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Integrating local malaria molecular monitoring into regular malaria indicator surveys on Bioko Island: high association between urban communities and low-density infections

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Abstract

Background Effective malaria control requires accurate identification of *Plasmodium* infections to tailor interventions appropriately. Rapid diagnostic tests (RDTs) are crucial tools for this purpose due to their small size and ease-of-use functionality. These tests typically target the *Plasmodium falciparum* histidine-rich protein 2 (HRP2) antigen. However, some strains of *P. falciparum* have deletions in the *hrp2* and *hrp3* genes, which may result in a false negative diagnosis using HRP2-based RDTs. Additionally, RDTs have a detection limit of 100 parasites per microlitre, insufficient for identifying low-density infections that sustain malaria transmission. This study explores integrating molecular monitoring using a novel cartridge-based PCR test, PlasmoPod, using samples from a malaria indicator survey (MIS) on Bioko Island, Equatorial Guinea to enhance detection of low-density infections and inform targeted malaria control strategies.

Methods The study utilized a combination of RDTs and the DiaxxoPCR device for molecular monitoring. The device DiaxxoPCR uses a prefilled cartridge system, termed PlasmoPod for a malaria-based assay that employs a qPCR assay targeting 18S rDNA/rRNA. Samples from the 2023 MIS were extracted from dried blood spots (DBS), qPCR run in duplicate on the PlasmoPod. Epidemiological data from the MIS were merged with molecular data and the association between MIS variables to malaria infection by qPCR, and low-density infections were measured.

Results The integration of molecular monitoring revealed a proportion of low-density infections that circumvented RDTs diagnosis. Notably, individuals in urban communities and those reporting recent fever were more

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likely to harbour low-density, asymptomatic malaria infections. Findings suggest that urban residents, although less associated to malaria infection than rural residents by both RDT and qPCR, may be serving as a transmission reservoir. The relationship between low-density infections and individuals who recently reported fever may reflect recent anti-malarial treatment or natural clearance, and thus have lingering parasites in their blood.

Conclusion The study highlights the limitations of HRP2-based RDTs in detecting low density infections and underscores the potential of molecular tools like PlasmoPod in malaria surveillance. By identifying elusive transmission reservoirs and tracking parasite importation, molecular monitoring can play a crucial role in achieving malaria elimination. The findings advocate for the broader implementation of molecular diagnostics in malaria programs, especially in areas with low transmission, to enhance the detection and targeting of hidden reservoirs of infection.

Keywords Malaria, *Plasmodium falciparum*, PlasmoPod, Low density infections, Bioko Island

Background

Essential to malaria control strategy is efficient, accurate identification of *Plasmodium* infection among human populations at risk to appropriately adjust targeted interventions. In 2022, an estimated 345 million malaria rapid diagnostic tests (RDTs) were directly distributed to national malaria programmes (NMPs), mostly in sub-Saharan Africa [1] with the purpose of providing quick diagnostic capability during clinical visits and field studies. These RDTs have become an essential tool for NMPs, and increasing their accessibility is associated with a decrease in child mortality [2]. The majority of RDTs target the *Plasmodium falciparum* histidine-rich protein 2 (HRP2) antigen, which can cross-react with the closely related *P. falciparum* HRP3 antigen in high-density infections [3]. Other malaria proteins targeted by the RDT are lactate dehydrogenase (pLDH) or aldolase to detect non-*falciparum* parasites [4, 5] and are more commonly used in combo RDTs with HRP2. *Plasmodium falciparum* accounts for the majority of malaria cases and deaths [1], as such HRP2-targeting RDTs are the most common diagnostic used globally and are much more sensitive than non-HRP2 RDTs [6]. However, evidence has been accumulating that some *P. falciparum* strains evade RDT diagnosis when the HRP2 target antigen becomes nonfunctional in the parasite genome as shown in strains that carry *hrp2* and *hrp3* deletions [4, 5]. These strains may become especially problematic in low-transmission areas, where selective pressures promote their expansion and compromise diagnosis/monitoring by RDT [7]. Additionally, RDTs are less sensitive for low density infections (< 100 parasites/µl) and, therefore, are more likely to misdiagnose these infections, allowing for continued transmission and hampering elimination efforts [8, 9]. Conversely, some proportions of RDT's results can also be false positive, usually due to the persistence of the HRP2 antigen post-treatment with anti-malarial drugs [10], and individuals may continue to have false RDT positive results for up to 15 or more days post treatment [11]. Especially for field studies in regions with year-round transmission, if false negative or positive results are significant, malaria prevalence measurements may be compromised, and bias downstream analysis, potentially affecting control strategy decisions. For NMPs approaching malaria elimination, it is essential to have sensitive, robust, fast and easy to deploy diagnostic methods with a low limit of detection and are not vulnerable to *hrp2* and *hrp3* deletions providing a granular and accurate measure of malaria prevalence.

Molecular malaria surveillance with higher sensitivity can identify transmission reservoirs that go undetected by RDTs [12]. Positive samples by molecular techniques can be quantified, unlike RDTs, using the *P. falciparum* WHO International Standard for nucleic acid amplification technique (NAAT) based assays [13], and assays have also been developed to distinguish between multiple *Plasmodium* species, as some can occur as low density co-infections [14–16]. Molecular monitoring further acts as an early-warning tool for detection of increasing prevalence of *P. falciparum* strains able to evade diagnostic tools [17, 18].

Quantitative reverse transcription polymerase chain reaction (RT-qPCR, but referred to as qPCR throughout this manuscript) has a lower limit of detection than RDT and can quantify infections [10, 19, 20] and thus makes it an ideal method for molecular surveillance. Implementation of the qPCR method typically requires specialized equipment, cold-chain sensitive and expensive reagents, advanced laboratory infrastructure and well-trained staff. To address these logistical limitations, a portable, cartridge-based, user-friendly qPCR instrument, Diaxxo-oPCR (Diaxxo AG, Zurich, Switzerland) using PlasmoPod cartridges, without the need for cold-chain or purchase of reagents, and shown to be as sensitive as currently used laboratory-based qPCR, was developed [21]. Cartridges are provided with all assay reagents in dried form within each well so with the addition of extracted nucleic material and immersion oil, qPCR results can be

generated within 30 min. Advantageous to the DiaxxoPCR technology platform is its versatility to detect various targets with an accessible assay design, including SARS-CoV2 variants of concerns [22]. Due to the novelty of PlasmoPod, its utility as a molecular monitoring method for malaria on a larger scale and incorporated into local malaria surveillance programmes has yet to be demonstrated.

Bioko Island, Equatorial Guinea, is a malaria endemic island off the coast of Cameroon in Central-West Africa with a population of approximately 270,000 inhabitants [23]. Malaria morbidity and mortality has been significantly reduced since intensive control interventions were implemented starting from 2004 by the Bioko Island Malaria Elimination Project (BIMEP) [23–25]. The majority of Bioko's residents live in Malabo city, the capital of Equatorial Guinea, in the northern part of the island. Despite BIMEP's progress, the island is vulnerable to malaria importation from its mainland neighbours [25, 26], and prevalence appears to have plateaued similar to other endemic regions across Africa since 2016 [1].

Here is described the outcome of a study of 1,500 dried blood spots (DBS) obtained from the BIMEP 2023 malaria indicator survey (MIS) on Bioko Island. These samples were analysed with the PlasmoPod assay at the local reference laboratory in Baney district. The qPCR results were compared to RDT results from the same sample set to provide insight into the added value of molecular malaria monitoring in the field.

