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Effect of low-dose primaquine treatment on *Plasmodium vivax* recurrence and transmission-blocking activity in southwest Ethiopia: a longitudinal cohort study

Hallelujah Getachew[1,2,3*], Kassahun Habtamu[4,5], Ashenafi Abossie[2,3,6], Assalif Demissew[7], Arega Tsegaye[2,3,8], Teshome Degefa[2,3], Daibin Zhong[9], Xiaoming Wang[9], Guofa Zhou[9], MingChieh Lee[9], James W. Kazura[10], Christopher L. King[10], Delenasaw Yewhalaw[2,3†] and Guiyun Yan[9†]

Abstract

Background Existing malaria control strategies for *Plasmodium vivax* are challenging due to its dormant and relapsing liver stages, as well as the early onset of gametocytogenesis. Primaquine (PQ) effectively eliminates dormant stages and can kill gametocytes; however, it necessitates repeated dosing. In this study, the effectiveness of chloroquine (CQ) plus low-dose of PQ on recurrence and its transmission-blocking activity was evaluated.

Methods Between September 2019 and July 2022, a prospective cohort study was conducted in the Jimma-Arjo and Dabo-Hanna districts of the Oromia region in Ethiopia. A total of 214 uncomplicated cases of *P. vivax* malaria were enrolled in the study. Participants were treated with either CQ alone (106) or CQ + PQ (108), based on whether their district was targeted for *P. vivax* elimination by the national malaria programme or not. After enrolment, participants were followed for clinical illness and parasitaemia on days 28, 42, and monthly for one year. To assess the effect of different treatment regimens on transmission-blocking activity, *Anopheles arabiensis* mosquitoes were used in direct membrane-feeding assays (DMFA) at baseline (pre-treatment) and on day 42 (post-treatment). Based on polymerase chain reaction (PCR) positivity, the time to the first recurrence was estimated using Kaplan–Meier survival analysis. Cox regression models were employed to assess risk factors for recurrence.

Results Of 3,590 individuals screened for malaria, 323 tested positive for *P. vivax*, and 214 were enrolled. Of these, 98.6% (211/214) completed the day 28 follow-up, and 67.3% (144/214) completed the one-year follow-up. Between days 28 and 42, 24% (95% CI 15.8–32.2%) of those individuals receiving CQ alone were PCR positive, and 10.3% (95% CI 4.5–16.0%) in those receiving CQ plus PQ. This represented a 57.3% reduction *P. vivax* recurrence in the CQ + PQ treatment group compared to CQ alone (risk ratio = 0.427, 95% CI 0.222–0.824, $p = 0.008$). During the year of follow-up at least one recurrence occurred in 70% (95% CI 59.1–80.2%) of the CQ alone and 46% (95% CI 35.5–58.1%) in the CQ + PQ treatment group ($p < 0.001$). Treatment regimen, high baseline parasitaemia and presence of gametocytaemia were risk factors for *P. vivax* recurrence. Compared to baseline DMFA at day 42, individuals showed an inhibition

†Delenasaw Yewhalaw and Guiyun Yan are senior authors contributed equally to this work.

*Correspondence:
Hallelujah Getachew
hgetachew4@gmail.com
Full list of author information is available at the end of the article

intensity of 39.0% in the CQ alone versus 77.8% in the CQ + PQ treatment group (p = 0.016), while inhibition prevalence was 35.2% in the CQ alone and 70.1% in the CQ + PQ treatment group (p = 0.021).

Conclusions This study demonstrate that with limited supervision of CQ + PQ treatment significantly lowers the risk of *P. vivax* recurrence in health clinics of southwest Ethiopia compared to CQ alone. Adding PQ to CQ also reduced *P. vivax* transmission to mosquito vectors relative to CQ alone but did not result in a complete transmission-blocking effect by day 42 post-treatment. Therefore, improved health education on treatment adherence and bed net use could enhance the effectiveness of PQ plus CQ. Higher doses of PQ for a shorter duration may be necessary to enhance treatment adherence, reduce recurrence rates, and decrease the risk of transmission.

Keywords *Plasmodium vivax*, Recurrent, Chloroquine, Primaquine, Transmission-blocking activity

Introduction

Vivax malaria has the widest global distribution, with an estimated 6.9 million cases by the year 2022 [1]. It has been overlooked in sub-Saharan Africa due to a relatively high burden of *Plasmodium falciparum* in most countries. In 2022, approximately 34.5% of global vivax malaria cases were attributed to Ethiopia, which accounted for 14% of the total cases [1]. In Ethiopia, *P. falciparum* and *Plasmodium vivax* parasites coexist. The proportion of *P. vivax* parasite reaches up to 40% [2].

Plasmodium vivax parasite has several distinct biological features, such as the formation of hypnozoites that persist in the liver and cause recurrences after the clearance of the acute blood-stage infection and early onset of gametocytogenesis before clinical symptoms occurs. These characteristics enhance its transmissibility to mosquito vectors and complicate *P. vivax* control strategies [3]. The recurrence of *P. vivax* can be due to treatment failure, reinfection, or reactivation of hypnozoites [4]. Differentiating between them has not been easy. The risk of relapse of *P. vivax* malaria without hypnozoitocidal drugs has been shown to contribute to up to 80% of all *P. vivax* blood-stage infections [5]. Primaquine (PQ) is the preferred hypnozoitocidal drug to eradicate the dormant liver stage of vivax malaria and is vital for the control and elimination of *P. vivax*.

The efficacy of PQ depends on the dose and quality of the drug as well as on the number of previously exposed activatable hypnozoites in the liver, the degree of immunity, and cytochrome P450 genetic polymorphisms [6]. The World Health Organization (WHO) recommends a 14-day course of PQ treatment (0.25–0.5 mg/kg/day) to eradicate the liver stage of the parasite and prevent relapse of the disease [7]. A high dose of PQ (0.5 mg/kg/day) is recommended for tropical Chesson strain, frequently relapsing *P. vivax* strains prevalent in East Asia and Oceania, while a lower dose (0.25 mg/kg/day) is recommended for temperate strains [7]. The Ethiopian Ministry of Health also recommended a standard treatment regimen as follows: CQ 10 mg base/kg on days 0 and 1, and 5 mg base/kg on day 2; PQ 0.25 mg/kg daily dose over 14 days starting on day 2 [2]. The major obstacle in PQ treatment is it toxicity in glucose- 6-phosphate dehydrogenase (G6PD) deficient patients and its poor adherence. The prolonged dosing reduced PQ toxicity associated with the administration of higher doses for shorter periods of time. If G6PD status is unknown or G6PD testing is not available, a decision to prescribe PQ must be based on risk benefit analysis of adding PQ as a treatment regimen [7]. In Ethiopia, PQ is rolled out without G6PD testing. In *P. vivax* endemic countries, except for Indonesia and Papua New Guinea, where CQ resistance has emerged [8], CQ remains the first-line treatment. In Ethiopia, CQ is still being used as a first-line drug for the treatment of vivax malaria [2] despite studies that documented reduced CQ efficacy [9, 10].

An effective anti-malarial treatment should prevent the human to mosquito and from mosquitos back to human transmission [11]. This includes CQ, a rapid schizontocidal drug that fails to kill gametocytes or hypnozoites. Primaquine is active against both parasite stages by generating effective toxic radicals against these non-replicating forms [12]. The effectiveness of transmission-blocking interventions, including PQ, can be evaluated in mosquito-feeding assays by detecting either oocysts within mosquito midgut or sporozoites in the salivary glands by microscopy [13]. This study aimed to assess the effect of low-dose primaquine treatment on *P. vivax* recurrence and transmission-blocking activity in health facilities in southwest Ethiopia.

