Journal of Infection 92 (2026) 106756

Contents lists available at ScienceDirect

Journal of Infection

journal homepage: www.elsevier.com/locate/jinf

Protozoa and Protozoal Diseases

Parasite and gametocyte dynamics and infectivity to mosquitoes during recurrent *Plasmodium vivax* infections: A longitudinal study from Ethiopia

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ARTICLE INFO

Article history:
Accepted 23 April 2026
Available online 28 April 2026

Keywords:
P. vivax
Recurrence infections
Direct membrane feeding assays
Infectious reservoir
Parasitemia
Gametocytemia

SUMMARY

Objectives: Plasmodium vivax relapses can lead to episodes of recurrent parasitemia. We longitudinally assessed parasite and gametocyte kinetics and infectivity to mosquitoes during P. vivax recurrent infections in Ethiopia.

Methods: Patients presenting with clinical P. vivax infections were enrolled at Shele health center in Arba Minch Zuria district, Ethiopia. All participants received chloroquine plus 14-day primaquine for radical cure, and were subsequently followed for one year with scheduled monthly visits. Parasite and gametocyte densities were quantified using molecular assays, and infectivity was assessed through mosquito feeding assays.

Results: Over the follow-up period, 54.6% (96/176) of patients experienced at least one recurrent parasitemia. Gametocyte density was similar between baseline and recurrent symptomatic episodes but lower during asymptomatic parasitemia ($p < 0.001$). Across all mosquito feeding assays, 167 (70.2%) of 238 feedings resulted in at least one infected mosquito, with similar proportions between baseline (117 [70.5%] of 166) and recurrent symptomatic infections (50 [69.4%] of 72). Mosquito infection rates were strongly associated with both asexual parasite density (ρ=0.29; $p < 0.0001$; n=166) and gametocyte density (ρ=0.32; $p < 0.0001$) at baseline. Genotyping a subset of recurrent infections indicated 45.7% (42/92) of recurrences were likely to be relapses. After adjusting for gametocyte density, mosquito infection rates appeared lower when feeding on blood of relapsing infections as opposed to new infections (Odds Ratio 0.48, 95% CI 0.26–0.87, p=0.016).

Conclusions: This comprehensive assessment of parasite kinetics and transmissibility over the course of infections uncovers a high frequency of recurring episodes of parasitemia that are accompanied by infectious gametocytes. This work further supports the need to strengthen radical cure to prevent relapsing infections and sustain malaria transmission control.

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Introduction

The successful scale-up of malaria control has halved the global burden of *Plasmodium falciparum* malaria in the last two decades yet the *Plasmodium vivax* burden remained relatively stable in co-endemic

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https://doi.org/10.1016/j.jinf.2026.106756
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settings.[1,2] Although, *Plasmodium vivax* is commonly referred to as the major cause of malaria-associated morbidity outside of sub-Saharan Africa,[3] Ethiopia forms an exception with vivax malaria being responsible for ~40% of its annual cases.[4]

P. vivax has several fundamental biological differences that affect its susceptibility to control.[5] Principal among these is the periodic activation of *P. vivax* hypnozoites that leads to repeated phases of parasitemia that do not involve infectious mosquito bites; this makes the community prevalence of *P. vivax* less responsive to vector control.[6] Activation of hypnozoites is estimated to be responsible for 40–77% of clinical *P. vivax* infections in Ethiopia, with seasonal variation.[7] The pyrogenic threshold may rise during relapsing *P. vivax* infections[8] and this may contribute to a considerable fraction of *P. vivax* infections being asymptomatic.[9]

Gametocyte density is the most important determinant of onward transmission potential to mosquitoes. *P. vivax* gametocytes occur within 2–3 days following asexual parasites,[10] which is considerably faster than *P. falciparum* (8–12 days).[11] As a consequence, clinical *P. vivax* infections with high parasite densities also have the highest densities of gametocytes and are highly infectious to mosquitoes. In natural *P. vivax* infections, gametocytes commonly make up ~10% of the total parasite biomass[12] with considerable variation between infections.[13] Importantly, onward transmission potential may not only be concluded based on gametocyte presence or density. Transmission-modulating immune responses may alter gametocyte infectivity over the course of (recurring) *P. vivax* infections and either reduce or enhance mosquito infection rates.[14–16]

It is currently unclear how *P. vivax* gametocyte production and infectivity vary over the course of natural infections or what the relative importance of recurrent infections is for onward transmission to mosquitoes. In this study, we assessed the kinetics of parasitemia and gametocytemia and infectivity to mosquitoes in naturally acquired *P. vivax* infections in Ethiopia.

Methods

Study design

We performed a longitudinal cohort study between December 2020 and September 2022 at Shele Health Center, Arba Minch Zuria district, Ethiopia. The district reported an average annual parasite incidence of 66 per 1000 population over the five-year period from 2018 to 2022. Both *P. falciparum* and *P. vivax* are endemic in the area[17,18] with seasonal transmission peaks typically occurring from May to August and November to February, following the two rainy seasons (Supplementary Figure 1). *Anopheles arabiensis* is the predominant malaria vector, while *An. stephensi* has recently been reported as an invasive species in Arba Minch town.[19]

Participants

Patients with uncomplicated clinical malaria attending Shele Health Center with microscopically confirmed *P. vivax* infection were eligible for recruitment if they were ≥2 years old. Recruitment was conducted on a rolling basis, with scheduled follow-up visits every four weeks for one year. Participants were also encouraged to attend the study clinic whenever malaria symptoms developed in between follow-up. Clinical episodes were defined as self-reported malaria symptoms combined with microscopy-confirmed parasitemia. These were treated according to national treatment guidelines with chloroquine (total dose of 25 mg base/kg for 3 days) plus 14-day primaquine (0.25 mg/kg body weight per day). Treatment with chloroquine and the first dose of primaquine were directly observed; patients were given instructions to continue primaquine treatment at home but no (proxy) measures of primaquine adherence such as pill counts were collected. Asymptomatic infections, identified retrospectively by microscopy and PCR, were not treated; this is in line with Ethiopian treatment guidelines that also take into consideration the cost, logistical and personal benefit implications of treatment strategies. Similarly, G6PD deficiency testing was not done prior to primaquine administration. This is in line with Ethiopian treatment guidelines[20] and informed by the very low prevalence of clinically relevant G6PD deficiency in Ethiopia. Written informed consent was obtained from all participants or their guardians. Ethical approval was granted by the AHRI/ALERT Ethics Review Committee (Ref. No. P042/18), the National Research Ethics Review Committee (Ref. No. MoSHE/RD/141/1097/19), and the London School of Hygiene and Tropical Medicine (LSHTM) (Ref. No. 22518).

