Malaria Journal

RESEARCH

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Post-treatment transmissibility of *Plasmodium falciparum* infections: an observational cohort study

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Abstract

Background Strengthening malaria control and expediting progress toward elimination requires targeting gametocytes to interrupt transmission. Artemisinin-based combination therapy (ACT) effectively clears *Plasmodium falciparum* asexual parasites and immature gametocytes but has a limited impact on mature gametocytes, which mosquitoes ingest during a blood meal. To address this gap, the World Health Organization recommends adding a single low dose of primaquine (PQ) to ACT regimens. This study assessed the efficacy of a single low-dose PQ for *P. falciparum* gametocyte clearance and evaluated mosquito infectiousness in Ethiopia.

Methods A prospective cohort study was conducted using passive case detection to enrol individuals with uncomplicated *P. falciparum* malaria at six health facilities. Participants were treated with either ACT alone or ACT plus 0.25 mg/kg single-dose PQ (ACT + PQ) and followed for 28 days with weekly visits. Blood smears for parasite counts, filter paper samples for DNA isolation, and whole blood for RNA preservation were collected on days 0, 7, 14, 21, and 28. On day 7, venous blood was obtained for membrane feeding assays using the Hemotek® system to assess mosquito infection. Logistic regression analysed mosquito infection predictors, while gametocyte prevalence was compared between treatment arms using χ^2 or Fisher's exact tests.

Results Of 304 screened patients, 192 were enroled, with a median age of 23 (IQR 17–30) years; 65.7% were male. Post-treatment, 11 human-to-mosquito transmission cases were identified on day 7. Participants receiving ACT + SLD-PQ were significantly less likely to be infectious on day 7 (OR 0.12, 95% CI 0.02–0.57, $p = 0.008$) and had a significantly reduced prevalence of gametocytes (OR 0.22, 95% CI 0.06–0.83, $p = 0.026$) compared to those receiving ACT alone.

Conclusion A single course of low-dose primaquine (PQ) given with ACT significantly decreases the prevalence of gametocytaemia. Furthermore, membrane-feeding assays show that this combination also considerably lowers mosquito infection, confirming existing knowledge and emphasizing the promise of low-dose PQ as a successful transmission-blocking strategy in managing malaria.

Keywords Artemisinin combination therapy, Single low dose primaquine, Membrane feeding assays, Gametocytes, *Plasmodium falciparum*, Ethiopia

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Background

Effective therapy is a critical element of malaria control and elimination, as it reduces both morbidity and mosquito transmission [1]. Artemisinin-based combination therapy (ACT), the first-line treatment for *Plasmodium falciparum* in sub-Saharan Africa, provides remarkable cure rates by rapidly clearing the parasite's asexual stages [2]. However, onward malaria transmission is not avoided due to the ineffectiveness of artemisinin and its companion drugs against mature gametocytes [3, 4]. If mature gametocytes are present before treatment, they survive following ACT, frequently at numbers below the detection threshold for conventional microscopy, and can allow malaria transmission to continue [5]. In addition, as asexual parasites give rise to gametocytes, patients who have clinical malaria will have gametocytes in their blood, increasing the likelihood of infecting mosquitoes [6–11]. Moreover, mature gametocytes persist after treatment and can maintain malaria parasite transmission [12].