Methods

Sample collection and selection

During the 2023 MIS conducted between August and October, DBS samples were collected from surveyed volunteers. At the start of each MIS visit to a household, participating adult volunteers provided informed consent and legal guardians gave consent for underage participants (< 18 years). Each volunteer then answered a questionnaire similar to previous MIS [27] and provided a blood sample from finger prick for RDT diagnosis (CareStart Malaria HRP2/pLDH Combo RDT) and a DBS on Whatman filter papers (GE healthcare Ltd, Forest farm, Cardiff UK). On each filter paper, four blood spots of 0.5 inches (1.27 cm) in diameter were produced, with each spot representing around 50 µl of blood. Selected survey data was used to investigate variable subgroups (Supplementary Table S1). Household sampling was based on population density of primary sampling units (PSUs) constructed from 1×1 km map-areas that make up Bioko's mapping grid [28].

Among all collected MIS samples (4998 households; 19,669 study participants), there were 12,583 total valid RDT results of which 1623 were RDT (+). A stratified random sampling approach was taken to select 1,400 DBS samples among RDT positive (+) individuals and 100 total DBS samples among RDT negative (−) individuals who shared a household with an RDT (+) member from each major district. The total time needed from extraction to results for 96 samples, for example, took 3 days approximately: 12–14 h for nucleic acid extraction from DBS, and 4.5 h for all qPCR runs (30 min for a single qPCR run) with PlasmoPod assuming 10 samples per cartridge and a single DiaxxoPCR device. Molecular results for all 1,500 samples were generated within 2–3 weeks after receiving DBS filter papers at the laboratory.

Nucleic acid extraction

A whole blood nucleic acid (DNA/RNA) extraction method developed by the Malaria Research Program at the University of Maryland, Baltimore, USA, was applied to collected filter papers [29]. Briefly, one full blood spot of the DBS was cut (~ 50 µl blood) and then incubated in lysis buffer (Guanidine thiocyanate, Triton X100, 0.5 M EDTA, 1 M trizma hydrochloride, isopropanol, 6 M HCl, and 2-mercaptoethanol) with gentle shaking for 2 h at 65–75 °C. Samples underwent two washes (Wash 1: same as lysis buffer without 2-mercaptoethoanol; Wash 2: ethanol and isopropanol (1:1 ratio), 1 M trizma hydrochloride, and 5 M NaCl) before eluting with 50 µl TE buffer by centrifugation. Extracted DNA was stored at − 80 °C until use. Suppliers of lysis and wash reagents have been published previously in supplementary materials [29].

Quantitative reverse transcription polymerase chain reaction

PlasmoPod 20-well cartridges for *Plasmodium* spp. targeting the 18S rDNA/rRNA by RT-qPCR supplied by Diaxxo AG (Zurich, Switzerland) [21] were used. Pspp primers used in the cartridges have been previously published, amplifying highly conserved rDNA and rRNA in chromosomes 1, 5, 7, 11, and 13 found only in the *Plasmodium* genus [30]. Briefly, 4.5 µl of extracted nucleic acid was added to each well, each sample run in duplicate, and covered in immersing oil, then run on the DiaxxoPCR device, outputting results in approximately 30 min. Results were generated automatically by the PlasmoPod reader's software, assigning positive and negative signals based on Cq curves, where any Cq < 40 was considered positive [21]. Serial dilutions of WHO standard were performed with PlasmoPod cartridges to quantify parasite densities [15]. Parasite density of infections was quantified by obtaining Cq values using PlasmoPod for known concentrations of serial WHO standard dilutions for DNA from NAAT-based assays from 5 to 500,000 parasites/µl (p/µl) [13]. From the

resulting standard curve ($R^2 = 0.86$), parasite densities were quantified using slope (m = -3.34) and y-axis intercept (y-intercept = 36.43) (Supplementary Fig. S1). Infections were considered low density if parasite density was under 100 p/µl and assumed to be below the limit of detection for RDTs.

To compare the Plasmopod results with a commercially available qPCR assay (RealStar Malaria PCR kit 1.0, Altona Diagnostics GmbH, Germany) targeting *Plasmodium* spp. DNA, a subset of samples evaluated with PlasmoPod (n = 60) was selected. Briefly, the conventional qPCR reagents were shipped on dry ice to the Baney Reference laboratory and required 10 µl of extracted sample. Briefly, the protocol is as follows: 1 cycle denaturation at 95 °C for 10 min, followed by 45 amplification cycles of 15 s at 95 °C, 45 s at 58 °C, and then 15 s at 72 °C. The sample selection was based on the parasite density estimate as determined by the WHO malaria standard established using Plasmopod. A stratified randomization strategy was applied, 20 samples were randomly selected with high density (> 5,000 p/µl), low density (< 100 p/µl), and negative outcome for PlasmoPod qPCR, each.

Data analysis

The generated qPCR data were linked to the MIS dataset using each volunteer's unique ID code. Socioeconomic status (SES) for each individual was generated based on household assets and utilities, scoring each household using principal components analysis (PCA). Assets include ownership of radio, television, VCR/DVD, computer, telephone, clock, watch, sofa, table, armoire, cabinet, fans, air conditioning, refrigerators, stove, washing machine, and car, as well as utilities such as public/private water source, protected/open water source, electricity source, toilet type and sanitation mechanism. All Cq results were generated in duplicate, and in the instance of high variance between duplicates (> 2 standard deviations separation from the samples' Cq mean), samples were excluded from the analysis. Statistical analysis and data visualization was conducted in R (v4.3.2) using the following packages: readxl, ggplot2, dplyr, reshape, pscl and car. Mann–Whitney p-values were used to compare the median parasite density values between groups. Binomial logistic regression univariate and multi-variate models were explored to produce odds ratios (OR) and 95% confidence intervals (CI). Multi-variate models were built from the univariate model, where each variable was included if the overall fitness of the model remained or improved. Model fitness was measured using McFadden's pseudo-R^2 values [31]. The variance inflation factor (VIF) was calculated between each variable to determine multi-co-linearity, where a variable was excluded from the regression model if it was highly correlated to another variable [32]. Wald test p-values were used to measure statistical significance between a variable's reference and comparison group within each regression model.

Results

Data descriptives

A total of 1,479 samples were included, of which recorded RDT diagnosis include 994 (67.2%) HRP2-only positive, 31 (2.1%) pLDH-only positive, 356 (24.1%) HRP2/pLDH positive, and 98 (6.6%) HRP2/pLDH negative, respectively (Table 1). PlasmoPod results were generated for all samples, of which 21 (1.4%) were excluded for high variance between Cq results. Selected samples included all Bioko districts (Malabo = 1034 (69.9%); Baney = 184 (12.4%); Luba = 103 (6.9%); Riaba = 158 (10.7%)), where 51.2% (n = 757) were collected in rural communities. There was a slightly higher proportion of selected samples from male volunteers (male = 51.5%; female = 48.5%), and the median age of all participants was 16 years (Range: 7 months–80 years). Initial SES results were grouped into quintiles of low (n = 320), low-middle (n = 334), middle (n = 313), middle-high (n = 294), and high (n = 218). The SES groups low and low-middle and middle-high and high were concatenated for subsequent analyses arriving at three SES groups namely low, middle, and high to reduce variability. A number of respondents in the selected sample set reported having fever in the previous 14 days (n = 223; 15.1%), of which 97.3% (217/223) had a positive RDT result. Travel history was reported within the previous 8 weeks, of which 90 volunteers (6.1%) reported travel activity, with the majority traveling to the continental region of Equatorial Guinea (83/90; 92.2%). Among malaria prevention behaviours, 31.0% (n = 459) of selected samples came from individuals who reported sleeping under a bed net the night before, and 64.8% receiving indoor residual spraying (IRS) within the previous 12 months (n = 959). Median parasite density across all selected samples was approximately 3862 p/µl (Cq = 24.4) with a range of 0.45–959,480 p/µl (Cq 16.2–37.8).