Procedures

Finger prick blood samples (~0.3 mL collected in microtainer EDTA tubes) were taken at scheduled monthly visits; venous blood samples (5 mL collected in vacutainer EDTA and Lithium Heparin tubes using Precision-Glide™ Multi-sample Needles) were taken at recruitment and at all subsequent clinical *P. vivax* episodes. On each occasion, a blood smear was prepared alongside dried blood spots (DBS) on 3MM Whatman filter paper (Whatman, Maidstone, UK). Venous blood samples collected in Heparin tubes were offered to locally reared *An. arabiensis* mosquitoes in direct membrane feeding assays (DMFA) following established methods.[21] DMFA was performed during clinical malaria episodes but not during asymptomatic episodes of parasite carriage; we previously demonstrated the transmission potential of asymptomatic malaria infections to the same mosquito colony in Adama, Ethiopia.[21,22]

Blood samples in EDTA tubes and DBS samples were used to extract genomic DNA using Kingfisher Flex robotic extractor (Thermo Fisher Scientific™). DNA was extracted from 50 µL whole blood using MagMAX™ magnetic bead-based technology DNA multi-sample kit following the manufacturer's protocol. Blood from 6 mm diameter punches of the DBS was first treated with Proteinase K (QIAGEN) (20 µL) containing Tissue Lysis Buffer (200 µL) (Roche Applied Sciences) at 56 °C overnight. Multiplex quantitative PCR (qPCR) targeting the 18S rRNA small subunit gene for *P. falciparum* and *P. vivax* was run using primer and probe sequences described before[23,24] using TaqMan Fast Advanced Master Mix (Applied Biosystems). *P. vivax*, parasite quantification was done using recombinant plasmid constructs to infer copy numbers by running serial dilutions (10^7–10^3 copies/mL). Blood samples (50 µL) preserved in protective buffer (250 µL RNA protect Cell Reagent; QIAGEN) were used to extract total RNA using the same MagMAX™ magnetic bead technology on a Kingfisher Flex robotic extractor (Thermo Fisher™). Reverse transcriptase quantitative PCR (RT-qPCR) targeting *Pvs25* transcript was done[23] using Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs, NEB). *P. vivax* gametocyte quantification was achieved using in vitro RNA constructs in serial dilutions (10^8–10^3 copies/mL). Four negative controls were included in each extraction batch in a 96 deep well format for both DNA and RNA extractions. Serial dilutions of the standard curves were generated in duplicate on each plate.

For individuals with symptomatic recurrences**,** samples from these recurrences and from recruitment were genotyped using the PvAmpSeq assay.[25] PvAmpSeq is a multiplex amplicon sequencing platform that analyzes 11 highly polymorphic loci located on different *P. vivax* chromosomes. Pooled libraries were normalized at 20 nM and sequenced on an Illumina NextSeq sequencer using a 2×250 paired-end strategy. The raw sequencing data were demultiplexed, and subsequent variant and haplotype calling were performed using AmpSeqR R Package https://github.com/bahlolab/AmpSeqR. To minimize technical noise and PCR artifacts, microhaplotypes with < 5 reads per locus or within-host haplotype frequency < 0.02 were excluded from the final analysis. The Multiplicity

Table 1
Characteristics of the study participants at baseline, categorized by whether they experienced recurrent infections.

	Patients experiencing no recurrence (n=80)	Patients experiencing at least one symptomatic recurrence (n=62)	Patients experiencing asymptomatic recurrence (s) only (n=34)	Total (n=176)
Sex				
Female	43 (53.8%)	19 (30.6%)	9 (26.5%)	71 (39.8%)
Male	37 (46.2%)	43 (69.4%)	25 (73.5%)	105 (60.2%)
Fever at recruitment				
No	39 (48.8%)	27 (43.6%)	16 (47.0%)	82 (46.6%)
Yes	41 (51.2%)	35 (56.4%)	18 (53.0%)	94 (53.4%)
Age				
< 15 years	39 (48.8%)	43 (69.4%)	22 (64.7%)	104 (59.1%)
15 years and above	41 (51.2%)	19 (30.6%)	12 (35.3%)	72 (40.9%)
Malaria in the past 3 months				
No	65 (81.3%)	37 (59.7%)	23 (67.7%)	125 (71.0%)
Yes	15 (18.7%)	25 (40.3%)	11 (32.3%)	51 (29.0%)
Malaria in the past 12 months				
No	47 (58.8%)	21 (33.9%)	17 (50.0%)	85 (48.3%)
Yes	33 (41.2%)	41 (66.1%)	17 (50.0%)	91 (51.7%)
Pv18S copies/µL, median (IQR)	58,963 (10,800–185,798)	159,032 (46,458–420,113)	92,308 (38,187–270,527)	9685 (18516–314,832)
Pvs25 transcript copies/µL, median (IQR)	18,391 (2260–92,170)	20,939 (3780–185,130)	30,551 (5402–143,898)	18,256 (2721–136,063)

IQR, interquartile range.

of Infection (MOI) was calculated by taking the maximum number of unique microhaplotypes detected at any of the 11 targeted loci within a single sample. It was calculated using the naïve estimator, defined as the maximum number of distinct haplotypes observed per marker per sample. However, it can be influenced by variability in amplicon sequencing depth, potentially leading to underestimation or overestimation of MOI in samples with uneven coverage. Relatedness based on identity by descent (IBD) was estimated using Dcifer.[26] Thereafter, it was classified as (high relatedness > 50%), intermediate (25–50%) or low (relatedness < 25%). Recurrence classification was performed using the Pv3Rs probabilistic model,[27] which computes posterior probabilities of re-infection, relapse and recrudescence. Priors were set at 45% re-infection, 45% relapse and 10% recrudescence; thereafter, a 50% probability threshold was used to classify recurrent infections as re-infection, relapse or recrudescence. However, our time-to-event model is constrained to a maximum of eight clones per sample, so that samples with higher than eight MOI are not included in the model and classification.