As malaria control programmes focus their efforts on local and global elimination, gametocyte clearance becomes crucial and can be targeted as part of the effort. Strategies aimed at disrupting the development of gametocytes and limiting their availability to blood-feeding mosquitoes can block the natural transmission of malaria [13]. Primaquine (PQ), a powerful anti-malarial drug, has been shown by researchers to rapidly eliminate *P. falciparum* gametocytes, which is a key component in reducing the transmission of malaria [14, 15]. The rapid clearance of gametocytes in less than a day, particularly when paired with ACT, demonstrates the capacity of primaquine to reduce the infectious reservoir and slow the development of malaria [16, 17]. The World Health Organization (WHO) recommended combining ACT with 0.25 mg/kg single-dose PQ (SLD-PQ) for malaria elimination in low transmission and to contain the risk of artemisinin resistance, without prior testing of glucose-6-phosphate dehydrogenase (G6PD) deficiency [18]. As a result of difficult logistic requirements, the effectiveness of SLD-PQ has only been studied in a small number of African countries to date [19–27]. These studies mostly used gametocyte carriage as a surrogate marker of SLD-PQ's potency to inhibit transmission potential, either by microscopy or TaqMan reverse transcriptase PCR (RT-PCR). This must be augmented with conducting studies on mosquito feedings to examine if gametocytes are sterilized by PQ treatment infectivity through membrane feeding [28, 29]. The infectiousness of the individuals treated with PQ can be directly assessed using a mosquito-feeding assay, which is considered the gold standard for evaluating transmissibility [30]. On top of that, Ethiopia has embraced the Global Technical Strategy (GTS) and created a strategic plan that aims to target districts with an annual parasite index of fewer than 10 by 2025 to achieve malaria elimination by 2030 [31, 32]. Moreover, this study targeted symptomatic patients because they are a significant part of the malarial infectious reservoir [33] and because addressing them is necessary for containment methods for malaria elimination [34].

In this study, the gametocyte state, infectiousness, and mosquito infectivity rates after feeding on post-treatment *P. falciparum* patients were evaluated. Subsequently, the study assessed how administering this SLD-PQ impacted the reduction of onward malaria transmission.

Implications of this study

This study adds value by employing the membrane feeding assay, the gold standard for mosquito infectivity, in conjunction with microscopy and the pfs25 assay to detect submicroscopic gametocytes. The addition of SLD-PQ caused the gametocyte prevalence to decrease more quickly, preventing the mosquito from infection. Furthermore, this study presents the first evidence that an SLD-PQ added to ACT blocks transmission by day 7 following therapy. Additionally, the results of this study add to the growing body of evidence that using ACT in conjunction with an SLD-PQ regimen is an effective way to stop the spread of *P. falciparum*. The results of this study confirm the advice given by WHO that ACT be used in conjunction with an SLD-PQ to halt the spread of malaria in regions that seek to eliminate malaria.

Methods

Study area, participants, and study design

The study was conducted in southwestern Ethiopia's Arjo-Didessa sugarcane irrigation area (Fig. 1) [35]. In this malarious environment, *P. falciparum*, *Plasmodium vivax*, and *Plasmodium ovale* coexist and transmission is seasonal peaking during September–November [36]. This study was part of a primaquine cohort in the International Center of Excellence for Malaria Research (ICEMR) research project, which divided the irrigation region into eleven clusters, or agricultural commands. Of the eleven clusters from three districts—Jimma Arjo district (Abote Didessa), Bedele District (Command 5), and Dabo Hana district (Kerka and Sefera Tabiya)—six clusters were chosen at random. In this prospective cohort

study, patients with uncomplicated *P. falciparum* malaria who presented at six healthcare facilities were identified by passive case detection. Patients at Sefera-Tabiya Health Centre and Kerkha Health Post got ACT alone since PQ medication had not yet been introduced in the two districts where these facilities are located throughout the study period. These patients served as the control group. On the other hand, ACT + SLD-PQ was used to treat patients at the remaining four health facilities: Abote-Dedessa Health Post, Arjo Sugar Factory Clinic, Command 2 Health Post, and Command 5 Health Post. If there were PQ stockouts, however, ACT was used alone. The study health facilities were selected based on their proximity to the PQ cohort study. The study's health facilities include Abote-Dedessa Health Post, Arjo Sugar Factory Clinic, Karkaha Health Post, and Sefera-Tabiya Health Centre.

Patients with symptomatic, uncomplicated *P. falciparum* infections detected by microscopy were included in an observational cohort study from healthcare facilities between September 2019 and November 2022 for this prospective cohort study. Individuals were initially enlisted irrespective of their asexual parasite count and microscopic gametocyte status. Patients with comorbid illnesses, severe malaria symptoms, or treatment protocol contraindications were excluded. Blood transfusions within the last 90 days, anti-malarials taken within the last 48 h, PQ usage within the last four weeks, known allergy to study drugs, haemoglobin levels below 5 g/dL, infants under one year old, pregnant or breastfeeding mothers, and non-falciparum malarial infections at screening were also excluded. To confirm the microscopy results, PCR confirmation of *P. falciparum* infection was performed after fieldwork.