Methods

Study design

A prospective cohort study was conducted from September 2019 to July 2022 to evaluate the effectiveness of unsupervised low-dose CQ + PQ treatment in preventing the recurrence of *P. vivax* and its transmission-blocking activity. Study participants who provided consent were followed for one year. The eligibility criteria included: a microscopic confirmation of uncomplicated *P. vivax* mono-infection, being older than one year, no signs of

severe malaria, being non-pregnant and non-lactating, and a willingness to comply with the study protocol.

Study site

This study was carried out in Arjo-Didessa Sugar factory clinic, Abote Didessa Health Post, Command 2 Health Post, Command 5 Health Post, Hunde Gudina Health Post, Kerka Health Post, and Sefera Tabiya Health Post selected health facilities of Jimma-Arjo and Dabo-Hanna districts of Oromia Region, located in southwest Ethiopia (8°36′0" N, 36°24′0" E). A detailed description of the study site has been published elsewhere [14, 15]. Malaria transmission in the study site is largely seasonal and unstable, with peaks from September to December and from April to June. *P. vivax* accounted for 42.4% of health facilities visits for malaria in the past 10 years (2008–2017) [14]. However, *P. vivax* was predominant over *P. falciparum* for three consecutive years (2014, 2015, and 2016). According to a community based repeated cross-sectional study in the area, the prevalence of malaria was 2% [15]. The Ethiopian Ministry of Health launched malaria elimination efforts in 2018 in 239 districts within five regions, including Oromia. In *P. vivax* elimination-targeted districts, patients received CQ with a 14-day course of low-dose (0.25 mg/kg daily) PQ [16]. The rest of the non-elimination districts were not targeted for eliminating received CQ alone treatment. Four health facilities from the Jimma-Arjo district that provided CQ + PQ and three health facilities from the Dabo-Hanna district that provided CQ alone were included in this study. These health facilities were selected based on whether they were within the elimination targeted districts or in the non-elimination targeted district, and proximity to the study research centre (Arjo-Didessa International Center for Malaria Research, ICEMR) with facilities for membrane feeding.

Sample size calculation

In this study, the sample size was initially calculated to detect a 20% difference in treatment efficacy rate between the CQ alone and CQ + PQ treatment group with 80% power and a 5% significance level. The expected proportion of treatment efficacy was 65% for CQ alone and 85% for CQ + PQ treatment group at day 28 [17]. Thus, the sample size was 83 study participants in each treatment group. By including 20% of the calculated sample size to compensate for an expected loss to follow-up and withdrawal, the sample size was estimated to require 100 individuals in each of the two study treatment groups.

Study procedures

For *P. vivax,* the standard regimen was CQ (Candela Pharmaceuticals (Ethiopia) PLC) 10 mg base/kg on days 0 and 1, and 5 mg base/kg on day 2; PQ (Remedica Limited, Limassol Industrial Estate, Cyprus) 0.25 mg/kg daily dose over 14 days commencing on day 2. It was prescribed according to national guidelines [2] (Supplementary Table S1). The participants were given the anti-malarial treatment and instructed on how to take the medications and potential side effects. To track adherence, the number of PQ tablets left on the 3rd, 7 th, and 13 th day were recorded by health facilities personnel. Since there was no direct observation of the participants taking the medication, the administration was classified as limited supervision. The existing procedures established by the Ethiopian Ministry of Health were adopted to best emulate actual drug administration in health facilities.

During enrolment, trained data collectors administered a questionnaire to consenting study participants and collected capillary blood samples for blood film preparation, and dry blood spot (DBS) preparation on filter paper for polymerase chain reaction (PCR) analysis. Venous blood was also collected at baseline (pre-treatment) from each participant involved in the transmission-blocking activities of the infection experiment. Participants were asked to return to the clinic for scheduled visits and to report any symptoms consistent with malaria. If patients failed to attend their scheduled visits, the research team, along with community leaders and health extension workers, traced them to their homes using their addresses. During follow-up visits, capillary blood was collected for blood film preparation, rapid diagnostic tests (RDT), and DBS preparation on days 28 and 42 and monthly thereafter until one year. Venous blood was collected from randomly selected study participants for the infection experiment on day 42 (post-treatment). At each appointment, participants'axillary body temperature and other malaria symptoms were assessed.

The pregnancy test was done on all reproductive-age female study participants because PQ is contraindicated in pregnancy. All follow-up patients were tested for parasites by CareStart™ Malaria (PfHRP2/PvLDH) Ag Combo RDT (Access Bio Ethiopia, INC.), and all slides were examined by two independent microscopists. Blood smears were confirmed by nested PCR [18]. Recurrent infection was confirmed by quantitative PCR [19]. According to the established protocol, DMFA was conducted using a Hemotech membrane-feeding apparatus (PS- 6 System, Discovery Workshops, Accrington, UK) [20].

Recurrence was a positive thick blood smear or PCR for *P. vivax* between days 29 and 360, with or without clinical symptoms [4]. In all recurrences, participants were treated according to the national treatment guidelines and continued the follow-up as scheduled until day 360. Oocyst intensity was defined as the average number

of visible oocysts in all mosquitoes fed on the same blood source, and oocyst prevalence was defined as the proportion of mosquitoes harbouring oocyst [21].

Nested PCR

A modified nested PCR amplification was performed as previously published protocol based on 18S rRNA gene [18, 22]. The genus-specific (nested- 1) PCR was carried out in 25 µL reaction mixture with 5 µL of genomic DNA and with *Plasmodium* genus specific primers rPLU5 and rPLU6. For the species-specific (nested- 2) PCR was also carried out in 25 µL reaction volume with 2 µL of amplicon product from nested- 1 and *P. vivax* specific primers rVIV1 and rVIV2. PCR condition for nested- 1 included an initial denaturing step at 95 °C for 10 min and 35 cycles of 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1.5 min, and a final extension for 10 min. The nested- 2 used similar condition with nested- 1 except which used 30 cycles for the reaction. Sequences of primers were shown in Supplementary Table S2 A.

Multiplex qPCR

The amplification reaction of genomic DNA of each sample was carried out in 12 µL qPCR mixture, which included 2 µL DNA sample, 6 µL of PerfeCTa (2X), and 0.4 µL each of forward and reverse primers specific to *P. vivax* and *P. falciparum* (primers Pv- 1, Pv- 2, F-F and F-R) by targeting the 18S rRNA genes. Additionally, the reaction contained 0.5 µL each of Pv-vic and Pf-fam, *P. vivax* and *P. falciparum* TaqMan probe, in a final volume made up to 12 µL with double-distilled water [19, 23]. To perform this procedure a Quant Studio 3 Real-Time PCR system from Applied Biosystems was used, with an initial hold stage at 50 °C for 2 min and 95 °C for 2 min, followed by 45 cycles of qPCR amplification stage at 95 °C for 3 s and 60 °C for 30 s. (See the sequences of primers and probes in Supplementary Table S2 B).