Statistical analysis

Recurrent symptomatic infections during follow-up were defined as microscopy-confirmed parasitemia with accompanying symptoms after at least one preceding qPCR-negative visit. Recurrent asymptomatic infections were defined as microscopy- or qPCR-positive infections without symptoms, detected after at least two consecutive qPCR-negative visits (i.e., ≥two months with no detectable parasitemia). This conservative definition was chosen because asymptomatic infections often occur at low parasite densities, fluctuating around the detection thresholds, and reliance on a single negative time point could lead to misclassification of persistent infections as cleared.[28] Recurrent parasitemia was defined as the cumulative of parasite positivity at any time point after treatment during follow-up period, and includes above defined recurrent symptomatic infections and recurrent asymptomatic infections.

Proportions were compared using one-sample proportion test, and Pearson's chi-square test or Fisher's exact test for independent observations. Equality tests on unmatched data such as parasite and gametocyte density with categorical variables were tested by two-sample Wilcoxon rank-sum (Mann-Whitney) test. Spearman's rank correlation coefficient (ρ) was used to assess associations between continuous variables. Continuous data were summarized as medians with interquartile ranges (IQRs) or as geometric means.

Time to any subsequent recurrence was estimated using Kaplan-Meier event-free probabilities, starting from the date of symptomatic recruitment, symptomatic recurrence or asymptomatic recurrence. Survival curves were compared using the log-rank test. The stratified Cox proportional hazards shared frailty model was used to assess the effects of selected risk factors on the gap times between recurrences. Stratified baseline hazards for the category of the previous infection were used, to allow different hazard rates while assuming the same covariate effects. Random intercepts were used to account for the within-individual correlation. Statistical analyses were performed using STATA (version 17.0, Stata Corp., TX, and USA) and R (version 4.3.1).

Results

Between December 2020 and September 2022, 191 patients with signs and symptoms of malaria and microscopy-confirmed *P. vivax* infection were enrolled on a rolling basis. Fifteen patients were lost to follow-up before the first monthly scheduled visit and were excluded from the analyses, resulting in a study population of 176 patients. Among these, the majority were male (60.2%; 105/176), and 59.1% (104/176) were under 15 years of age. At recruitment, approximately half (53.4%; 94/176) of the patients had a measured fever (axillary temperature ≥37.5 °C), and 51.9% (91/176) of patients reported a malaria episode in the 12 months preceding enrollment (Table 1).

Overall, 54.6% (96/176) of patients experienced recurrent parasitemia following enrollment and treatment. Symptomatic recurrences were detected in 35.2% (62/176) of patients, accounting for a total of 91 episodes. An additional 55 recurrent asymptomatic infections were detected during scheduled monthly follow-up visits, with 19.3% (34/176) of patients experiencing only asymptomatic recurrences (Table 1). Across all follow-up visits, parasitemia was detected 375 times, occurring in the presence of fever in 117 instances and in its absence in 258 instances. The maximum number of parasite-positive observations per participant during follow-up was 11. Among the 258 asymptomatic parasite-positive time points, 23.3% (60/258) were detected by microscopy, while the remaining were detected by qPCR only (Fig. 1A; Supplementary Figure 2).

Symptomatic recurrent infections were more frequent in male (69.4%, 43/62) than females (30.6%, 19/62; p=0.001), in children