Proportion of positivity rate per subgroup

Stratifying by RDT results, 26.5% of RDT (-) were positive by qPCR (i.e. suspected RDT false negatives), and 74.7% of RDT (+) results were positive for qPCR, suggesting 25.3% were suspected false RDT positives. RDT (−) had lower parasite density (median parasite density = 121 p/µl) than all RDT (+) groups (HRP2 median parasite density = 1176 p/µl; pLDH RDT median parasite density = 7970 p/µl; and HRP2/pLDH RDT median parasite density = 28,516 p/µl) (Table 1). To corroborate qPCR results by PlasmoPod, a subset

Table 1 Distribution and positivity rate of variable subgroups by 18S RT-qPCR (qPCR). Median parasite density (parasite per microlitre) is reported.

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Variable	Subgroup	N (%)	qPCR (+) %	Median p/µl
RDT	Negative	98 (6.6)	26.5	121
	HRP2	994 (67.2)	71.5	1,176
	pLDH	31 (2.1)	71.0	7,970
	HRP2/pLDH	356 (24.1)	83.4	28,516
Variable	**Subgroup**	**N RDT(+) / N RDT(-)**	**qPCR(+) % by RDT(+)**	**qPCR(+) % by RDT(-)**
Age group	<5	116 / 12	71.6	16.7
	5-14	500 / 31	71.8	25.8
	15-45	673 / 42	75.8	31.0
	45+	92 / 13	85.9	23.1
District	Malabo	1009 / 25	73.3	20.0
	Baney	159 / 25	77.4	28.0
	Luba	79 / 24	86.1	20.8
	Riaba	134 / 24	74.6	37.5
Sex	Female	662 / 56	71.3	25.0
	Male	719 / 42	77.7	28.6
SES	Low	615 / 39	78.4	28.2
	Middle	294 / 19	73.5	31.6
	High	472 / 40	70.6	22.5
Community	Rural	699 / 58	78.7	29.3
	Urban	682 / 40	70.5	22.5
Sick (14 days)	No	1126 / 88	75	27.3
	Yes	249 / 9	72.7	22.2
Fever (14 days)	No	1156 / 90	74.7	26.7
	Yes	217 / 6	73.7	33.3
Slept under net	No	867 / 76	76.9	23.7
	Yes	439 / 20	69.0	35.0
Travel (8 weeks)	No	1237 / 92	74.9	27.2
	Yes	85 / 5	74.3	20.0
IRS	No	488 / 32	75.0	25.0
	Yes	893 / 66	74.5	27.3
HH density	low (<1 person/room)	736 / 42	75.0	23.2
	high (>1 person/room	645 / 56	74.3	31.0

of 60 samples was run with a validated qPCR assay (RealStar Malaria PCR kit) showing 100% agreement among high density infections (> 5000 p/µl), and 42.9% and 57.9% for low density and negative samples, respectively (Supplementary Table S2). RDT(+) seniors (45 + years) had the highest proportion of qPCR(+) results among all age groups (85.9%), while RDT(−) adults (age range 15–45 years) had the highest proportion of qPCR(+) tests (Table 1). In both, RDT(+) and RDT(−) groups, males had a higher proportion of qPCR(+)s than females.

Parasite density stratified by RDT negative versus RDT positive

Parasite quantification of positive qPCR results stratified by RDT results (negative *versus* positive) illustrate suspected false negative RDTs typically were lower mean parasite density infections compared to RDT positives (121 p/µl *versus* 4137 p/µl; p = 0.05) (Fig. 1). When stratifying by sample set variables, median parasite density of RDT(−)s was lower than RDT(+)s in most subgroups, although some subgroups were of small sample size (Supplementary Table S3).

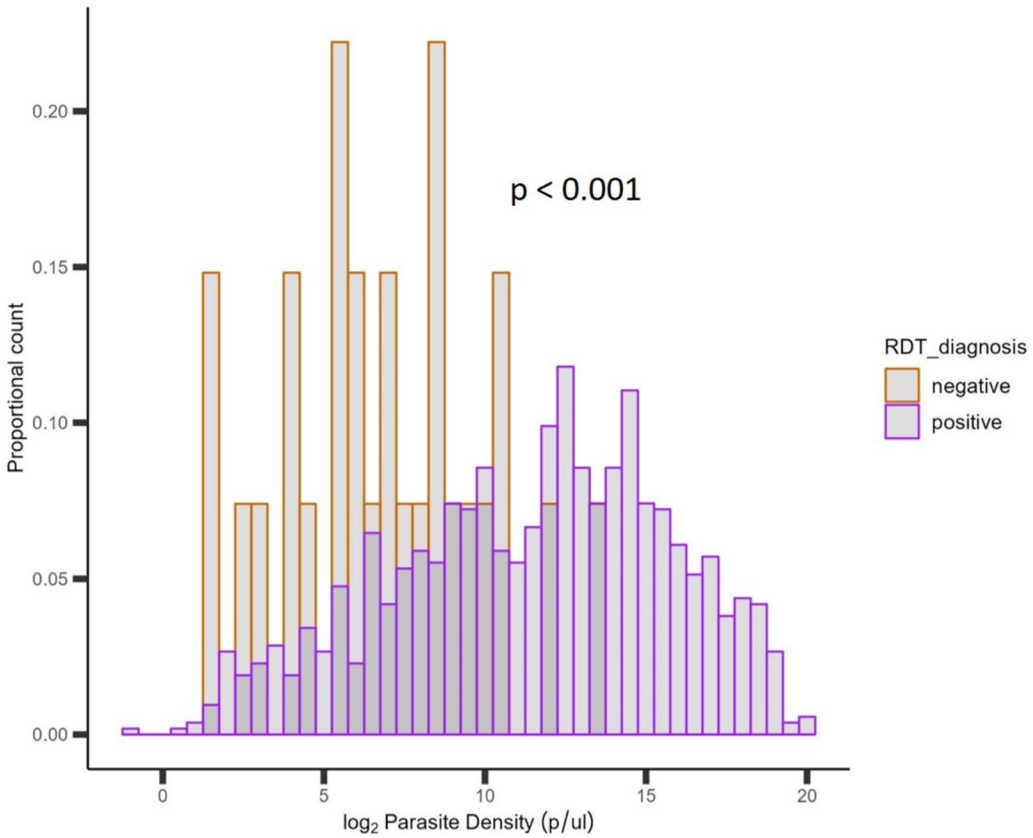

Fig. 1 Distribution of qPCR results by RDT diagnosis. Histogram of log-scaled parasite density by qPCR (p/µl) stratified by RDT results (negative and positive). Orange represents parasite density of false negative RDTs and purple represented parasite density of RDT positives. Y-axis values are scaled to show the proportion count per group. P-values were calculated using Mann-Whitney method.