Overall, 304 patients were screened and 247 were enroled in the cohort study, with 109 in ACT alone and 138 participants in ACT + SLD-PQ treatment group (Fig. 2) using passive case detection. The investigators did not participate in the assignment of research groups, and patients were treated in compliance with Ethiopia's government malaria treatment guidelines. Participants in the study were grouped into study arms according to the healthcare facility they visited and the treatments they received.

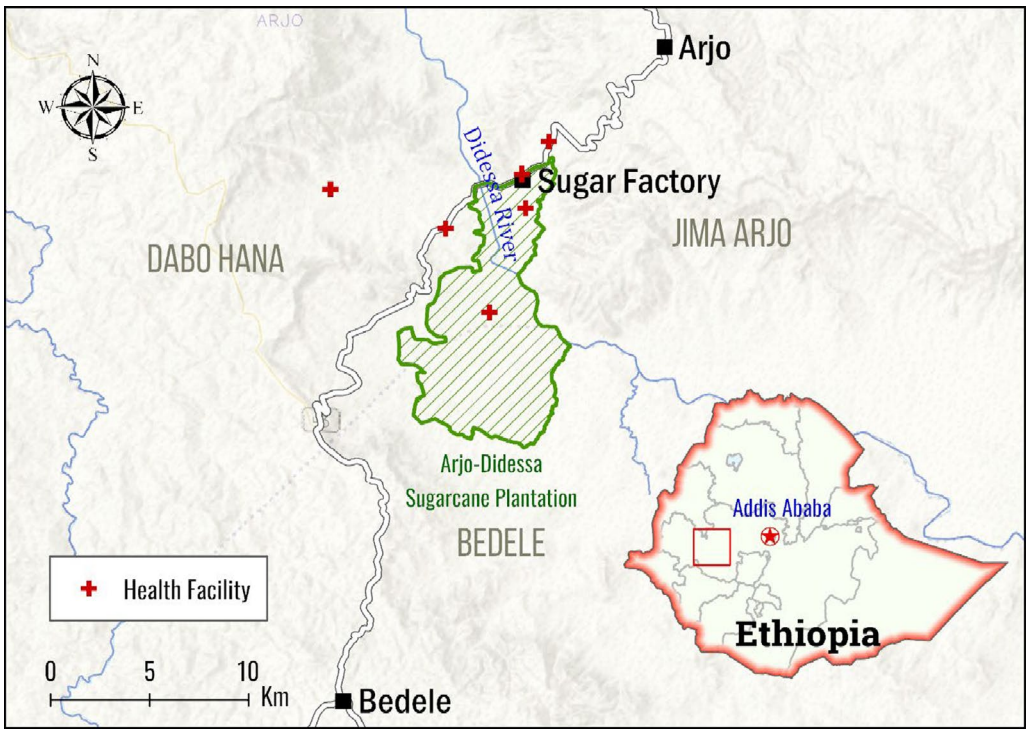

Fig. 1 Map of the study area

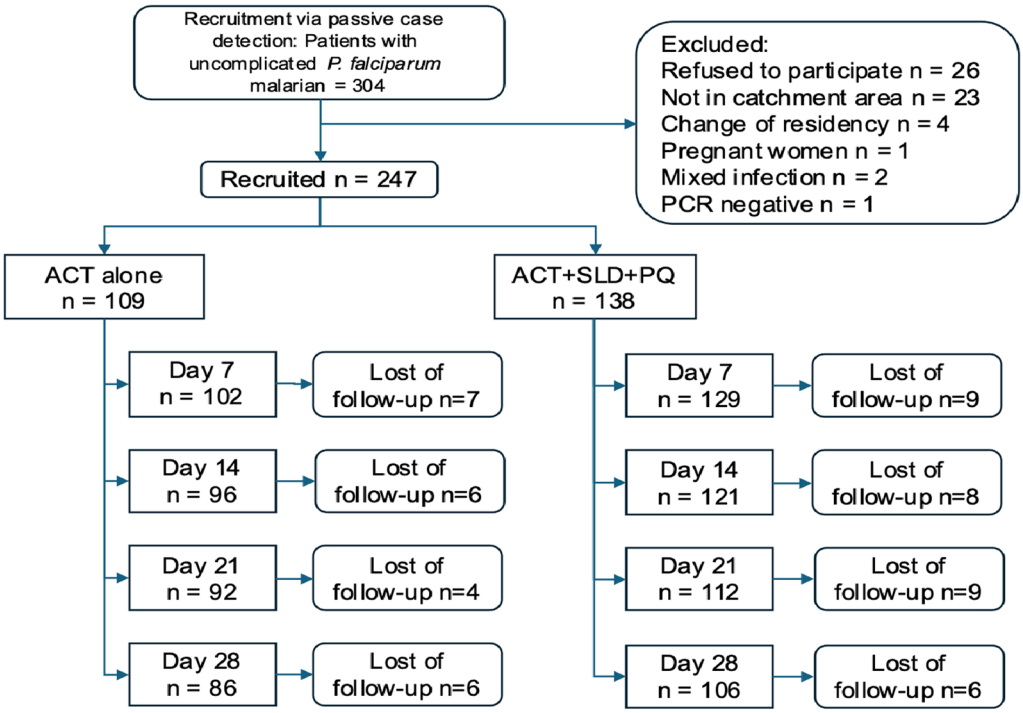

Fig. 2 Flow chart of the prospective cohort study design

Sample size calculation

The sample size was determined by using the two-proportion Z-test formula, which is suitable for comparing two independent proportions. Previous data indicated that 80% of individuals infected at least one mosquito, with a maximum infection rate of 25% among mosquitoes, i.e., a 0.2 estimated likelihood of infection [20]. The calculation aimed at a 90% reduction in infectivity with 80% power at a significance level of 0.05, a sample size of approximately 43 participants per group was required, or 48 participants in each group after allowing for a 10% loss to follow-up for the first hypothesis. The sample size needed to test the second hypothesis was determined to determine whether ACT alone and ACT + SLD-PQ treatments differed in gametocyte prevalence on day 7. Assuming ACT alone treatment results in a 50% gametocyte prevalence by Day 7, the addition of PQ at 0.25 mg/kg will reduce the prevalence to 25% (50% reduction) [22], to achieve 80% power at a 5% significance level, 55 participants are required in each treatment