Membrane feeding assay

An insectary for the *Anopheles arabiensis* mosquito colony was established at the Arjo-Didessa ICEMR in 2019. The adult *An. arabiensis* mosquitoes were sourced from the Tropical and Infectious Disease Research Center (TIDRC) at Jimma University in Sekoru, Ethiopia [24]. In this study, randomly selected consenting participants infected with *P. vivax* from seven selected health facilities were recruited for an experimental infection study at Arjo-Didessa ICEMR. A 6 mL venous blood sample was collected from these participants using lithium heparin tubes (Vacutainer®; BD, New Jersey, USA) before they received any antimalarial treatment (baseline) and again after treatment with CQ alone and CQ + PQ on day 42. Direct membrane feeding assays (DMFA) were conducted using a Hemotech membrane-feeding apparatus (PS-6 System, Discovery Workshops, Accrington, UK) by trained laboratory technicians. Prior to the membrane feeding experiments, three to 5-day-old adult female *An. arabiensis* mosquitoes were prepared by placing them in small paper cups covered with mesh (30 mosquitoes per cup) and starving them for 9–12 h. The heparinized venous blood samples were prepared for the experiment immediately after collection or within 2–4 h by placing the samples in a water bath maintained at 37 °C [25]. Then, the Hemotek blood reservoir unit was enclosed with a parafilm membrane, and a 2 mL heparinized venous blood sample was transferred into it. Finally, the Hemotek blood reservoir and the control arm are connected to the power supply by placing the Hemotek blood reservoir on the top of the paper cup containing the mosquitoes. The feeding took place in a dark room for 25 min with the feeder temperature maintained at 37 °C. Unfed and partially fed mosquitoes were removed from the paper cup, leaving fully fed mosquitoes undisturbed. Fully fed mosquitoes were maintained for 8 days in a temperature and humidity-controlled room using a 10% sucrose solution. Mosquitoes were dissected, and the presence of oocyst was examined microscopically after staining with 1.0% mercurochrome according to established protocol [20]. The feeding cups with mosquitos were performed in triplicate for each blood sample.

Outcome and predictor variables

The study's primary outcomes included the recurrence of *P. vivax* malaria after anti-malarial treatment as measured by qPCR between 28 and 42 days after enrolment and throughout a one-year follow-up. Another primary outcome was the impact on transmission to mosquitoes, assessed by transmission-blocking or the reduction in the number of oocysts at day 42 following treatment compared to pre-treatment levels. Secondary outcomes identified potential risk factors associated with the recurrence of *P. vivax* malaria, such as types of anti-malarial treatment, age, sex, occupation, education level, baseline parasitaemia, presence of gametocytes at baseline, *P. falciparum* infection during the follow-up period, insecticide-treated net (ITN) ownership, indoor residual spraying (IRS), duration of residence in the area, and season of enrolment. In this study, occupation was categorized into outdoor and indoor activities. Outdoor activities included factory workers, drivers, construction workers, agricultural workers, herdsmen, and security guards, while indoor activities comprised office workers, housewives, students, and non-school-aged children.

Data analysis

Data was analysed using STATA (version 17) and GraphPad Prism (version 9.5.1). The cumulative incidence of the first recurrence was calculated using Kaplan–Meier survival analysis. The association of potential risk factors with first recurrence and multiple events per participant was examined using a Cox proportional hazards model (Cox PH) and the Cox extension proposed by Prentice, William, and Peterson's total time model (PWP-TT), respectively [26, 27] (Supplementary Table S3). Sensitivity analysis was performed by comparing the distribution of demographic characteristics and *P. vivax* recurrence predictors between study participants who completed the follow-up visits (complete-cases) and all study participants who had at least one follow-up visit (Supplementary Table S4).

The proportion of mosquitoes infected using DMFA at day 42 was compared with the baseline using Fisher's exact test. Similarly, comparisons of oocyst intensity at baseline compared to day 42 were carried out using the Kruskal–Wallis test and Dunn's multiple comparisons test. The inhibition parameters are the percentage reduction (relative to baseline) in the number of oocysts per mosquito and mosquito infection rate at the given time points. Inhibition prevalence is the transmission-blocking activity estimator, while inhibition intensity is the transmission-reducing activity estimator [11].

Ethical clearance

Ethical clearance was obtained from the National Research Ethics Review Committee (NRERC) with reference number 3.10/131/2018. Additionally, the local health authorities, specifically the Jimma-Arjo District Health Office (Ref. No. 0178/JA/2019) and the Dabo-Hanna District Health Office (Ref. No. WF/662/19) granted permission for the study. Each participant provided written informed consent or assent after receiving a detailed explanation of the study's objectives and follow-up procedures, as well as their right to withdraw from participation at any time without penalty. For minors under 12 years old, parents or guardians signed the informed consent; while individuals aged 12 to 17 were asked to provide their individual assent.

Results

Baseline characteristics

A total 3590 patients were screened for malaria in the selected seven health facilities during the study period. Of these 323 *P. vivax* symptomatic cases were eligible and 214 uncomplicated *P. vivax* mono-infected patients were enrolled in this study. Of these, 106 received CQ alone and 108 received CQ + PQ. Of the 214 enrolled patients, 98.6% completed day 28 follow-up schedule (CQ alone: n = 104; CQ + PQ: n = 107), and 67.3% completed follow-up for an entire year (CQ alone: n = 70; CQ + PQ: n = 74) (Fig. 1). However, comparing the distribution of demographic characteristics and other predictor variables between study participants completed the follow-up (complete-case) and all study participant, found the difference was minimum (similar pattern) (Supplementary Table S4). Age, sex, occupation, education, ITN ownership, duration in the study area, and baseline parasitaemia and gametocytaemia were equivalent between treatment groups (CQ alone: 3198 parasite/μL; CQ + PQ: 3648 parasite/μL, $p = 0.988$), or between the proportion of infections with gametocytes (CQ alone: 72.6%; CQ + PQ: 75.0%, $p = 0.6947$) (Table 1).

Treatment efficacy

Between days 28 and 42, 25 of 104 (24.0%, 95% CI 15.8–32.2%) recurrent infections were detected among those treated with CQ alone and 11 of 107 (10.3%, 95% CI 4.5–16.0%) among participants receiving CQ + PQ. Thus CQ + PQ treatment reduced the risk of recurrence by 57.3% compared to CQ alone (risk ratio = 0.427, 95% CI 0.222–0.824, $p = 0.008$).

Concerning one-year follow-up, at least one *P. vivax* recurrence was observed in 57.6% (83/144) of all study participants who completed 1-year follow-up by qPCR (Fig. 2). Those receiving CQ alone 49 of 70 (70.0%, 95% CI 59.1–80.2%) had a recurrent *P. vivax* infection, and 34 of 74 (46.1%, 95% CI 35.5–58.1%) in those treated with CQ + PQ ($p < 0.001$). Treatment with the CQ + PQ reduced the risk of recurrence by 34.4% (risk ratio = 0.656 (95% CI 0.491–0.878, $p = 0.0035$) compared to CQ alone. Notable was the marked difference in treatment groups risk within the first 60 days of follow-up (Fig. 2). In this study, it was observed that most of the *P. vivax* recurrence occurred between 29 to 180 days: 83.7% (41/49) and 85.3% (29/34) in CQ alone and CQ + PQ treatment groups, respectively. *Plasmodium vivax* asymptomatic infections were detected in 91.7% of CQ alone and 75.5% of CQ + PQ treatment groups, $p = 0.022$ by qPCR (Table 2). During the follow-up period, 22 participants were infected with *P. falciparum,* and three had mixed infections (*P. vivax* and *P. falciparum*).