Adults and males had a higher association to malaria infection by qPCR

To measure malaria prevalence by qPCR among subgroups, a multivariate logistic regression model was constructed (Fig. 2). To minimize selection bias, all RDT negative results were excluded from the model. The categorical variable for age groups was condensed to optimize the sample sizes of subgroups ($N_{<14}=616$; $N_{15+}=765$). The reported sickness variable was excluded from the model due to high multi-co-linearity with reported fever (VIF > 10). Within the sample set, subgroups among RDT(+) individuals with a statistically significant association to malaria infection by PlasmoPod (i.e. True RDT result) compared to their respective reference group include: individuals over 15 years of age, individuals from Baney, males, high SES, urban communities, and bed net users (Supplementary Table S4). Older RDT(+) individuals (over 15) had higher odds of being qPCR positive compared to younger RDT(+) individuals (OR = 1.31; 95% CI 1.01–1.71; p = 0.04). Further, those RDT(+) individuals from Baney had 53% increased odds of being qPCR positive compared to individuals from Malabo (OR = 1.53; 95% CI 1.02–2.36; p = 0.05). Interestingly, RDT(+) males appeared to have 43% higher odds of being qPCR positive than females (OR = 1.43; 95% CI: 1.11–1.85; p = 0.006). RDT(+) individuals in urban communities were less likely to be qPCR positive compared to rural communities (OR = 0.55; 95% CI 0.41–0.75; p < 0.001). Further within the selected sample set, RDT(+) individuals who slept under a bed net the previous night (i.e. bed net users) had lower odds of being qPCR positive compared to non-bed net users (OR = 0.65; 95% CI 0.50–0.85; p = 0.001).

Individuals from urban communities and recent reported fever had higher association to low density infections

To measure the association of variable subgroups and low density infections (< 100 p/µl), a multivariate logistic regression model was generated with only qPCR (+) samples (n = 1057) (Fig. 3). As observed previously, the variable of reported sickness was excluded from the model due to high multi-co-linearity with reported fever (VIF > 10). Variables with a significant association to low density infections include: false negative RDT results,

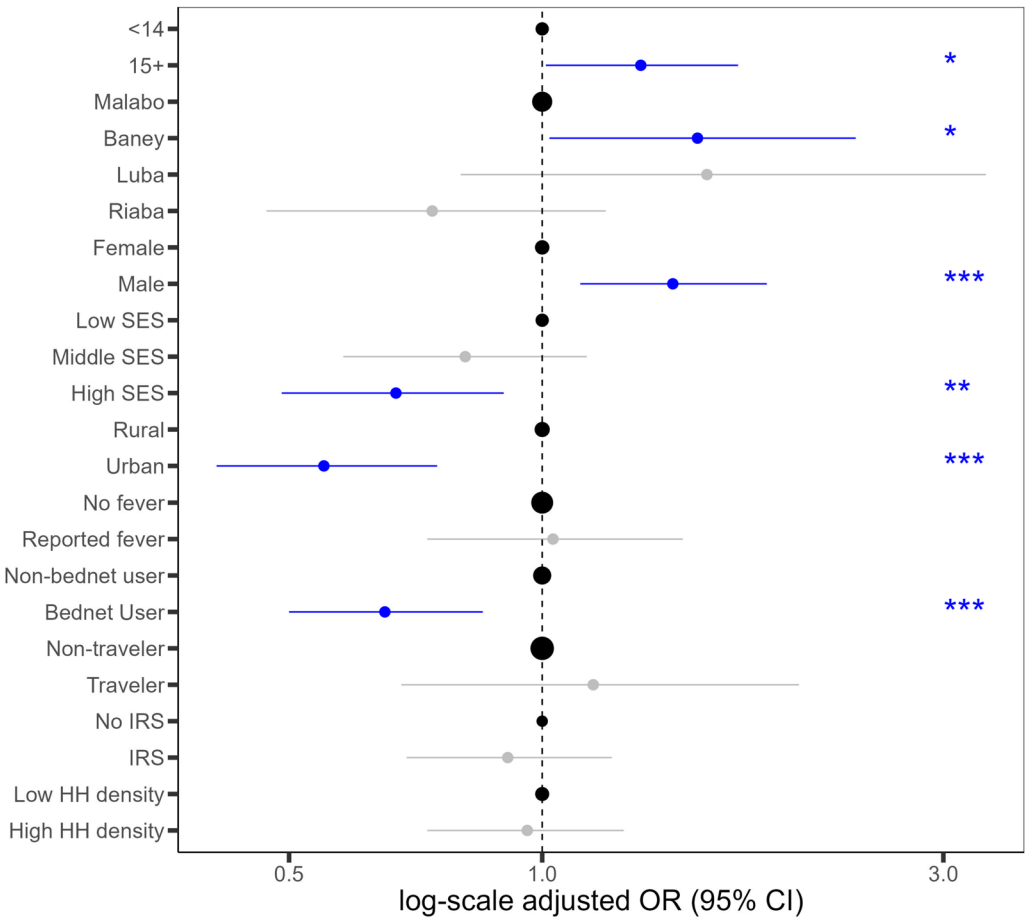

Fig. 2 Association between infection by qPCR and subgroups among RDTs(+). Association of subgroups to qPCR (+) reported as adjusted odds ratios (OR) estimated from a multivariate logistic regression model among RDT(+) individuals from a MIS on Bioko Island, Equatorial Guinea. OR values are reported on a log scale. Dotted line represents the reference value (OR = 1). Black dots represent reference subgroups and are sized according to sample size. Lines on points represent 95% confidence intervals, and * symbols represent Wald p-values (* < 0.05; ** < 0.01; *** < 0.001). Statistically significant associations are marked in blue.

urban communities, reported fever, and high-density households (Supplementary Table S5). As expected, individuals with a false negative RDT had over 4 times the odds of having a low-density infection compared to RDT positive individuals (OR = 4.63; 95% CI 1.92–11.04; p < 0.001). Individuals from urban communities had 1.84 times the odds of having a low-density infection compared to rural communities (95% CI 1.22–2.80; p = 0.004). Individuals with a reported fever in the previous 14 days had higher odds of having a low-density infection compared to individuals with no reported fever (OR = 1.68; 95% CI 1.08–2.56; p = 0.02). Individuals in higher density households (> 1 person/room) had 64% higher odds of having a low-density infection compared to lower density households (OR = 1.64; 95% CI 1.14–2.38; p = 0.008).

Discussion

The BIMEP aims to eliminate malaria from Bioko Island in the near future, and the continuous surveillance of circulating parasites is essential for prevention of parasite resurgence from low density reservoirs or strains circumventing RDT diagnosis by lacking functional expression of *hrp2* or *hrp3* [5, 12, 33, 34]. Molecular monitoring techniques can help identify those infections missed by RDTs, such as qPCR, which is typically a specialized, resource-rich laboratory technique. PlasmoPod was designed as a more rapid, cost-efficient and more accessible qPCR tool for malaria monitoring in the field [21]. This study is the first report of PlasmoPod use on a large scale using DBS samples for *Plasmodium* spp. from the 2023 MIS on Bioko Island, Equatorial Guinea. With PlasmoPod, 45 PCR cycles can be run in under 30 min [35]. Most of the study's associated labor was dedicated to extracting DNA from