group, or 70 participants in each group which allows for a 20% loss of follow-ups. However, recruitment was extended due to a higher-than-expected influx of patients, particularly in the PQ arm. The ACT-alone arm had a reduced sample size because of the countrywide use of PQ, which restricted the number of patients who could be enroled to receive only ACT. The logistical challenges of mosquito husbandry also restricted the collection of data in this group. Conversely, because PQ was incorporated into standard malaria treatment practices, enrolment in the PQ treatment arm was higher than expected, resulting in a larger sample size. Despite these challenges, the study had sufficient statistical power to detect the desired reduction in infectiousness. Moreover, participant characteristics were similar across the treatment groups (Table 1).

Patient treatments

All enroled participants were treated with a standard 3-day regimen of oral artemether-lumefantrine (AL) (Coartem®; Ipca Laboratories Ltd, Mumba, India) in doses based on age, following twice daily for three consecutive days with the standard six-dose regimen according to national malaria treatment guideline of Ethiopia for uncomplicated *P. falciparum* malaria [37]. This was a fixed dose combination of 20 mg of artemether and 120 mg of lumefantrine in a tablet. A full course of AL consisted of 6 doses given twice daily (8 hourly apart on Day 0 and 12 h apart on days 1 and 2). The dosing was divided into four age and weight groups: children under 4 months received 1 tablet; those aged 4 months to 2 years received 1 tablet; children aged 3 to 7 years received 2 tablets; those aged 8 to 10 years received 3 tablets; and individuals aged 10 years and older received