Risk factors of *P. vivax* recurrences

In the univariable analysis of the Cox proportional hazards (PH) model (first recurrence) and the PWP-TT model (all recurrent events), several potential risk factors were identified. These included treatment group, occupation, level of education, ownership of insecticide-treated nets (ITNs), indoor residual spraying (IRS), and duration of stay in the area. Additionally, the presence

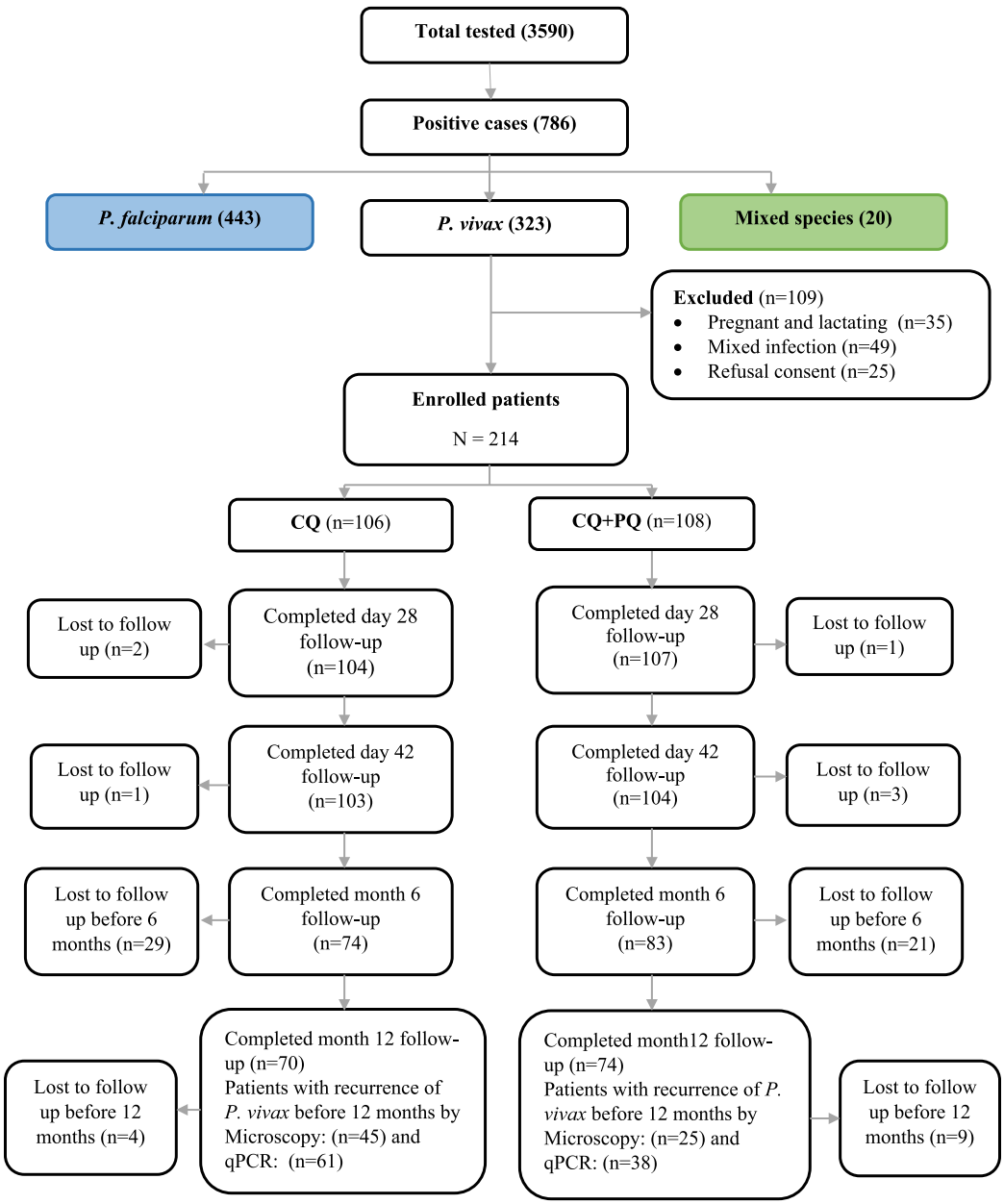

Fig. 1 Flow chart of *Plasmodium vivax* patient screening and recruitment for CQ alone and CQ + PQ treatment groups, in Arjo-Didessa sugar development site and its surrounding, southwest Ethiopia

of gametocytes at baseline and age were identified as risk factors in the PWP-TT model (see Supplementary Table S5).

After adjusting for potential confounders (occupation, IRS, and duration of stay), treatment, baseline level of parasitaemia, level of education, and ITN ownership remained significant predictors in the multivariable Cox PH model analysis of first recurrence. The risk of recurrence in the CQ + PQ treatment group was 52% lower than in the CQ alone (AHR = 0.48; 95% CI 0.30–0.77, p = 0.002). Individuals with parasitaemia levels between 1001 and 5000 or > 5000 parasite/μL had a two-fold increased risk of recurrence compared to those with < 1000 parasite/μL (AHR = 2.13; 95% CI 1.00–4.54, p = 0.049) and (AHR = 2.09; 95% CI 1.05–4.16, p = 0.036), respectively. Similarly, individuals who did not own ITNs were 3.3 times more at risk of *P. vivax* recurrence than those who owned ITNs (AHR: 3.3; 95% CI 1.71–6.44, p < 0.001).

Table 1 Baseline characteristics of the 214 patients enrolled in the cohort study of Arjo-Didessa sugar development site and its surrounding, southwest Ethiopia (2019–2022)

Variable	CQ		CQ + PQ	
	N	%	N	%
Subject enrolled	106		108	
Age (in years)				
Median	18		20	
Mean ± SD	20.5 ± 12.9		22.0 ± 12.6	
< 5	14	13.2	5	4.6
5–15	25	23.9	33	30.5
> 15	67	63.2	70	64.8
Sex				
Female	45	42.4	34	31.9
Male	61	57.5	74	68.5
Occupation				
Indoor activity	56	52.8	48	44.4
Outdoor activity	50	47.2	60	55.6
Level of education				
≥Secondary	26	24.5	39	36.1
Primary	42	39.6	38	35.2
Never attended school	38	35.8	31	28.7
ITN ownership(at list one ITN)				
Yes	55	51.9	62	57.4
No	51	48.1	46	42.6
IRS in past 12 months				
Yes	31	29.2	41	37.9
No	75	70.7	67	62.0
Duration of stay in the area				
> 3 years	40	37.7	51	47.2
1–3 years	31	29.2	31	28.7
< 1 year	35	33.0	26	24.1
Baseline haemoglobin level (g/dL)				
Mean ± SD	11.6 ± 1.8		12.3 ± 1.7	
Baseline parasite density/μL				
< 1000	22	20.7	16	14.8
1001–5000	29	27.3	32	29.6
> 5000	55	51.9	60	55.5
Infections with gametocytes (baseline)	77	72.6	81	75.0
Asexual parasites/μL (geometric mean) 95%CI	3198 [2405–4254]		3648 [2769–4808]	
Gametocytes/μL (geometric mean) 95%CI	366 [281–477]		441 [337–576]	

Moreover, individuals who never attended school and those with only a primary education were at 3.5 and 2.9 times increased risk of recurrent infection compared to individuals with more than a secondary education level (AHR: 3.5; 95% CI 1.6–7.67, $p < 0.001$) and (AHR: 2.98; 95% CI 1.39–6.41, $p < 0.001$), respectively (Table 3).