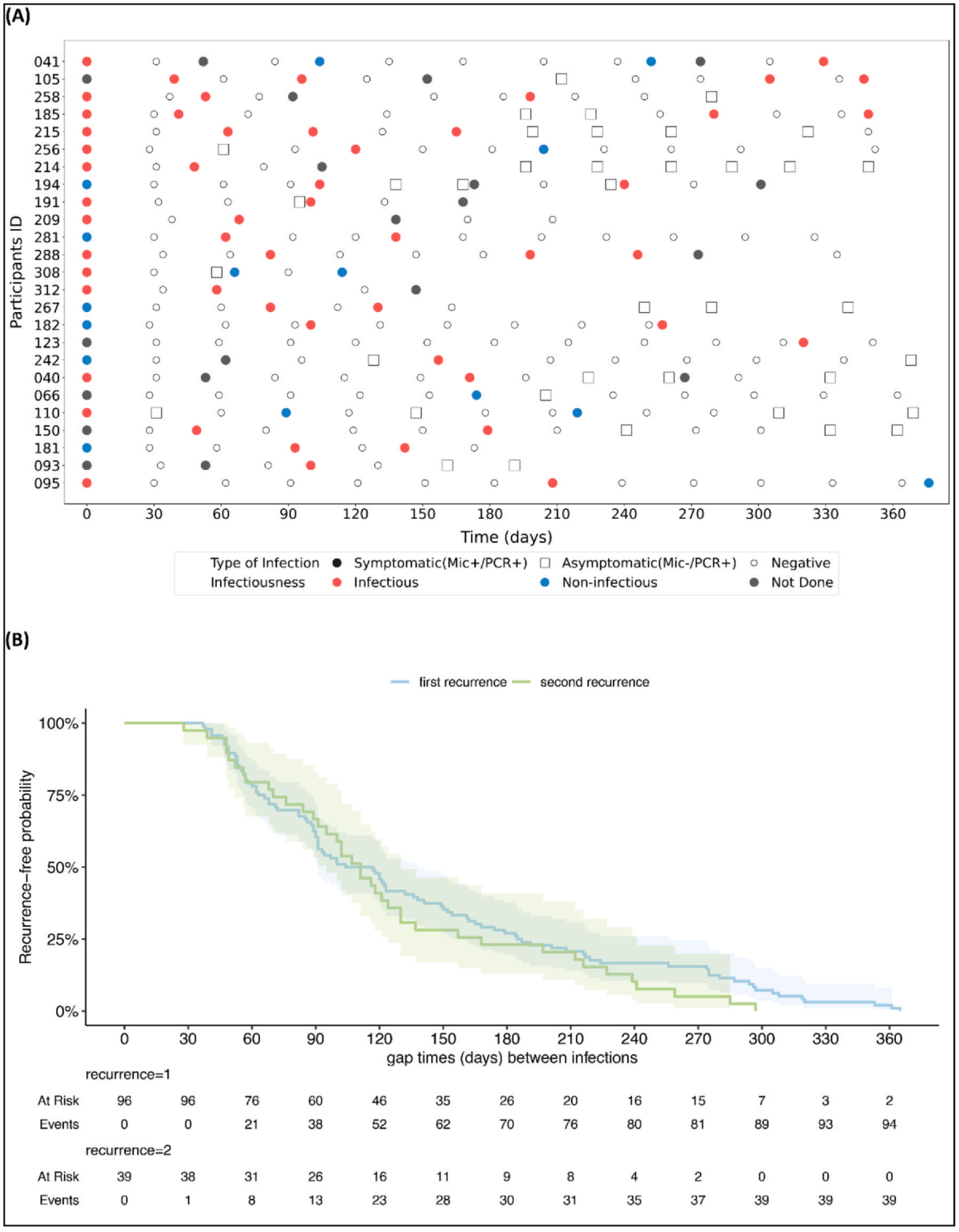

Fig. 1. Individual and recurrence-free survival probability (Km plot) over time. (A) Individual profile plot for patients with at least two symptomatic recurrent infections during the study period. The x-axis shows follow-up time (in days since enrollment; the date of enrollment differs between individuals); the y-axis shows patient id. Patients (n=23) were ordered from top to bottom based on the total number of symptomatic recurrences they experienced with the participant with the highest number of symptomatic recurrences presented at the top of the graph. Symptomatic parasite-positive observations are represented with filled circles; asymptomatic microscopy negative PCR positive infections are represented with empty rectangles. Of the symptomatic parasite-positive observations (filled circles), red filled circles show infectious feedings, blue filled circles show non-infectious feedings, and black filled circles indicate that membrane feeding assays were not performed at that instance, and small open circles show instances samples were negative by both microscopy and PCR. As example, participant 041 was infectious at baseline and experienced 5 episodes of recurrent infection during follow-up, all of which were clinical and of which 1 was infectious, 2 were non-infectious and 2 had no DMFA performed. In addition, this participant had 7 parasite-free timepoints; (B) Recurrence-free survival probability following first and second recurrent infections. Kaplan Meier curves show the time between recurrent infections in days, comparing the recurrence-free probability after the first recurrence (light blue) and second recurrence (light green). Shaded areas represent 95% confidence intervals. The number of individuals at risk and the number of events over time are shown below the plot.

under 15 years (69.4%, 43/62) compared to adults (30.6%, 19/62; $p < 0.0001$), and in participants reporting a history of malaria in the 12 months preceding enrollment (59.7%, 37/62) vs those without prior infection (40.3%, 25/62; p=0.016; Table 1). The median time to first recurrence among individuals with the first recurrent infections was 110 days (95% CI: 91–138), and the median time from first to second recurrence among individuals with the second recurrent infections was 111 days (95% CI: 95–130; Fig. 1B).

In a multivariate model adjusting for sex and age, the likelihood of recurrent infections was not associated with higher parasite density at the preceding infection (Hazard Ratio: 1.55; 95% CI: 0.89–2.69, p=0.1202). However, male participants were more likely to experience recurrent infections than females (HR 1.85; 95% CI: 1.16–2.94, p=0.0095; Table 2; Supplementary Table 1). While self-reported malaria episodes in the 12 months prior to enrollment were associated with recurrent parasitemia in univariate analyses, this association lost statistical significance after adjusting for covariates.

Parasitemia during the clinical malaria episode at recruitment (median 96,185 18S copies/µL; IQR 23,568–321,411; n=191) was similar to that detected during symptomatic recurrent infections (117,472; IQR 46,250–347,024; n=91; $p < 0.001$) but substantially higher than at time-points of asymptomatic parasitemia (82 18S copies/µL; IQR 20–356; n=55; $p < 0.001$; Fig. 2A). Overall, 185 (96.8%) of 191 patients were gametocyte positive at recruitment, as determined by *Pvs25* RT-qPCR. Higher median gametocyte densities were detected in both symptomatic baseline (22,521 transcripts/µL, IQR 2980–130,467; n=185) and symptomatic recurrent infections (10,237 transcripts/µL, IQR 775–71,985; n=88) compared to time-points with asymptomatic parasitemia (237 transcripts/µL, IQR 20–1904; n=30; p=0.0001; Fig. 2C). Median parasite density (18S copies/µL) and gametocyte density (*Pvs25* transcripts/µl) did not appear to change with increasing numbers of recurrent infections (Fig. 2 B and D). Gametocyte density was positively associated with total parasite density at all time-points (ρ=0.53; $p \leq 0.0001$; Fig. 3A).

Mosquito membrane feeding assays were conducted at recruitment for 166 (86.9%) of 191 participants. Additional feedings (n=72) were performed on samples from 50 (53.6%) of 93 individuals who experienced a recurrent symptomatic episode. Most individuals participated in these additional feeding assays during one recurrence (n=50), with some participating during two or more recurrences (n=22). On average 80–100 mosquitoes were exposed per feeding experiment but only median of 30 (IQR: 30–32) randomly picked mosquitoes were dissected and the rest was discarded. Overall, 167 (70.2%) of 238 feedings resulted in at least one infected mosquito, with no significant difference in successful infections between recruitment (70.5%, 117/166) and symptomatic recurrent infections (69.4%, 50/72). At recruitment, the proportion of mosquitoes infected was positively associated with both parasite density (ρ=0.28; $p < 0.0003$; n=166) and gametocyte density by qPCR (ρ=0.22; $p < 0.0053$; n=165). These associations persisted for parasite density for recurrent symptomatic infections (ρ=0.34; $p < 0.0032$; n=72) but were not observed for gametocyte density (ρ=0.051; $p < 0.6762$; n=70). (Fig. 3B and C). After adjusting for gametocyte density, the proportion of infected mosquitoes was higher for individuals above 15 years compared to younger individuals (Odd Ratio [OR]: 1.82, 95% CI: 1.64–2.02, $p < 0.0001$) and lower for individuals with fever at the time of mosquito feeding (OR: 0.80, 95% CI 0.72–0.89; $p < 0.0001$). Similarly, after adjusting for gametocyte density, age, and fever, there was no difference in the proportion of infected mosquitoes between enrollment and recurrent infections (OR:1.04, 95% CI 0.93–1.18; p=0.4840; Fig. 4).

To explore whether recurrent symptomatic infections represented relapses or re-infections, we genotyped 98 symptomatic recurrent infections alongside 58 recruitments infections using PvAmpSeq. These markers exhibited moderate diversity (mean expected heterozygosity [He]=0.63; Fig. 5A). Multiplicity of infection (MOI), calculated using the naïve estimator (maximum number of distinct haplotypes per marker per sample), indicated multiple clones in 96.6% of recruitment samples (56/58; maximum MOI=6) and 93.9% of symptomatic recurrent infections (92/98) with similar MOI estimates (Fig. 5B). IBD analysis comparing recruitment and paired recurrent infections showed that 39.8% (39/98) of samples had low relatedness (IBD < 0.25), 9.2% (9/98) moderate relatedness (IBD 0.25–0.5), 16.3% (16/98) high relatedness (IBD 0.5–0.95), and 34.7% (34/98) were clonal or nearly identical (IBD≥0.95; Fig. 5C and D). For context, recruitment samples from different participants had low relatedness (IBD=0.142), reflecting overall high population diversity.