4 tablets. To successfully eliminate gametocytes, the Ethiopian Malaria Treatment Guideline recommends a single dose of PQ (0.25 mg/kg) or 15 mg for patients who weight more than 60 kg (Primaquine phosphate, Remedica, Ltd., Limassol, Cyprus) was administered on Day 0 in parallel with the first dose of AL. according to national malaria treatment guidelines: for children aged 7 months to 5 years, ½ tablet of 7.5 mg was given; for ages 5 to 7 years, 1 tablet of 7.5 mg; for ages 8 to 10 years, 1 tablet of 7.5 mg; for ages 11 to 13 years, 1 ½ tablets of 7.5 mg; and those 14 years and older, 2 tablets of 7.5 [38]. After enroling, participants were closely observed by researchers to make sure they took the recommended dosage of PQ. To verify treatment completion on day 7, self-reported adherence to the ACT regimen was recorded. This approach made sure that every participant in the study had finished the entire course of treatment as needed.

Follow-up, sample collection, and preservation

All enroled patients were followed for 28 days with scheduled weekly visits. The administration of treatments was started on D0 (the day of enrolment). During follow-up visits, clinical and safety assessments were performed, a blood slide for parasite count was taken, filter paper blood sample for PCR analyses, haemoglobin levels were measured and to preserve RNA for gametocyte detection, 50 µL of the sample was taken into 250 µL of RNAprotect® Cell Reagent (Qiagen, Hilden, Germany) on each follow-up day. The thick and thin blood films were made to screen for *Plasmodium* parasites under a microscope. On D7 venous blood was collected for mosquito feeding assay. The patients and their parents or guardians were also informed to return on any day if the symptoms returned or any other adverse signs were present. Patients who did not attend the scheduled visits or could not be found despite all possible efforts were classified as lost to follow-up.

Gametocyte detection and assessment

To determine the prevalence of gametocytes, blood samples were taken on Days 0, 7, 14, 21, and 28 at enrolment and after therapy. The microscopy directly measured gametocyte presence using Giemsa-stained thick and thin blood smears. Furthermore, subpatent gametocytes—undetectable under a microscope but can nonetheless contribute to transmission—were found using RT-PCR-based techniques. A more sensitive measurement of gametocyte presence is made possible by the addition of RT-PCR, particularly in patients with low-density infections.

Microscopy examination of blood films

To determine the prevalence of gametocytes, blood samples were taken on Days 0, 7, 14, 21, and 28 at enrolment and after therapy. The microscopy directly measured gametocyte presence using Giemsa-stained thick and thin blood smears. For each visit, thick and thin blood smears were prepared and stained with 10% Giemsa solution for eight to ten minutes using the WHO-recommended malaria microscopy standard operating procedure (MM-SOP-07A) [39]. After detecting asexual parasites on the thick film, all participants' parasite densities were computed using the WHO easy assumption of 8.0×10^9/L of blood for WBC counts using the WHO-recommended malaria microscopy standard operating procedure (MM-SOP-09) [40]. The thin films determined the different malaria species in the study population. Two separate microscopists looked at each blood smear. The slides were examined for malaria parasites by both microscopists separately. If their readings differed, the disagreements were resolved by consulting a third microscopist. The consensus reading served as the basis for determining each sample's outcome. If no asexual or sexual forms of the parasites were seen after 2500 WBCs or 200 high power fields (HPFs), the slide was declared negative [41].

qPCR confirmation of malaria infection

PCR testing was carried out at the follow-up and screening phases. During the screening process, it ensured accurate population characterization by verifying *Plasmodium* species and detecting possible mixed infections that microscopy might miss. PCR testing added additional validation, even if therapy was given based on microscopy data, such as species identification and parasitaemia levels. PCR was used to confirm parasite clearance and identify submicroscopic parasitaemia throughout follow-up, providing a more sensitive assessment of the effectiveness of ACT in conjunction with SLD-PQ in gametocyte clearance and transmission reduction.

The DNA was extracted using the Chelex-saponin protocol described elsewhere by Wooden et al. [42]. Briefly, DNA was extracted from filter paper dry blood spot samples using the standard Chelex protocol (Bio-Rad Laboratories, Hercules, CA, USA). The details of the molecular analyses were described in the Supplementary Materials (Supplement Material S1). The multiplex real-time PCR assay was used for genus-specific amplification targeting the 18S rRNA genes of *Plasmodium*. Sequences of primers and probes are given in Supplement File S1 and Table S1, and the composition of reaction mixes and thermo profiles are shown in Supplement File S2 [43, 44].