With adjustment of the above risk factors in the PWP-TT model, those treated with CQ + PQ had a 45% lower risk of *P. vivax* recurrence than those treated with CQ alone (AHR = 0.55; 95% CI 0.39–0.79, $p < 0.001$). However, individuals with parasitaemia levels between 1001 and 5000 parasite/μL had a 1.9-fold increased risk of recurrence compared to the parasitaemia level < 1000 parasite/μL (AHR = 1.91; 95% CI 1.14–3.21, $p = 0.014$). In addition, individuals with gametocytaemia on enrolment (baseline) were at a 61% higher risk of *P. vivax* recurrence than those without gametocytaemia (AHR = 1.61; 95% CI 1.01–2.56, $p = 0.045$). Similarly, study participants who

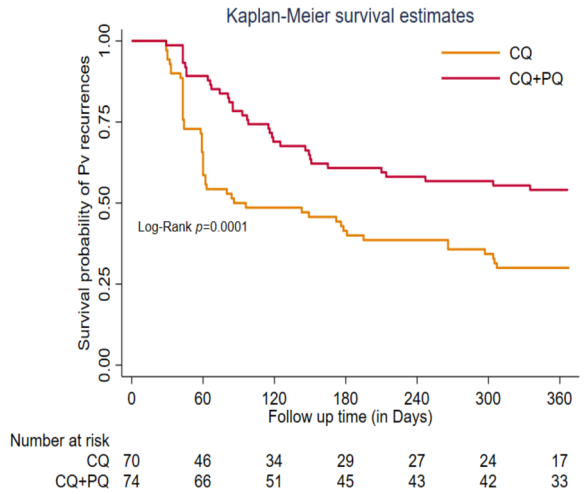

Fig. 2 The overall Kaplan–Meier Survival estimate between CQ alone and CQ + PQ treatment groups for complete-cases

Table 2 *Plasmodium vivax* recurrence in CQ alone and CQ + PQ treatment groups in Arjo-Didessa sugar development site and its surrounding, southwest Ethiopia (2019–2022)

Recurrences	CQ		CQ + PQ		*p*-value
	n	%	n	%	
Patients with no recurrence	21	30.0	40	54.0	0.0035
Patients with recurrence:	49	70.0	34	45.9	
1 recurrence only	14	28.6	11	33.3	0.8209
2 recurrence	12	24.5	10	30.3	
3 recurrence	15	30.6	8	24.2	
> 4 recurrence	8	16.3	4	12.1	
Time intervals from enrolment to first recurrence:					
29–180 days	41	83.7	29	85.3	0.8417
> 180 days	8	16.3	5	14.7	
Number of recurrences/individual (Median [IQR]; max)	(2 [1–3]; 5)		(2 [1–3]; 8)		
Median time from enrolment to first recurrence (days), [IQR]	60[29–146]		95[29–149.2]		
All vivax malaria by diagnostic methods					
Microscopy and PCR	89	74.2	47	61.0	
qPCR	31	25.8	30	38.9	0.0518
All qPCR detected recurrence by symptom					
Symptomatic	10	8.3	16	20.5	
Asymptomatic	110	91.7	62	79.5	0.0218

did not own ITN and those who never attended school or primary education were significant predictors of recurrence of *P. vivax* infection. However, individuals with outdoor activities had a 40% lower risk of vivax malaria recurrence than those with indoor activities (Table 3). To assess whether loss of participants prior to completing the one-year follow-up might have biased the primary outcome of *P. vivax* recurrence, the Cox proportional hazard analysis was performed on all enrolled individuals with at least one follow-up visit (Supplementary Table S6). The adjusted hazard ratio (AHR = 0.51; 95%CI 0.34–0.77) was similar to that of those participants that completed the one year follow-up (AHR = 0.48; 95%CI 0.30–0.77) (Table 3).

Transmission-blocking activity of PQ

For membrane feeding assays, 54 randomly selected study participants were included; 26 from the CQ alone and 28 from the CQ + PQ treatment group. Seven (7) from the CQ alone and eight from the CQ + PQ treatment groups were excluded due to incomplete PQ treatment, change of residence, or the COVID- 19 pandemic lockdown. Therefore, 19 (CQ alone) and 20 (CQ + PQ) paired assay were included in the analysis for the baseline and for day 42. Most study participants were adults above 15 years of age (78.9% vs 70.0%) and males (73.7% vs 70%) in CQ alone and CQ + PQ treatment group, respectively (Table 4). The geometric mean of asexual parasitaemia at the baseline was 3366 parasite/μL in CQ alone (Fig. 3A) and 4710 parasite/μL in CQ + PQ treatment groups (Fig. 3B). Geometric mean gametocytaemia at baseline was 544 gametocyte/μL in CQ alone (Fig. 3C) and 415 gametocyte/μL in those treated with CQ + PQ (Fig. 3D). At the baseline, all study participants had gametocytaemia and they were infectious to at least one mosquito. On day 42, those treated with CQ alone, 7/19 (36.8%), had detectable gametocytes, and the CQ + PQ treated group, 5/20 (25%), had gametocytes (Table 4). Notable was 73.6% in CQ alone, and 50.0% of CQ + PQ treated participants were infectious to mosquitoes, indicating participants without detectable gametocytes were also infectious to mosquitoes (Fig. 4A). The mean gametocytaemia declined between baseline and day 42 in both treatment groups (CQ alone and CQ + PQ) (Fig. 3C, D). Mean asexual parasitaemia declined in the CQ + PQ treatment group, but not in the CQ alone at the baseline and day 42 (Fig. 3A, B).

From the total dissected mosquitoes, oocyst infections were observed in 50.1% of the mosquitoes that fed on the blood of CQ alone treated individuals and 43.5% of CQ + PQ treated group at the baseline. While, at day 42 the oocyst infection rate was 36.5% and 21.5% in CQ alone and CQ + PQ treated group, respectively. The Kruskal Wallis test showed that the mean oocyst per mosquito midgut was significantly decreased from the baseline to day 42 in CQ + PQ treated group (43.6 vs 5.3, $p < 0.0001$)

Table 3 Multivariable risk factor analysis for first recurrence using Cox PH model and for all recurrences using PWP-TT model (n = 144)

Variable	First recurrence (Cox PH model)			All recurrence (PWP-TT Model)		
	n/N	Risk ratio	AHR [95% CI]	n/N	Risk ratio	AHR [95% CI]
Overall	83/144	0.57		198/336	0.59	
Treatment group						
CQ alone	49/70	0.70	1.00	120/187	0.64	1.00
CQ + PQ	34/74	0.46	0.48 [0.30–0.77]***	78/149	0.52	0.55 [0.39–0.79]***
Age (in year)						
< 5	10/14	0.71	0.44 [0.17–1.10]	23/36	0.64	0.90 [0.42–1.96]
5–15	29/45	0.64	0.72 [0.40–1.32]	79/122	0.65	0.72 [0.47–1.10]
> 15	44/85	0.52	1.00	96/178	0.54	1.00
Occupation						
Indoor activity	50/76	0.66	1.00	132/204	0.65	1.00
Outdoor activity	33/68	0.48	0.65 [0.35–1.20]	66/132	0.50	0.60 [0.39–0.82]***
Level of education						
> Secondary	14/40	0.35	1.00	27/66	0.41	1.00
Primary	30/50	0.60	2.98 [1.39–6.41]***	71/119	0.59	1.95 [1.03–2.67]*
Never attended school	39/54	0.72	3.50 [1.60–7.67]***	100/151	0.66	1.85 [1.03–3.32]*
Baseline parasite density/μL						
< 1000	11/25	0.44	1.00	27/51	0.53	1.00
1001–5000	26/44	0.59	2.13 [1.00–4.54]*	69/112	0.61	1.91 [1.14–3.21]**
> 5000	46/75	0.61	2.09 [1.05–4.16]*	102/173	0.59	1.52 [0.92–2.54]
Baseline gametocyte						
Absent	17/34	0.50	1.00	35/67	0.52	1.00
Present	66/110	0.60	1.79 [0.95–3.37]	163/269	0.60	1.61[1.01–2.56]*
ITN ownership(at list one ITN)						
Yes	24/64	0.37	1.00	53/116	0.45	1.00
No	59/80	0.74	3.32 [1.71–6.44]***	145/220	0.66	1.93 [1.14–3.25]**
IRS sprayed the past 12 months						
Yes	12/33	0.36	1.00	27/59	0.46	1.00
No	71/111	0.64	0.50 [0.20–1.25]	171/277	0.62	0.83 [0.42–1.63]
Duration of stay in the area						
> 3 years	17/47	0.36	1.00	37/83	0.44	1.00
1–3 years	31/49	0.63	1.18 [0.56–2.45]	76/123	0.62	0.88 [0.51–1.53]
< 1 year	35/48	0.73	2.09 [0.97–4.51]	85/130	0.65	1.01 [0.52–1.97]
Season of recruitment						
Dry	24/37	0.65	1.00	51/88	0.58	1.00
Wet	59/107	0.55	0.84 [0.48–1.45]	147/248	0.59	0.76 [0.53–1.08]

AHR adjusted hazard ratio; $p \leq 0.05$*, $p < 0.01$ **, $p < 0.001$***

while, no significant difference was observed between baseline and day 42 in CQ alone (34.0 vs 20.6, $p = 0.060$). There was a significant decreased in mean oocyst per mosquito midgut between CQ alone and CQ + PQ treatment group at day 42 (20.6 vs 5.3, $p = 0.038$) (Fig. 4B).