Using Pv3Rs classification, 91.8% (90/98) of symptomatic recurrent infections were classified as either relapses or reinfections: of which 46.7% (42/90) as relapses and 53.3% (48/90) as reinfections. To assess the robustness of our classifications, we performed sensitivity analyses using different prior assumptions; even including an extreme prior of 70% recrudescent infections did not meaningfully alter infection classifications (Supplementary Figure 5), indicating stability of the results across prior assumptions. These classifications broadly aligned with IBD-based relatedness (Fig. 5C and D; Supplementary Figure 3A–C). We then used these classifications to explore whether our previous estimates of transmissibility may have been different between relapses and reinfections. Among individuals with genotyping data and membrane feeding assays, parasite densities were slightly higher in relapses (median; 271,097 18S copies/µl; IQR 80,855–752,275; n=32) than reinfections (median; 122,238 18S copies/µl; IQR 64,130–352,068; n=36), however the difference was not statistically significant (p=0.2169). A similar trend was observed for gametocyte densities: relapses (median; 23,933 transcripts/µl; IQR 295–127,372; n=32) compared to reinfections (median; 9036 transcripts/µl; IQR 917–66,880; n=36); again, it was not statistically significant (p=0.3472; Supplementary Figure 4).

Mosquito feeding results were available for 75.6% (68/90) of genotyped symptomatic recurrent infections. No significant difference was observed in the proportion of infected mosquitoes between relapses (68.8%, 22/32) and reinfections (80.6%, 29/36; p=0.3995; Supplementary Table 2). After adjusting for age and gametocyte density, relapse infections were associated with a lower proportion of infected mosquitoes compared to reinfections (OR=0.48, 95% CI 0.26–0.87, p=0.016; Supplementary Table 3).

Table 2
Hazard ratio of recurrent infections with and without adjustment for covariates.

	HR (Hazard ratio) (95% CI, *p*-value)	Adjusted hazard ratio (95% CI, *p*-value)
Male sex	1.85 (1.16, 2.94, p=0.0095)	9.65 (2.22,41.97, p=0.0025)
Age above 15 years	0.77 (0.57, 1.02, p=0.0701)	0.86 (0.65, 1.14, p=0.3066)
Parasite density (log10)	1.55 (0.89, 2.69, p=0.1202)	1.22 (0.72, 2.08, p=0.4635)
Self-reported malaria in 3 months pre-enrollment	1.43 (1.08, 1.88, p=0.0111)	.
Self-reported malaria in 12 months pre-enrollment	1.30 (0.99, 1.70, p=0.0563)	.

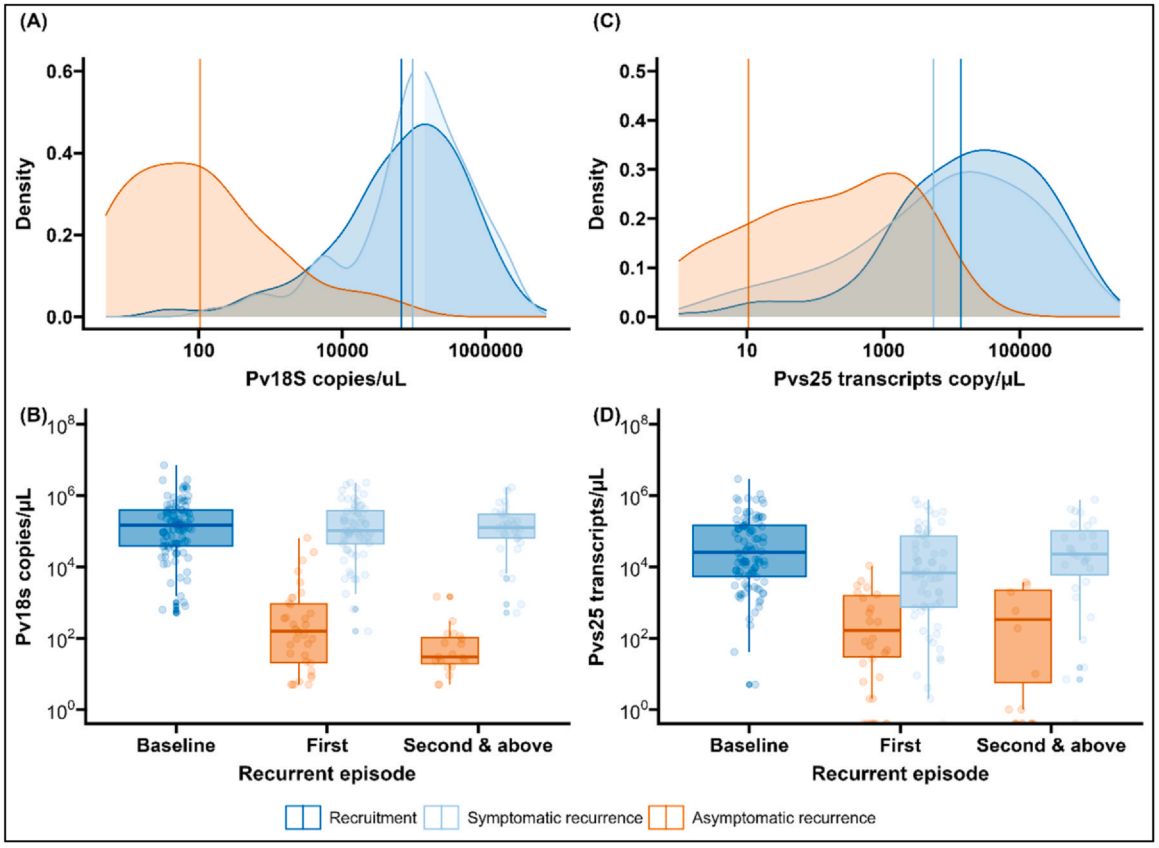

Fig. 2. Distribution of parasite and gametocyte densities at recruitment and at time-points with recurrent infections. The density plot in (A) indicates distribution of total parasite density (Pv18S copies/µL) at recruitment, symptomatic, and asymptomatic recurrent infections. Shown in (B) are boxplots for parasite densities detected at recruitment, symptomatic or asymptomatic recurrent infections. The density distribution in (C) indicates gametocyte density for recruitment, symptomatic, and asymptomatic infections as determined using quantification of gametocyte specific transcript Pvs25 (copies/µL) with boxplots in (D) showing the distribution of gametocyte densities for symptomatic first recurrence (n=59), and second and above recurrence instance (n=26); and asymptomatic first (n=23) and second recurrence instances (n=7). The lines in the density plots (A) and (C) indicate geometric mean and the box plot indicates the interquartile range (IQR), with whiskers extending to 1.5 times the IQR, and the median indicated by a horizontal line.