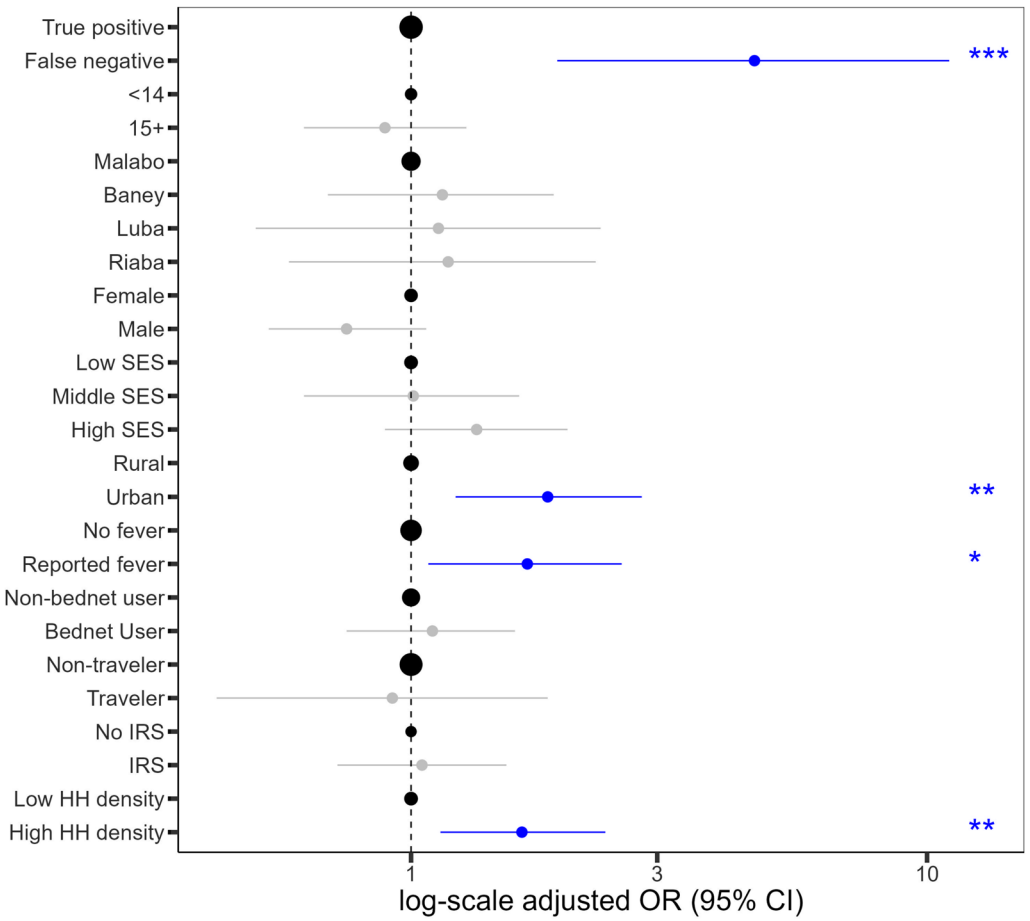

Fig. 3 Association between low density infections and variable subgroups. Association of variables to low density infections (less than 100 p/µl) reported as adjusted odds ratios (OR) estimated from a multivariate logistic regression model among MIS subgroups on Bioko Island, Equatorial Guinea. OR values are reported on a log scale. Dotted line represents the reference value (OR = 1). Black dots represent reference subgroups and are sized according to sample size. Lines on points represent 95% confidence intervals, and * symbols represent Wald p-values (* < 0.05; ** < 0.01; *** < 0.001). Statistically significant associations are marked in blue.

DBS samples. Overall, data was generated slightly faster with PlasmoPod than a similar sample size using a mobile qPCR device [36], and at lower cost as other conventional qPCR assays [37]. The distinct advantage of PlasmoPod is the use of prefilled cartridges that come ready to use without the need for expensive cold chain logistics and reagent storage, also eliminating handling steps required for preparation of PCR primers, mastermix and probes in the lab. These assay characteristics will minimize the potential for introduction of human errors and reagent batch to batch variation effects when running the qPCR.

The majority of samples were selected from individuals with a positive RDT result to optimize the rate of successful extractions from DBS as it was the first time being performed at the Baney Reference laboratory. RDT (-) samples were selected from households with an RDT (+) member, so the actual rate of RDT false negatives overall is likely lower. This selection criteria for RDT (-) individuals was done to improve identification of low density infections that cluster at higher rates in such households [38]. Although these false negatives were not tested for *hrp2* or *hrp3* deletions, RDT (-) results from Equatorial Guinea have been linked to strains with HRP2 and HRP3 deletions [10, 39, 40]. However, most infections are polyclonal on Bioko so their expansion is likely hampered by a co-infection with a non-deletion carrying strain [10, 41]. These strains may become a larger issue for BIMEP should Bioko reach low-transmission levels.

The presence of suspected false positive RDTs may overestimate malaria prevalence, and is possibly linked to lingering parasite proteins post-clearance of *P. falciparum* [10, 11, 42, 43]. Despite qPCR as a more sensitive assay, qPCR false positives may appear, although less likely, since they can amplify both RNA and DNA, thereby also reflecting residual parasite DNA [10, 44]. The presence

of qPCR false positives may further be reflected in the regression models as RDT(-) individuals reporting a fever in the previous 2 weeks had much higher odds of having a low-density infection. It can be assumed some of these individuals may be recovering from a recent *Plasmodium* infection and may have recently taken anti-malaria treatment [42, 43]. Also, considering malaria exposure remains high on Bioko, some false positive RDT results may occur among individuals who have spontaneously cleared parasites through naturally acquired immunity [45]. Recent anti-malarial treatment is a question included in the MIS, but was not included in the analysis here since the question was not collected for all age groups and the data had high missingness. In low-transmission areas, differentiating between false positive RDT due to recent anti-malarial treatment or spontaneous clearance may offer greater detail on transmitting parasites as it may be a reflection of parasite virulence. Overall, using only RDT diagnostic methods to measure prevalence during an MIS is useful, but may overestimate malaria burden, while also underestimating low density infections contributing to transmission [46, 47]. Application of a molecular assay into malaria surveillance activities can offer a finer-scaled perspective of malaria burden.

Among RDT(+) individuals, older individuals (15+) had a stronger association to a positive qPCR result compared to younger individuals. This may reflect shifts in age-specific malaria burden, which may occur by delaying naturally acquired immunity as prevention, surveillance and treatment protect younger at-risk populations [48, 49]. Further, younger cases may be more likely to be treated (i.e. lingering parasite proteins) as malaria infections among younger age groups develop symptomatically [50, 51]. However, older individuals were not more associated to low density infections as their acquired immunity over decades of exposure may allow for increased tolerability to higher density infections [52, 53]. As higher density infections are more likely to result in gametocytemia [54], this group may be an important reservoir of asymptomatic carriers maintaining transmission. Further, older children (6–15 years) often have low density infections [48], however this study's sampling strategy and regression models (concatenated age groups) could not look into this relationship, but is of interest in future studies. Of interest, RDT(+) males having a higher likelihood of infection may reflect an increased occupational exposure to malaria-transmitting mosquitos as well as cultural characteristics [55–57], where on Bioko, males more frequently work outside in the jungle and outdoor biting rates of malaria-transmitting *Anopheles* mosquitos have increased [24]. This is supported by the negative association between RDT(+) individuals in urban communities to malaria infection by qPCR compared to rural communities, as historically there is a higher observed prevalence of malaria in rural areas on Bioko measured by RDT [27]. Although individuals in urban communities had lower odds of being a true RDT(+) result than those in rural settings, they did have a higher probability of having lower density infections. This is somewhat contrary to expectations considering urban environments on Bioko are subject to increased human density and migration to and from the mainland of Equatorial Guinea facilitating malaria transmission [26, 58]. The low density infections in urban communities may also be a reflection of greater accessibility to health clinics and anti-malarial treatment, provided free of charge in Equatorial Guinea [59], subduing prevalence in urban environments despite its potentially higher transmission intensity. In general, low density infections, albeit accounting for a smaller proportion of overall infections on Bioko, likely contribute to transmission [60], and strategies to target these urban subgroups may need to be expanded once BIMEP achieves low-transmission levels of malaria [61, 62]. In addition to adjusting control strategies to account for imported cases, a focus on those hidden malaria transmission reservoirs will be required to effectively eliminate malaria from Bioko Island.