In study participants who were infectious at day 42, the inhibition intensity was 39.0% (range; −42.81 to 100.00) in CQ alone and 77.8% (− 19.04 to 100.00) in CQ + PQ treated group and the difference was significant ($p = 0.016$) (Fig. 4C). Moreover, the mean inhibition prevalence at day 42 was 35.2% (range; −60.00 to 100.00) in CQ alone while, 70.1% (range; 5.00–100.00) in CQ + PQ treated group and the difference was significant ($p = 0.021$) (Fig. 4D).

Discussion

The recurrence of *P. vivax* poses a considerable challenge to vivax malaria control and elimination. Primaquine (PQ) is one of the approved anti-malarial treatments to prevent relapses, and it is efficacious under supervision

Table 4 Membrane feeding assay at the baseline and day 42 in Arjo-Didessa sugar development site and its surrounding, southwest Ethiopia (2019–2022)

Characteristics	CQ		CQ + PQ	
	Baseline n = 19	Day 42 n = 19	Baseline n = 20	Day 42 n = 20
Age (in years), n (%)				
Median	18	18	21	21
Mean ± SD	23.0 ± 9.8	23.0 ± 9.8	24.7 ± 12.6	24.7 ± 12.6
5–15	4 (21.0)	4 (21.0)	6 (30)	6 (30)
> 15	15 (78.9)	15 (78.9)	14 (70)	14 (70)
Sex, n (%)				
Female	5 (26.3)	5 (26.3)	6 (30)	6 (30)
Male	14 (73.7)	14 (73.7)	14 (70)	14 (70)
Asexual parasite/μL (geometric mean) 95%CI	3366 (1823–6211)	6332 (4167–9623)	4710 (2631–8431)	948 (454–1976)
Gametocytes/μL (geometric mean) 95%CI	544 (329–899)	737 (397–1369)	415 (254–675)	159 (55–457)
No. of gametocyte positive individuals	19 (100.0)	7 (36.8)	20 (100.0)	5 (25.0)
Mosquitoes				
Exposed	1700	1614	1584	1464
Fed	1093	1101	918	722
Patient infectious to mosquito, n (%)	19 (100)	14 (73.6)	20 (100)	10 (50.0)
Infected mosquitoes, n (%)	356/710 (50.1)	250/684 (36.5)	275/632 (43.5)	86/400 (21.5)
Mean oocyst/mosquito (range)	34.0 (1–216)	20.6 (0–214)	43.6 (1–215)	5.3 (0–82)
Range of oocyst intensity	33–2974	0–1548	14–3725	0–423

in clinical trials; however, its effectiveness remains uncertain in a real-world setting. Therefore, this study demonstrates its effectiveness and transmission-blocking ability in real-world practice.

In this study, it was not observed early treatment failure based on the absence of parasite on day 28. However, by day 42 *P. vivax* infections started to reoccur was 24% of participants treated with CQ alone and in 10.3% of those treated with CQ + PQ. The recurrence was higher than CQ alone (18.7%) and CQ + PQ treatment group (1.2%) a clinical trial study that PQ admiration under directly observed treatment (DOT) at day 2, 3, 7, 10 and 14 from central Ethiopia [28]. Additionally, the recurrence after CQ + PQ treatment was higher than in a systematic review and individual patient data meta-analysis (1.5%) with good PQ adherence (≥ 90% PQ treatment adherence) [29]. However, it was comparable *P. vivax* recurrences (7.4% vs 10.9%) after CQ + PQ administration under DOT till day 3 and self-administered thereafter in Northwest Ethiopia [30] and South Ethiopia, respectively [31]. The recurrence after CQ treatment was lower than (31.8%) another study conducted in central Ethiopia [32]. The discrepancy in CQ + PQ treatment group might be due to treatment adherence [33, 34] studies showed low recurrence rate in good adherence (supervised) [28, 29] than in poor adherence (semi-supervised the first 3 days and unsupervised) [29, 35]. Another plausible explanation could be there might be a significant drop in CQ level or level of protection decline after 28 days in participants' blood.

Furthermore, this study showed that the addition of PQ to CQ substantially reduced the risk of recurrence by 34.4% compared to CQ alone over a one-year follow-up period. In contrast, 70% of those in the CQ alone treated experienced recurrence, whereas only 46% in the CQ + PQ treated group did. This finding was consistent with other studies [28, 36, 37]. For instance, study conducted by Yeshiwondim et al. [17], in Ethiopia, documented *P. vivax* recurrence in 61.8% and 26.3% in CQ and CQ + PQ arm, respectively. Another study in Ethiopia by Abreha et al. [28], reported *P. vivax* recurrence was 61.7% and 20.5% in CQ and CQ + PQ arm, respectively. In Thailand-Myanmar border, Chu et al. [37], found *P. vivax* recurrence in 74% of CQ and 18% of CQ + PQ arm. According to an Afghan study by Awab et al. [36], the overall recurrence was 29.9% in CQ arm and.13.1 in CQ + PQ arm. Even though, the addition of PQ to CQ had a substantial reduction of recurrence, when CQ + PQ treatment group compared to other studies there was still 2 to threefold higher recurrence rate in this study. The variation in recurrence rate between CQ + PQ treatment might be due to treatment adherence, baseline antimalarial immunity, duration of follow-up, primaquine