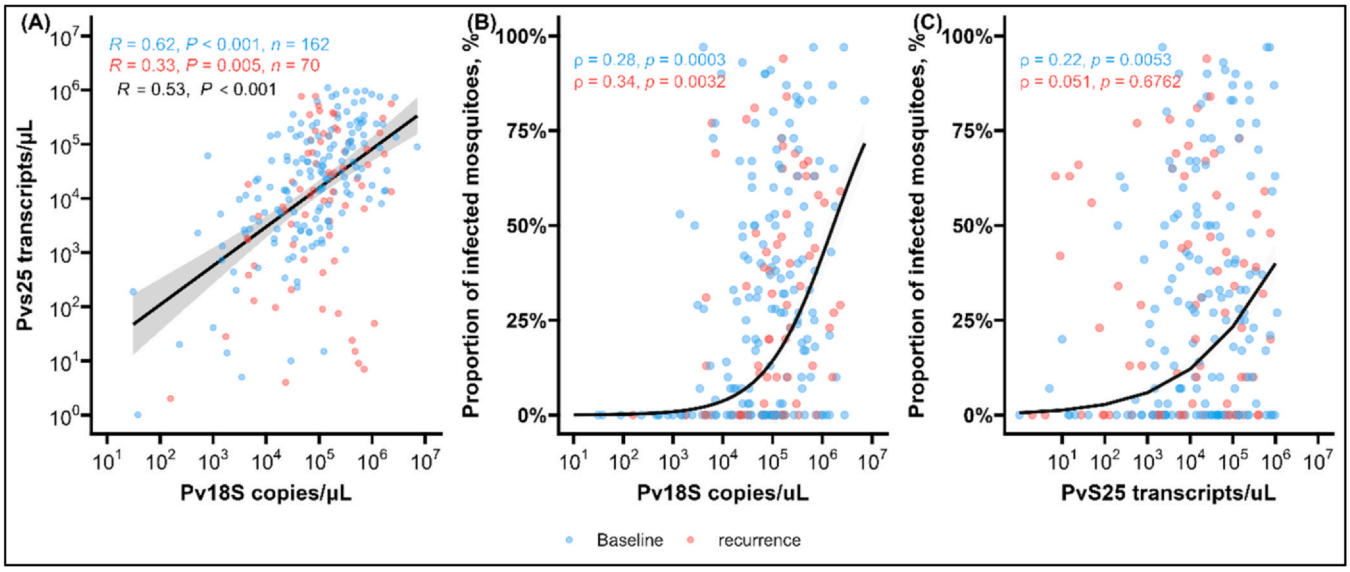

Fig. 3. Association between parasite and gametocyte density, and their correlation with proportion of infected mosquitoes. Indicated in (A) is the association between total parasite density (Pv18s copies/µL, X-axis) and gametocyte density (Pvs25 transcripts copy/µL, Y-axis) is indicated for baseline clinical infections, symptomatic recurrent infections, (B) Proportion of infected mosquitoes versus parasite density for baseline and symptomatic recurrent infections, (C) Proportion of infected mosquitoes versus gametocyte density for baseline and symptomatic recurrent infections.

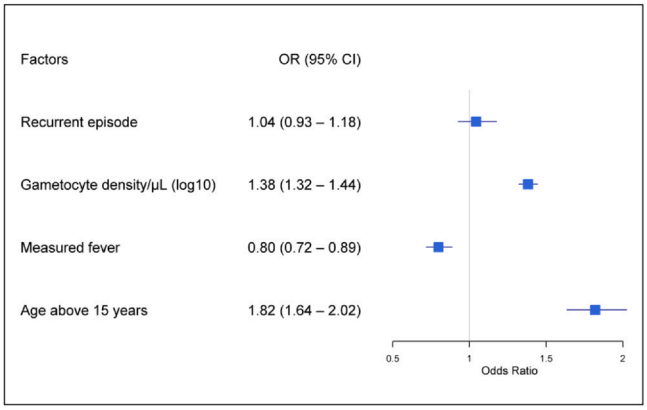

Fig. 4. Forest plot showing adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for predictors of mosquito infection in individuals with *P. vivax*, estimated from a generalized additive model (GAM) with a binomial family. Squares represent point estimates of the ORs, and horizontal lines indicate 95% confidence intervals. The vertical line at OR=1.0 represents the null value. The predictors included are $\log_{10}$-transformed gametocyte density, age category (above 15 years vs. ≤15 years), fever status (presence vs. absence), and infection episode (recurrence vs. baseline).

Discussion

In this longitudinal cohort study, we monitored patients over 12 months following clinical *P. vivax* infection to quantify recurrent parasitemia, gametocyte carriage and transmissibility to mosquitoes. Our findings demonstrate that 35.2% of patients experienced one to four recurrent symptomatic infections with parasite densities, gametocyte densities and infectivity similar to their initial clinical infection. Notably, a large proportion of participants carried parasites asymptomatically for extended periods.

Recurrent parasitemia is a hallmark of *P. vivax* infection, primarily reflecting relapses from dormant liver-stage hypnozoites when radical cure is not provided.[7] In our study, 54.6% of patients experienced at least one episode of recurrent parasite carriage, either symptomatic or asymptomatic, consistent with observations from rural Amazonia[29] and Southeast Asia, where relapse rates without radical cure range from 50–75%. Previous studies have shown that directly observed primaquine treatment reduces relapse rates to < 10%.[30] Interestingly, a substantial number of asymptomatic (and sometimes submicroscopic) recurrences were observed in Vietnam after high-dose of directly observed 10-days primaquine treatment given.[31] The high recurrence rate in our cohort may partly reflect incomplete adherence to the 14-day primaquine regimen, which is known to be suboptimal in routine practice.[32] While poor primaquine metabolism due to cytochrome P-450 2D6 (CY2D6) polymorphisms can cause treatment failure,[33] ultra-rapid metabolizer genotypes are common in the Ethiopian populations linked with duplicated active genes of CYP2D6.[34] We did not determine CYP2D6 genotype or assess metabolizer status phenotypically and thus cannot rule out poor metabolization of primaquine in a subset of our study participants. However, we consider poor adherence to the 14-day primaquine regime, which is known to be common,[35] a more likely explanation for the high rate of recurrent infections.