Some limitations exist within this analysis. The majority of samples selected for qPCR were RDT (+) and therefore over-represent higher parasite density infections. To minimize the inherent bias, RDT positives and negatives were assessed separately as appropriate. Further, RDT (-) were selected from households with a RDT(+) member, increasing the probability of being false negative [38] and may overestimate the rate of suspected false negative RDTs. This selection criteria was used to optimize the probability of identifying positive and low density infections. This study's results complement historical observations of malaria burden on Bioko [24, 27, 38, 58], however overall malaria prevalence during the 2023 MIS was approximately 12.9%, therefore due to the sampling strategy, results should not be interpreted as representative of Bioko's malaria epidemiology. When comparing low-density and negative PlasmoPod results to a commercially available qPCR, there were some discrepancies in results, which neared qPCR's limit of detection [63]. Regardless, assay results suggest further verification of PlasmoPod with a validated qPCR assay would provide valuable confirmation of results. Ultimately, this study aimed to illustrate PlasmoPod's effectiveness as an accessible, robust and cost-efficient technique to complement malaria prevalence estimations by RDT in field studies. Here, the study focused primarily on RDT (+) results and investigated possible reservoirs of

low density infections among the residential population on Bioko Island.

Incorporating molecular monitoring in standard NMP surveillance has been recognized and is promoted by the WHO [64, 65]. Inherent within the successfully reduction in malaria transmission is the subsequent shift in malaria burden [50, 66] and the need to adjust surveillance efforts to find unidentified transmission reservoirs, which can be challenging relying solely on conventional RDTs. Molecular monitoring offers an accurate alternative to RDTs with increased sensitivity, but comes with its challenges [67]. PlasmoPod may offer a more accessible substitute to traditional laboratory techniques without significant investment in staff training, supply chain management and equipment but comparable performance. The lower limit of detection by qPCR using PlasmoPod was 0.2 p/µl [21], which is much more sensitive than HRP2 based RDTs [68]. Ultra-sensitive HRP2 based RDTs have been developed with a much lower limit of detection, but have not been able to reach the same level of sensitivity as molecular assays [37, 69, 70]. In future MIS studies, it would be valuable to compare their performance with the Plasmopod assay on the same biological sample side by side similarly to previous studies [37]. PlasmoPod is open source, and the qPCR assays run on the platform can be adapted to broader research questions like inclusion of drug resistance marker monitoring and contact tracing by targeting predefined SNPs [22]. Per 20-well cartridge, the laboratory team ran 8 samples performed in duplicate with accompanying positive and negative controls, also in duplicate. The application of PlasmoPod does not outperform the compatibility or speed of RDTs, but it could offer a more sensitive, complementary molecular technique for field studies at an affordable cost. Its operation forgoes the need for purchasing the reagents and materials needed for more conventional laboratory protocols. The data strongly suggests that it is feasible to embed PlasmoPod for regular malaria surveillance in moderate to low transmission settings. The qPCR assay can be run in medium throughput, takes only 30 min for obtaining results and in combination with automatized data analyses and uploading to cloud based storage may be very helpful for NMPs to arrive at highly accurate results close to real time for decision making.

Conclusion

To eliminate malaria, NMPs must be able to detect and target malaria transmission reservoirs that evade RDT detection, the most commonly used diagnostic method in sub-Saharan African countries. PlasmoPod is a novel qPCR device that warrants further investigation using field samples. Within the context of the 2023 MIS conducted by the BIMEP, 18S RT-qPCR highlighted malaria burden on Bioko at the molecular level. By the observed odds ratios within the selected sample set, individuals in urban communities or recent reported fever were more positively associated to low density parasite infections by PlasmoPod qPCR, suggesting that they could serve as a transmission reservoir. Individuals in urban communities may be under increased exposure, but have improved access to health clinics. For those individuals who reported recent fever events, this may reflect recent anti-malarial treatment or spontaneous clearance, which went undetected by RDT. This may be an interesting avenue for further investigation to differentiate between genomic characteristics of *P. falciparum* strains needing to be cleared by treatment *versus* those cleared via natural immunity. Ultimately, to address recent plateauing trends, BIMEP may need to rely more heavily on molecular monitoring in its surveillance strategy, identifying more elusive transmission reservoirs, trace parasite importation and the sources and sinks to continue their progress towards malaria elimination.

Abbreviations

BIMEP	Bioko Island Malaria Elimination Project
OR	Odds ratio
CI	Confidence intervals
DBS	Dried blood spots
HRP2	Histidine-rich protein 2
MIS	Malaria indicator survey
NMP	National malaria programme
NAAT	Nucleic acid amplification technique
OR	Odds ratio
PCA	Principal components analysis
pLDH	Plasmodium lactate dehydrogenase
PSU	Primary sampling units
RDT	Rapid diagnostic test
SES	Socioeconomic status
VIF	Variance inflation factor

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-025-05374-x.

Supplementary material 1.

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Author contributions

TCS and CD conceived and designed the study. TCS trained local laboratory staff, managed laboratory operations, performed data processing, analysis and wrote the manuscript. SH contributed to performing the analyses and

writing of the manuscript. EN managed laboratory staff and operations. JNG supported laboratory operations, contributed to data analysis and the writing of the manuscript. MKE, RNB, VMN, IEM, ALB, RMOB, JRB, EQR, AGN, VPI, SB, CNE performed all DBS extractions, and generated PlasmoPod results. MRR sponsored and oversaw the 2023 MIS. DSG managed the 2023 MIS and processed all associated data and contributed to downstream analysis. WPP supervised all BIMEP operations, contributed to supporting the Baney Reference laboratory. CAG contributed to the study design, supported interpretation of 2023 MIS epidemiological data, assisted in generating Bioko Island figures, and edited the paper. GAG contributed fundamental funding and resources to the study, contributed to the study design, and edited the paper. LM and TS provided technical support in installation, set up, and operating the DiaxxoPCR device. JCS contributed to the interpretation of results and edited the paper. CD conceived of the study, contributed essential resources to the study and wrote the paper. All authors read and approved the final version of the manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

Declarations

Ethics approval and consent to participate
Ethics approval for the 2023 MIS was provided by the Equatorial Guinea Ministry of Health and Social Welfare and the ethics committee of the London School of Hygiene and Tropical Medicine (approval number 5556). Written informed consent was sought from each participating adult and on behalf of participating children under 18 years of age.

Consent for publication
Not applicable.

Competing interests
LM and TS are employed by and shareholders of the ETH Zurich spin-off company, Diaxxo AG, the organization that produces the DiaxxoPCR device. All other authors declare that they have no competing interests.

