institute
GPS	Global positioning system
Hb	Haemoglobin
HH	Household
IQR	Interquartile range
MAX	Maximum value
MIN	Minimum value
MIS	Malaria indicator survey
MOE	Margin of error
nPCR	Nested polymerase chain reaction
OM	Optical microscopy
PDF	Portable document format
RBM	Roll Back Malaria
RDT	Rapid diagnostic test
rRNA	Ribosomal Ribonucleic acid
SAB	Autonomous sector of Bissau, the capital of Guinea-Bissau
SPSS	Statistical package for the social sciences
UV	Ultra-violet
VIF	Variance inflation factor
WHO	World Health Organization

Supplementary Information

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Additional file 1

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Author contributions

R.S., A.P.A. and M.M.M. designed the experiments. R.S., L.F.L. and M.M.M. generated data. R.S. and M.M.M. analysed data. A.P.A. and M.M.M. supervised data analysis. R.S. and M.M.M. wrote the main manuscript text. M.M.M. prepared all tables and supplementary material. L.F.L. prepared Figure 1. All authors reviewed the manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study is part of the SUBFAM protocol (*Submicroscopic infections of Plasmodium falciparum: an obstacle for malaria elimination*) led by MMM and approved by the National Committee for Ethics in Health of Guinea-Bissau under the code 035/CNES/INASA/2019.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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