Interestingly, we observed that male study participants were more likely to experience at least one recurrent infection. This association has been reported before[36] and might be associated with behavioral differences that may influence treatment adherence or practices that increase exposure to malaria-infected mosquitoes. Sex-related differences in infection duration, observed for *P. falciparum*,[28] might also have increased the likelihood of infection detection for males. The current study did not allow us to examine the reason underlying the difference between male and female participants in the incidence of recurrent infections.

A unique focus of our study was the quantification of gametocytes and transmission to mosquitoes over the course of recurrent infections. Gametocyte production appeared variable but not different between clinical infections detected at enrollment and during follow-up. For logistical reasons, we performed mosquito feeding assays at symptomatic episodes only. Having previously quantified the transmissibility of asymptomatic malaria infections in the region,[21,22] we hypothesized that, similar to *P. falciparum,* gametocyte production may vary over the course of infections and that human immune responses acquired in response to antigen exposure during the first infection may influence gametocyte maturation and infectivity.[37] Contrary to these hypotheses, we observed no evidence for lower gametocyte densities during recurrent infections or a reduced gametocyte density relative to the total parasite biomass.

In a subset of recurrent infections, we explored whether recurrent episodes were relapsing infections or a new infection using a panel of 11 genetic markers.[25] Approximately half of the infections (45.7%) were classified likely relapses, based on genetic relatedness to the baseline infection. Whilst one might hypothesize that (strain-specific) immunity could result in lower parasite and gametocyte densities in relapsing infections, our findings did not support this. We observed non-significantly higher parasitemia in relapsing infections compared to reinfections. Furthermore, the proportion of infected mosquitoes was strongly associated with both parasite and gametocyte densities at recruitment. However, at the time of recurrence, the association between mosquito infection rates and gametocyte density was weaker and not statistically significant, suggesting that peripheral gametocyte density alone may not fully capture transmission potential. After adjusting for age and gametocyte density, reinfections were associated with a higher proportion of infected mosquitoes compared to relapses. This indicates that factors beyond gametocyte density may influence infectivity. Notably, naturally acquired antibodies to gametocyte antigens have been associated with reduced transmission of *P. vivax* gametocytes to mosquitoes,[38] and other factors such as gametocyte maturity or genetic diversity, may influence infectivity remains to be determined and will be the subject of a separate manuscript. These findings underscore the complexity of *P. vivax* transmission and highlight the need for detailed and controlled studies to accurately assess the contributions of relapses and reinfections to malaria transmission. Although the liver has long been considered the primary site of *P. vivax* relapse, emerging evidence suggests that most parasite biomass is concentrated in deep erythropoietic tissues, particularly the spleen and bone marrow.[39–41] These reservoirs may sustain chronic infections by shielding parasites from drug action and immune clearance, thereby complicating relapse dynamics and limiting detectability in peripheral blood. This underscores the need for refined models that capture parasite persistence across multiple tissue compartments".

Our study has several limitations. We did not perform mosquito feeding assays at time-points when parasites were detected in the absence of symptoms. Whilst gametocyte densities were markedly lower at these time-points, we can therefore not compare infectivity between symptomatic and asymptomatic infections, especially since fever is associated with lower mosquito infection rates, as previously reported for *P. falciparum*.[42] In addition, we did not directly observe the full 14 days of primaquine with the intent of evaluating parasite dynamics and infectivity under operational conditions. This leaves uncertainty about the effect of treatment adherence on recurrent episodes of parasitemia and it is possible that we mis-attributed the cause of relapses. Future studies should ideally directly observe treatment and may include direct measurements of either drug plasma levels and/or genotyping or phenotyping participants for CYP2D6 metabolizer status. In addition, we only examined mosquito infection rates for clinical malaria cases and used one mosquito species only. Vector species may differ in susceptibility and