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References

1. WHO. World malaria report 2023. Geneva, World Health Organization; 2023.
2. Zhang H, Fink G, Cohen J. Malaria rapid tests, febrile illness management, and child mortality across sub-Saharan African countries. JAMA. 2024;332:1270–81.
3. Kong A, Wilson SA, Ah Y, Nace D, Rogier E, Aidoo M. HRP2 and HRP3 cross-reactivity and implications for HRP2-based RDT use in regions with *Plasmodium falciparum* hrp2 gene deletions. Malar J. 2021;20:207.
4. Thomson R, Parr JB, Cheng Q, Chenet S, Perkins M, Cunningham J. Prevalence of *Plasmodium falciparum* lacking histidine-rich proteins 2 and 3: a systematic review. Bull World Health Organ. 2020;98:558-568F.
5. Gendrot M, Fawaz R, Dormoi J, Madamet M, Pradines B. Genetic diversity and deletion of *Plasmodium falciparum* histidine-rich protein 2 and 3: a threat to diagnosis of *P. falciparum* malaria. Clin. Microbiol.Infect. 2019;25:580–585.
6. WHO. Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs: round 6 (2016–2018). Geneva, World Health Organization; 2018.
7. Verma AK, Bharti PK, Das A. HRP-2 deletion: a hole in the ship of malaria elimination. Lancet Infect Dis. 2018;18:826–7.
8. Kavanaugh MJ, Azzam SE, Rockabrand DM. Malaria rapid diagnostic tests: literary review and recommendation for a quality assurance, quality control algorithm. Diagnostics (Basel). 2021;11:768.
9. Watson OJ, Sumner KM, Janko M, Goel V, Winskill P, Slater HC, et al. False-negative malaria rapid diagnostic test results and their impact on community-based malaria surveys in sub-Saharan Africa. BMJ Glob Health. 2019;4: e001582.
10. Hosch S, Yoboue CA, Donfack OT, Guirou EA, Dangy J-P, Mpina M, et al. Analysis of nucleic acids extracted from rapid diagnostic tests reveals a significant proportion of false positive test results associated with recent malaria treatment. Malar J. 2022;21:23.
11. Dalrymple U, Arambepola R, Gething PW, Cameron E. How long do rapid diagnostic tests remain positive after anti-malarial treatment? Malar J. 2018;17:228.
12. Stone W, Gonçalves BP, Bousema T, Drakeley C. Assessing the infectious reservoir of falciparum malaria: past and future. Trends Parasitol. 2015;31:287–96.
13. Padley DJ, Heath AB, Sutherland C, Chiodini PL, Baylis SA; Collaborative Study Group. Establishment of the 1st World Health Organization International Standard for P*lasmodium falciparum* DNA for nucleic acid amplification technique (NAT)-based assays. Malar J. 2008;7:139.
14. Schindler T, Robaina T, Sax J, Bieri JR, Mpina M, Gondwe L, et al. Molecular monitoring of the diversity of human pathogenic malaria species in blood donations on Bioko Island. Equatorial Guinea Malar J. 2019;18:9.
15. Kamau E, Alemayehu S, Feghali KC, Saunders D, Ockenhouse CF. Multiplex qPCR for detection and absolute quantification of malaria. PLoS ONE. 2013;8: e71539.
16. Gimenez AM, Marques RF, Regiart M, Bargieri DY. Diagnostic methods for non-falciparum malaria. Front Cell Infect Microbiol. 2021;11: 681063.
17. Agaba BB, Smith D, Travis J, Pasay C, Nabatanzi M, Arinaitwe E, et al. Limited threat of *Plasmodium falciparum* pfhrp2 and pfhrp3 gene deletion to the utility of HRP2-based malaria RDTs in Northern Uganda. Malar J. 2024;23:3.
18. Rogier E, Battle N, Bakari C, Seth MD, Nace D, Herman C, et al. *Plasmodium falciparum* pfhrp2 and pfhrp3 gene deletions among patients enrolled at 100 health facilities throughout Tanzania: February to July 2021. Sci Rep. 2024;14:8158.
19. Guirou EA, Schindler T, Hosch S, Donfack OT, Yoboue CA, Krähenbühl S, et al. Molecular malaria surveillance using a novel protocol for extraction and analysis of nucleic acids retained on used rapid diagnostic tests. Sci Rep. 2020;10:12305.
20. Okell LC, Ghani AC, Lyons E, Drakeley CJ. Submicroscopic infection in *Plasmodium falciparum*-endemic populations: a systematic review and meta-analysis. J Infect Dis. 2009;200:1509–17.
21. Bechtold P, Wagner P, Hosch S, Gregorini M, Stark WJ, Gody JC, et al. Development and evaluation of PlasmoPod: a cartridge-based nucleic acid amplification test for rapid malaria diagnosis and surveillance. PLoS Glob Public Health. 2023;3: e0001516.
22. Bechtold P, Wagner P, Hosch S, Siegrist D, Ruiz-Serrano A, Gregorini M, et al. Rapid identification of SARS-CoV-2 variants of concern using a portable peakPCR platform. Anal Chem. 2021;93:16350–9.
23. Fries B, Guerra CA, García GA, Wu SL, Smith JM, Oyono JNM, et al. Measuring the accuracy of gridded human population density surfaces: a case study in Bioko Island. Equatorial Guinea PLoS One. 2021;16: e0248646.