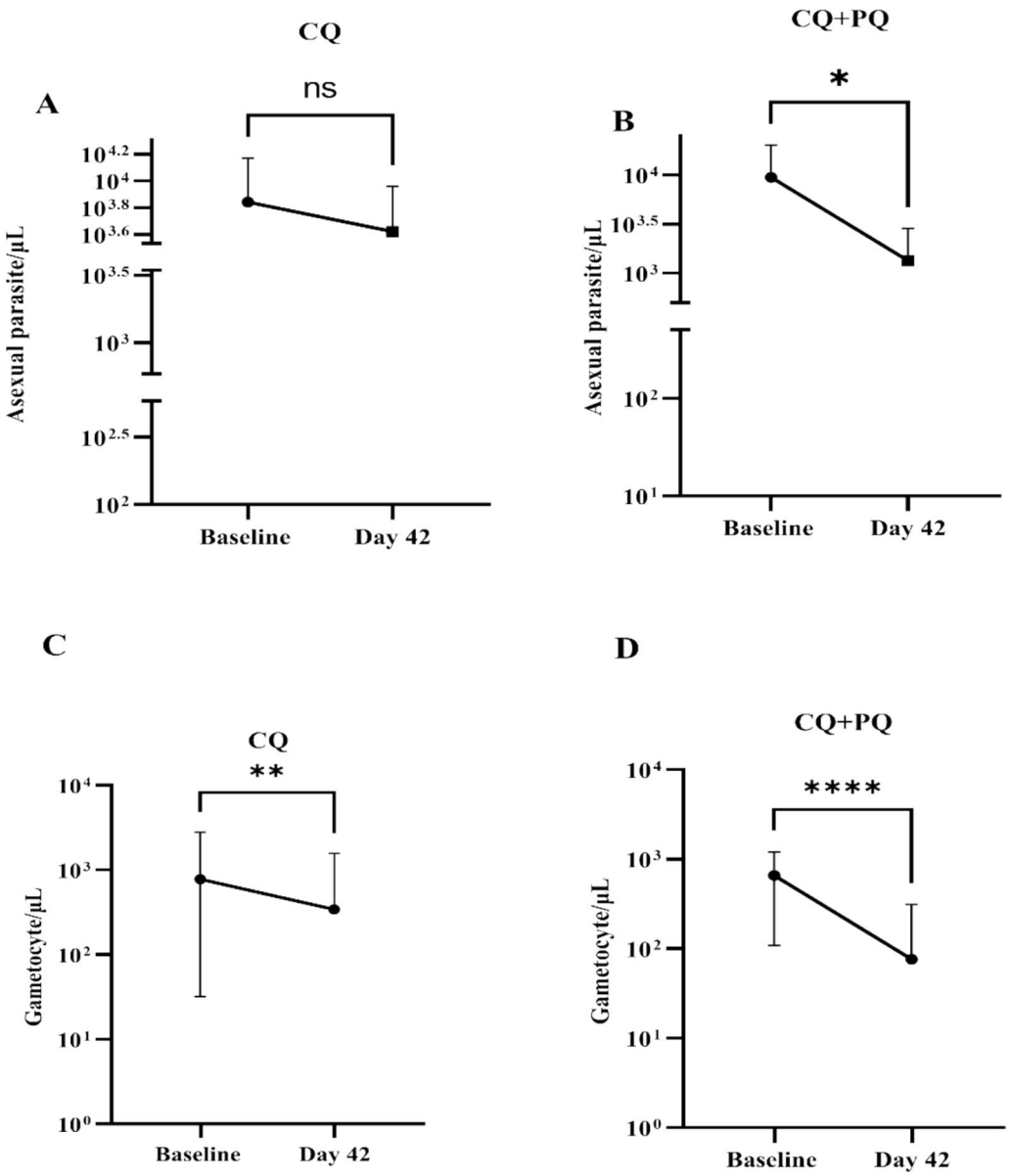

Fig. 3 The mean asexual parasitaemia (**A** and **B**) and mean gametocytaemia (**C** and **D**) at baseline and day 42 of CQ alone and CQ + PQ treatment group, respectively. The small star or asterisks (*) indicate significant difference in mean asexual parasitaemia and mean gametocytaemia at day 42 against the baseline. Error bars indicate standard error of mean. Key: ns: not significant; * $p < 0.05$; ** $p < 0.001$; **** $p < 0.0001$ significant level

dosing regimens, differences in study design, patient populations, and geographic location [5].

This study also investigated the transmission-blocking effects of CQ alone and the combination of CQ with primaquine (CQ + PQ) on *P. vivax* transmission to mosquito vectors. In low and moderate transmission areas, WHO recommends assessing the efficacy of anti-malarial treatments up to day 42 to detect late treatment failures [38, 39]. Therefore, direct membrane feeding assays were performed at baseline and day 42 in this research. The findings indicated that neither treatment achieved complete inhibition by day 42. However, the level of inhibition increased significantly in CQ + PQ treatment group, with a 39.0% inhibition intensity for CQ alone and a 77.8% for CQ + PQ. The inhibition prevalence at day 42 also showed a significant increase in CQ + PQ treatment group, with 35.2% for CQ alone and 70.5% for CQ + PQ.

The addition of PQ to CQ reduced the proportion of individuals able to transmit *P. vivax* infection and, if infected, resulted in fewer oocysts. Overall, this study

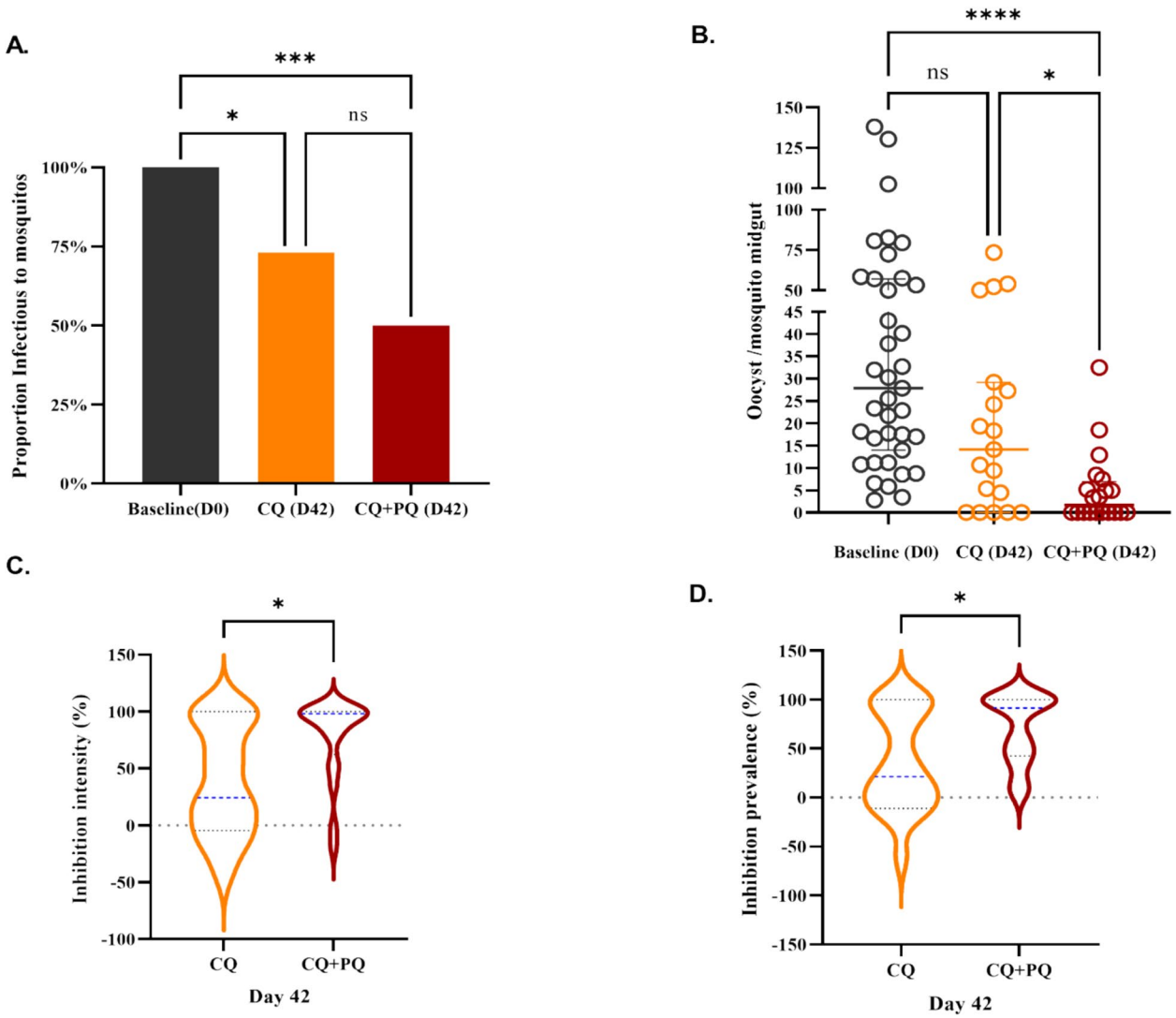

Fig. 4 **A** The proportion of individual's infectiousness to mosquitoes at the baseline and day 42 post treatment of CQ alone and CQ + PQ treatment group. **B** Mean oocyst/mosquito midgut at the baseline and day 42 of CQ alone and CQ + PQ treatment group. **C** the inhibition intensity between CQ alone and CQ + PQ treatment group by day 42. **D** the inhibition prevalence between CQ alone and CQ + PQ treatment group by day 42. Violin plot showing the blue horizontal dot lines indicate median, the black horizontal dot line indicates interquartile range; and spikes indicate upper and lower adjacent values. Asterisks (*) indicate the significant difference against the antimalarial treatment group