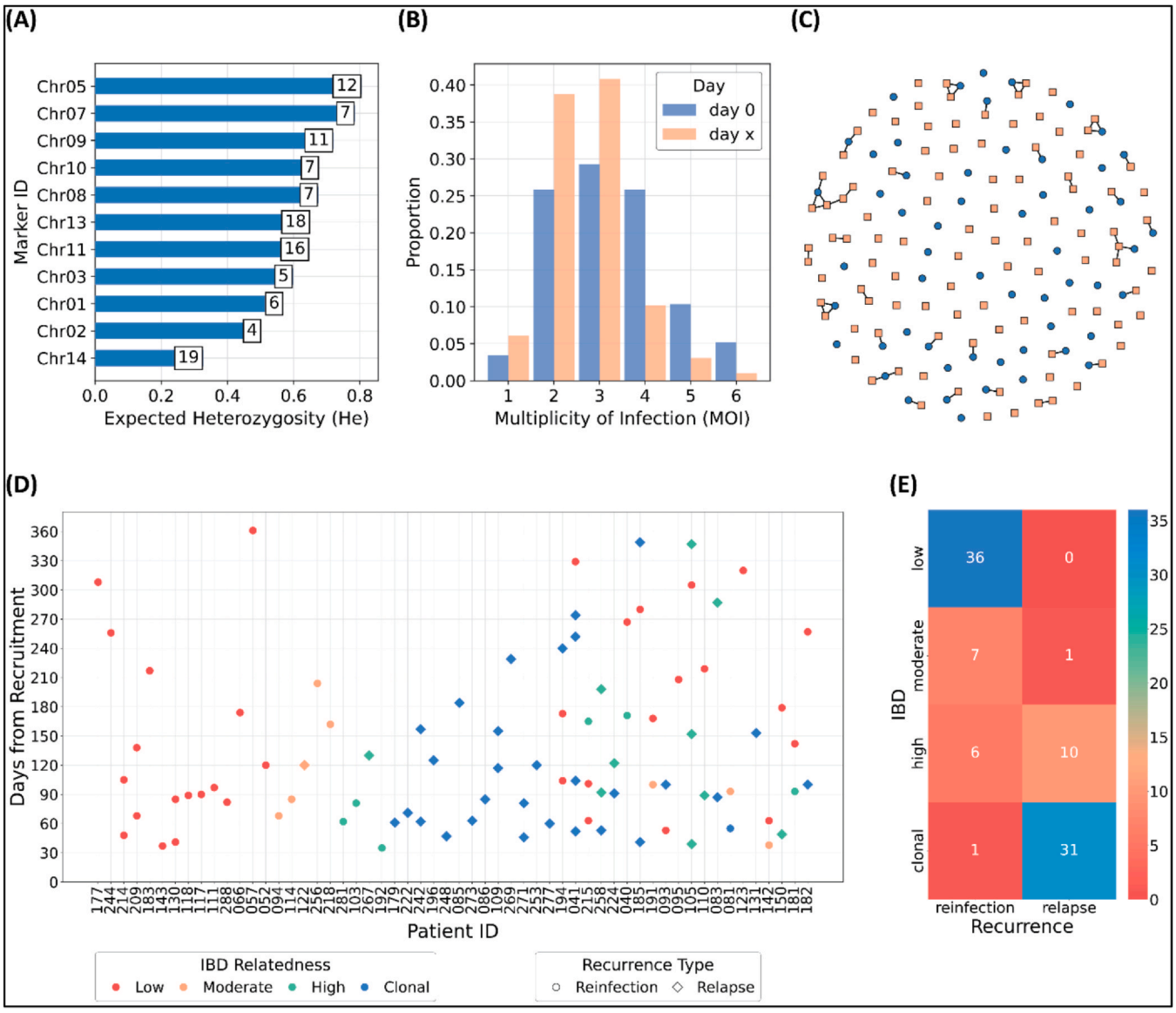

Fig. 5. Population and within-host diversity, and recurrence classifications. (A) illustrates the expected heterozygosity (He) of each marker (Chromosomes), sorted in descending order. The number inside each bar indicates the number of observed microhaplotypes for that marker across 156 samples (58 enrollment samples and 98 symptomatic recurrent infection samples with first (n=56) and second and above (n=42) samples. (B) shows the distribution of multiplicity of infection (MOI) in day 0 (58 samples) and day x (98 samples). Bars represent the proportion of samples for each MOI level for baseline and recurrent infections. For baseline, 2 samples had MOI of 1, 15 samples had MOI 2, 17 samples had MOI 3, 15 samples had MOI 4, 6 samples had MOI 5, and 3 samples had MOI 6. For recurrent infections, 6 samples had 1 MOI, 38 samples had MOI 2, 40 samples had MOI 3, 10 samples had MOI 4, 3 samples had MOI 5, and 1 sample had MOI 6. (C) illustrates IBD-based relatedness, between paired day 0 (orange circles) and day x (blue circles) samples. Nodes are connected by an edge if the samples belong to the same patient and share an identity-by-descent (IBD) value ≥0.95, indicating clonal or highly related parasites. Of the total of 58 patients, 46.6% (27/58) had at least one pair of samples with IBD ≥0.95. These patterns suggest that the recurrent infections are genetically similar parasites, probably due to relapse or recrudescence. (D) shows the IBD patterns of individual patients across follow-up days combined with the Pv3Rs-based recurrence classification results. Each row represents a patient, with columns showing sample time points. Color indicates relatedness categories: low (relatedness < 0.25), moderate (0.25 ≥ relatedness < 0.5), high (0.5 ≥ relatedness < 0.95), Clonal (≥0.95). Patients are grouped by their relatedness category. Shape presents classifications-based Pv3Rs' results comparing symptomatic recurrences to recruitment (day 0 vs. day x). Of the first recurrent instances (n=54), 29 were classified as reinfections, and 25 were classified as relapses. Of the second and above recurrent instances (n=38), 21 were classified as reinfection, and 17 were classified as relapse 17. Patients are grouped by classification outcome: all reinfection, all relapse, or mixed recurrence types (relapse at some timepoints, reinfection at others). (E) shows the comparison between Dcifer-based relatedness classification (y-axis) with PV3Rs-based recurrence classification (x-axis) of recurrent infections into relapse or reinfection. Each cell represents the number of paired recurrence samples (day x vs. day x+1), with color intensity reflecting the count. Higher counts in the cells corresponding to relapse with high IBD, and reinfection with low IBD suggesting good agreement between the two approaches.

transmission efficiency for different parasite strains, which can alter local transmission dynamics. However, in this study we used *A. arabiensis*, the primary vector in the area, while *A. stephensi*, the invasive vector expanding in the country is recently reported in the study area.[19] *A. stephensi* is a competent vector for *P. vivax* and may sustain transmission in urban settings as indicated in our previous works where we compared it with *A. arabiensis*.[43] Our results are most applicable to moderate-transmission settings in Ethiopia, where relapse rates and transmission intensity are balanced. We acknowledge that patterns may differ in low-transmission regions, with lower levels of immunity and less frequent re-infections.

Our findings have potential implications for treatment policy. We find a high rate of recurrent parasitemia that contributes significantly to onward transmission to mosquitoes. In line with current guidelines, asymptomatic *P. vivax* infections were not treated in our study. Our findings highlight the potential role of asymptomatic

carriers and putative recrudescent infections in sustaining transmission. Specifically targeting such infections alone through test and treat approaches is likely to be ineffective compared with those that target the hypnozoite reservoir such as MDA or serological test and treatment[44]; https://www.pvstatem.eu/). Ultimately any approach will need to explicitly address operational feasibility, safety considerations, and cost-effectiveness. Treatment guidelines would then balance these factors alongside the potential public health benefits of reducing clinical burden and transmission.

Funding

The study was funded by a Wellcome Trust Early Career Fellowship to FGT (DRIVAX: 226350/Z/22/Z) and Gates Foundation to FGT, CD, and TB (INDIE OPP1173572 A Wellcome Trust Early Career Fellowship funded the study). TB is further supported by a European Research Council (ERC) Consolidator Grant (ERC-CoG 864180; QUANTUM).

Declaration of Competing Interest

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

Acknowledgements

We would like to thank the study participants who volunteered to participate in the study. We also thank the study team members at field site, Shele Health Center, Arbaminch Zuria district, and malaria program officers at the regional, zonal, and district health bureau who supported the execution of the study.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2026.106756.

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