24. García GA, Janko M, Hergott DEB, Donfack OT, Smith JM, Mba Eyono JN, et al. Identifying individual, household and environmental risk factors for malaria infection on Bioko Island to inform interventions. Malar J. 2023;22:72.
25. Guerra CA, Citron DT, García GA, Smith DL. Characterising malaria connectivity using malaria indicator survey data. Malar J. 2019;18:440.
26. Bradley J, Monti F, Rehman AM, Schwabe C, Vargas D, Garcia G, et al. Infection importation: a key challenge to malaria elimination on Bioko Island. Equatorial Guinea Malar J. 2015;14:46.
27. Cook J, Hergott D, Phiri W, Rivas MR, Bradley J, Segura L, et al. Trends in parasite prevalence following 13 years of malaria interventions on Bioko island, Equatorial Guinea: 2004–2016. Malar J. 2018;17:62.
28. García GA, Hergott DEB, Phiri WP, Perry M, Smith J, Osa Nfumu JO, et al. Mapping and enumerating houses and households to support malaria control interventions on Bioko Island. Malar J. 2019;18:283.
29. Zainabadi K, Adams M, Han ZY, Lwin HW, Han KT, Ouattara A, et al. A novel method for extracting nucleic acids from dried blood spots for ultrasensitive detection of low-density *Plasmodium falciparum* and *Plasmodium vivax* infections. Malar J. 2017;16:377.
30. Kamau E, Tolbert LS, Kortepeter L, Pratt M, Nyakoe N, Muringo L, et al. Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for detection and quantitation of *Plasmodium* by amplifying RNA and DNA of the 18S rRNA genes. J Clin Microbiol. 2011;49:2946–53.
31. McFadden D, Zarembka P. Frontiers in econometrics. New York: Academic Press; 1974.
32. Montgomery DC, Peck EA, Vining GG. Introduction to linear regression analysis. John Wiley & Sons; 2021.
33. Schneider KA. Charles Darwin meets Ronald Ross: a population-genetic framework for the evolutionary dynamics of malaria. In: Teboh-Ewungkem MI, Ngwa GA (eds.); Infectious Diseases and Our Planet. Cham. Springer Int Publ; 2021:149–191
34. Schneider KA, Salas CJ. Evolutionary genetics of malaria Front Genet. 2022;13:1030463.
35. Gregorini M, Mikutis G, Grass RN, Stark WJ. Small-size polymerase chain reaction device with improved heat transfer and combined feedforward/feedback control strategy. Industrial Engineer Chem Res. 2019;58:9665–74.
36. Carlier L, Baker SC, Huwe T, Yewhalaw D, Haileselassie W, Koepfli C. qPCR in a suitcase for rapid *Plasmodium falciparum* and *Plasmodium vivax* surveillance in Ethiopia. PLoS Glob Public Health. 2022;2: e0000454.
37. Mpina M, Stabler TC, Schindler T, Raso J, Deal A, Acuche Pupu L, et al. Diagnostic performance and comparison of ultrasensitive and conventional rapid diagnostic test, thick blood smear and quantitative PCR for detection of low-density *Plasmodium falciparum* infections during a controlled human malaria infection study in Equatorial Guinea. Malar J. 2022;21:99.
38. Hergott DEB, Balkus JE, García GA, Cruz KR, Seilie AM, Masters H, et al. Clustering of subpatent infections in households with asymptomatic rapid diagnostic test-positive cases in Bioko Island, Equatorial Guinea independent of travel to regions of higher malaria endemicity: a cross-sectional study. Malar J. 2021;20:313.
39. Berzosa P, González V, Taravillo L, Mayor A, Romay-Barja M, García L, et al. First evidence of the deletion in the pfhrp2 and pfhrp3 genes in *Plasmodium falciparum* from Equatorial Guinea. Malar J. 2020;19:99.
40. Molina-de la Fuente I, Pacheco MA, García L, González V, Riloha M, Oki C, Benito A, Escalante AA, Berzosa P. Evolution of pfhrp2 and pfhrp3 deletions in Equatorial Guinea between the pre– and post–RDT introduction. Malar J. 2024;**23:**215.
41. Sepúlveda N, Phelan J, Diez-Benavente E, Campino S, Clark TG, Hopkins H, et al. Global analysis of *Plasmodium falciparum* histidine-rich protein-2 (pfhrp2) and pfhrp3 gene deletions using whole-genome sequencing data and meta-analysis. Infect Genet Evol. 2018;62:211–9.
42. Iqbal J, Siddique A, Jameel M, Hira PR. Persistent histidine-rich protein 2, parasite lactate dehydrogenase, and panmalarial antigen reactivity after clearance of *Plasmodium falciparum* monoinfection. J Clin Microbiol. 2004;42:4237–41.
43. Markwalter CF, Gibson LE, Mudenda L, Kimmel DW, Mbambara S, Thuma PE, et al. Characterization of *Plasmodium* lactate dehydrogenase and histidine-rich protein 2 clearance patterns via rapid on-bead detection from a single dried blood spot. Am J Trop Med Hyg. 2018;98:1389–96.
44. Imwong M, Hanchana S, Malleret B, Rénia L, Day NPJ, Dondorp A, et al. High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias. J Clin Microbiol. 2014;52:3303–9.
45. Roe MS, O'Flaherty K, Fowkes FJI. Can malaria parasites be spontaneously cleared? Trends Parasitol. 2022;38:356–64.
46. Kojom Foko LP, Moun A, Singh V. Addressing low-density malaria infections in India and other endemic part of the world—the opportune time? Crit Rev Microbiol. 2025;51:229–45.
47. Laban NM, Kobayashi T, Hamapumbu H, Sullivan D, Mharakurwa S, Thuma PE, et al. Comparison of a PfHRP2-based rapid diagnostic test and PCR for malaria in a low prevalence setting in rural southern Zambia: implications for elimination. Malar J. 2015;14:25.
48. Kigozi SP, Kigozi RN, Epstein A, Mpimbaza A, Sserwanga A, Yeka A, et al. Rapid shifts in the age-specific burden of malaria following successful control interventions in four regions of Uganda. Malar J. 2020;19:128.
49. Griffin JT, Ferguson NM, Ghani AC. Estimates of the changing age-burden of *Plasmodium falciparum* malaria disease in sub-Saharan Africa. Nat Commun. 2014;5:3136.
50. Nkumama IN, O'Meara WP, Osier FHA. Changes in malaria epidemiology in Africa and new challenges for elimination. Trends Parasitol. 2017;33:128–40.
51. Li J, Docile HJ, Fisher D, Pronyuk K, Zhao L. Current status of malaria control and elimination in Africa: epidemiology, diagnosis, treatment, progress and challenges. J Epidemiol Glob Health. 2024;14:561–79.
52. Doolan DL, Dobaño C, Baird JK. Acquired immunity to malaria. Clin Microbiol Rev. 2009;22:13–36.
53. Barry A, Hansen D. Naturally acquired immunity to malaria. Parasitology. 2016;143:125–8.
54. Gonçalves BP, Kapulu MC, Sawa P, Guelbéogo WM, Tiono AB, Grignard L, et al. Examining the human infectious reservoir for *Plasmodium falciparum* malaria in areas of differing transmission intensity. Nat Commun. 2017;8:1133.
55. Tanner M, Vlassoff C. Treatment-seeking behaviour for malaria. a typology based on endemicity and gender. Soc Sci Med. 1998;46:523–32.
56. Bates I, Fenton C, Gruber J, Lalloo D, Lara AM, Squire SB, et al. Vulnerability to malaria, tuberculosis, and HIV/AIDS infection and disease. Part 1. determinants operating at individual and household level. Lancet Infect Dis. 2004;4:267–77.
57. Okiring J, Epstein A, Namuganga JF, Kamya EV, Nabende I, Nassali M, et al. Gender difference in the incidence of malaria diagnosed at public health facilities in Uganda. Malar J. 2022;21:22.
58. Guerra CA, Kang SY, Citron DT, Hergott DEB, Perry M, Smith J, et al. Human mobility patterns and malaria importation on Bioko Island. Nat Communications. 2019;10:2332.
59. Rehman AM, Mann AG, Schwabe C, Reddy MR, Roncon Gomes I, Slotman MA, Yellott L, Matias A, Caccone A, Nseng Nchama G, Kleinschmidt I. Five years of malaria control in the continental region. Equatorial Guinea Malar J. 2013;12:154.
60. Drakeley C, Gonçalves B, Okell L, Slater H. Understanding the importance of asymptomatic and low-density infections for malaria elimination. In:Manguin S, Dev V (eds.); Towards Malaria Elimination-A Leap Forward. IntechOpen. 2018.
61. Doumbe-Belisse P, Kopya E, Ngadjeu CS, Sonhafouo-Chiana N, Talipouo A, Djamouko-Djonkam L, et al. Urban malaria in sub-Saharan Africa: dynamic of the vectorial system and the entomological inoculation rate. Malar J. 2021;20:364.
62. WHO. Global framework for the response to malaria in urban areas. Geneva: World Health Organization; 2022.
63. Roth JM, Korevaar DA, Leeflang MMG, Mens PF. Molecular malaria diagnostics: a systematic review and meta-analysis. Crit Rev Clin Lab Sci. 2016;53:87–105.
64. WHO. A framework for malaria elimination. Geneva: World Health Organization; 2017.
65. WHO. Global technical strategy for malaria 2016–2030 update. Geneva: World Health Organization; 2021. p. 2021.
66. O'Meara WP, Mangeni JN, Steketee R, Greenwood B. Changes in the burden of malaria in sub-Saharan Africa. Lancet Infect Dis. 2010;10:545–55.
67. Nsanzabana C. Strengthening surveillance systems for malaria elimination by integrating molecular and genomic data. Trop Med Infect Dis. 2019;4:139.

68. Das S, Peck RB, Barney R, Jang IK, Kahn M, Zhu M, et al. Performance of an ultra-sensitive *Plasmodium falciparum* HRP2-based rapid diagnostic test with recombinant HRP2, culture parasites, and archived whole blood samples. Malar J. 2018;17:118.
69. Danwang C, Kirakoya-Samadoulougou F, Samadoulougou S. Assessing field performance of ultrasensitive rapid diagnostic tests for malaria: a systematic review and meta-analysis. Malar J. 2021;20:245.
70. Yimam Y, Mohebali M, Abbaszadeh Afshar MJ. Comparison of diagnostic performance between conventional and ultrasensitive rapid diagnostic tests for diagnosis of malaria: a systematic review and meta-analysis. PLoS ONE. 2022;17: e0263770.

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