showed the potential effect of PQ in reducing *P. vivax* transmission was substantial. Studies examining the transmission-blocking effects of CQ on *P. vivax* malaria within 0–72 h following anti-malarial treatment have shown inconsistent results. While Popovici et al. [40], noted that patients remained infectious to mosquitoes 24 h after the initial dosage, Jeffery demonstrated that patients continued to be infectious after 48 h of CQ treatment [41]. Klein et al. [42], indicated that nearly all mosquitoes feeding on patients following the third dose of CQ treatment were free of *P. vivax* oocysts within 24 h. Another study on mosquito infections reported that when a *P. vivax* positive sample was incubated with a high concentration of CQ and then fed to mosquitoes, the mean oocyst count decreased by 1.40-fold, and the mean sporozoite count decreased by 1.34-fold [43]. However, a handful studies have shown complete inhibition of mosquito infection after 4 h [41] and 24 h [11] when CQ was combined with a high dose of PQ.

In this study, high baseline parasitaemia was associated with a 1.9-fold increased risk of *P. vivax* recurrence compared to low parasitaemia at enrolment. This finding aligns with studies conducted elsewhere [33, 34, 36, 44, 45]. Similarly, individuals with gametocytaemia

at enrolment had a 1.6-fold increased risk of recurrent *P. vivax* infection compared to those without gametocytaemia. Other studies have also documented similar findings [36, 46]. This may be attributed to the tropical strain, which can produce approximately equal quantities of hepatic schizonts and hypnozoites that may be reactivated promptly after the initial infection [47, 48]. This ratio remains persistent regardless of the initial number of sporozoites introduced [49]. Consequently, the total number of hypnozoites is likely to increase along with the parasite count, thereby raising the likelihood of recurrence (relapse). This could be more common if an individual has lower naturally acquired immunity to primary *P. vivax* infection and the lower immunity might result in recurrence of infection. As partial malaria immunity acquired through repeated exposure to *Plasmodium* infection. When the exposure decline the waning of immunity will occur.

In this study, the ownership of insecticide-treated nets (ITNs) was significantly associated with the recurrence of *P. vivax* malaria. Participants who did not own an ITN had an increased risk of recurrence compared to those who did. However, the effect of ITNs on *P. vivax* recurrence is not as straightforward as it is for *P. falciparum* infections. ITNs can reduce primary *P. vivax* infections by serving as a physical barrier and killing mosquitoes. This can lead to fewer primary infections, resulting in fewer individuals carrying hypnozoites in the liver. Consequently, ITNs indirectly impact the overall burden of *P. vivax* relapses by decreasing the number of primary infection. Additionally, this study found that socioeconomic factors, such as occupation and education level, were significant predictors of recurrent *P. vivax* malaria. Individuals engaged in outdoor occupations had a 40% reduced risk of recurrent infection compared to their counterparts. This finding aligns with a study conducted in Brazil, which identified domestic work activities as an increased risk factor for recurrence [50]. However, inconsistent result with a study conducted in Thailand-Myanmar border [51]. In many areas where *P. vivax* predominated, vectors bite early in the evening, obtain blood meals outdoors and rest outdoors [52–55] but it is not clear that why individual involved in outdoor job activity had reduced risk of recurrence than indoor job in this study. The result of this study also showed level of education significantly associated with recurrence. These results in line with other studies conducted elsewhere [50, 51]. All these risk factors are known to increase exposure to malaria infection and probably increase the hypnozoite burden.

There were several limitations to this study. The primary limitation was the unsupervised administration of PQ, and another potential limitation was that the recruited participants with CQ + PQ came from a different area than those receiving CQ alone. *Plasmodium vivax* transmission may differ between these sites, which could confound the results, although the malaria infection parameters appeared similar. The third limitation was the lack of available information to differentiate between recrudescence, relapse, and re-infection. The fourth limitation was that nadir or day 7, 14, and 21 haemoglobin measurements were not done to study the safety of PQ during the follow-up period. Additionally, only 67.3% (144/214) of the enrolled patients completed the full one-year follow-up due to the COVID- 19 pandemic during data collection. However, the sensitivity analysis between complete-cases and all study participants did not reveal much difference in the outcomes.

Conclusion

This study demonstrate that the addition of PQ to CQ significantly reduces the recurrence of *P. vivax*. Furthermore, CQ + PQ resulted in a higher prevalence of inhibition of *P. vivax* in mosquito vectors compared to CQ alone. However, neither CQ nor CQ combined with a low dose of PQ exhibited a complete transmission-blocking effect at day 42 post-treatment. On the other hand, the main risk factors for *P. vivax* recurrence included high baseline parasitaemia, the presence of gametocytaemia at enrolment, ownership of ITNs, and sociodemographic factors such as education level and outdoor occupation. Therefore, strengthening malaria control and elimination efforts is essential by ensuring proper health education on treatment adherence and vector control tools for *P. vivax* patients. In addition, higher doses of PQ administered shortly may be necessary to reduce the recurrence rate and improve the transmission risk. Ensuring adequate coverage and proper utilization of vector control tools and monitoring *P. vivax* -positive individuals within six months to capture recurrent infections may also be needed.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-025-05365-y.

Supplementary material 1: Table S1, S2, S3, S4, S5 and S6. The Ethiopia malaria treatment protocol, Primer and probe sequences used in this study, Model selection criteria for Cox extension, Comparison of distribution of demographic characteristics and predictors between complete-cases and all study participants, Univariable analysis of complete-cases and Multivariable risk factor analysis of all study participants, respectively.

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Author contributions
H.G, C.L.K, J.W.K, D.Y and G.Y conceived and designed the study. H.G, K.H, A.A, A.D, G.Z, A.T, T.D, and M.C.L involved in data collection, field supervision and data analysis. H.G, M.C.L, J.W.K, D.Y and G.Y involve in data curation. H.G, D.Z, K.H, A.D, A.A, and X.W involved in nested and qPCR analysis. H.G, K.H, A.T and A.A performed membrane feeding assay. H.G drafted the manuscript. J.W.K, C.L.K, D.Y, and G.Y critically reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Deidentified participant datasets used for the current study will be available immediately after publication from the corresponding author or the project data manager team on reasonable request.

Declarations

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Medical Laboratory Technology, Arbaminch College of Health Sciences, Arbaminch, Ethiopia. [2]Department of Medical Laboratory Sciences, Institute of Health, Jimma University, Jimma, Ethiopia. [3]Tropical and Infectious Diseases Research Center (TIDRC), Jimma University, Jimma, Ethiopia. [4]Menelik II Medical & Health Science College, Addis Ababa, Ethiopia. [5]Department of Microbial, Cellular & Molecular Biology, Addis Ababa University, Addis Ababa, Ethiopia. [6]Department of Medical Laboratory Sciences, College of Medicine and Health Science, Arbaminch University, Arbaminch, Ethiopia. [7]Department of Medical Laboratory Sciences, College of Medicine and Health Sciences, Ambo University, Ambo, Ethiopia. [8]Department of Biology, College of Natural Science, Jimma University, Jimma, Ethiopia. [9]Program in Public Health, University of California at Irvine, Irvine, CA 92697, USA. [10]Center for Global Health and Diseases, Case Western Reserve University, Cleveland, OH, USA.

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