Pfs25 mRNA qRT-PCR for detection of gametocyte

The female-specific pfs25 assay was used to detect submicroscopic gametocytes, which may nevertheless contribute to transmission even though they are not detectable under a microscope. Using reverse-transcription quantitative PCR (RT-qPCR), which targets the Pfs25 genes and was previously reported by Schneider et al. [45], the presence of mRNA transcript was confirmed. Table S1 (Supplement File S1) contains a list of primers and probes. To check for stage-specific *P. falciparum* mRNA that differentiates mature (stage V) gametocytes from other stages, RT-qPCR was performed on the preserved RNA of every PCR-positive subject. The Supplementary Material (Supplement File S2) described the gametocyte detections.

Mosquito colony preparation

A colony of *Anopheles arabiensis* was established in the field insectary of the JU-UCI Joint ICEMR Laboratory in Arjo-Didessa, Ethiopia, in 2019. The strain of *An. arabiensis* used for the membrane feeding assay (MFA) was obtained from the Tropical and Infectious Diseases Research Center (TIDRC) Sekoru insectary at Jimma University. This colony has been effectively utilized for transmission tests in the past ten years [46]. The experiment was carried out in four separate areas, each with a distinct purpose. The first room, which was 4 m by 3 m and had a 12:12 light/dark cycle, was used to grow larvae. It was kept at 27 ± 1 °C and 70 ± 10% relative humidity. A temperature of 27 ± 2 °C and a relative humidity of 80 ± 10% were established in the second 4 m × 3 m room for the preservation of adult mosquitoes. The second 4 m × 3 m room was set for adult mosquito maintenance, with a temperature of 27 ± 2 °C and RH of 80 ± 10%. Feeding experiments occurred in a 3 m × 3 m- dark area, under the same conditions as the adult maintenance room, and mosquito dissections were conducted in a 3 m × 4 m space that operated under ambient temperature conditions. This setup ensured a controlled environment for mosquito development and experimentation stages [47].

Mosquito membrane feeding experiments and oocyst detection

Mosquito infectivity was evaluated in a subpopulation of the study participants, regardless of gametocyte level, using a Hemotek® (electrically heated feeder reservoir membrane feeding system). On D7 post-treatment, 96 participants from each arm were randomly selected for MFAs, following an established methodology [29], to detect biological evidence of gametocyte clearance. In brief, 3–4 ml venous blood samples were drawn and placed in a heparin-containing tube (BD Vacutainer®, Sodium Heparin^{N} (NH) 158 & 75 USP Units (Lot # 0289426, Becton, Dickinson, and Company, USA) kept at 37 °C water bath. This sodium heparinized blood was immediately transferred to the Hemotek feeder (Discovery Workshops, Lancashire, United Kingdom). Then the Hemotek feeder was attached to a warm power source which maintains the blood at 37.5 °C. Before the studies, mosquitoes were starved overnight. There were 60–90 mosquitoes, with 20–30 colony-reared mosquitoes (3–5 days old) in each of the three paper cups. A Hemotek membrane feeder allowed the mosquitoes to feed on the participant's blood samples for 15 to 20 min. Following the protocol by Ouédraogo et al. [48], only fully fed mosquitos were placed into a holding cage in the insectary provided with 10% glucose solution. Blood-fed mosquitoes were dissected for oocysts 7–8 days post blood meal. All survived mosquitoes were anaesthetized using cold exposure, then dissected for microscopic detection of oocysts in the midgut after being stained with 1% mercurochrome (Mercury dibromo fluorescein disodium salt, Lot # 028k0724V, Sigma-Aldrich).

Outcomes

The primary outcomes were mosquito infection metrics seven days after treatment, as determined by membrane feeding and oocyst presence in mosquitoes that were dissected on the eighth day after feeding. Three metrics were used to measure mosquito infectivity, i.e., the proportion of blood samples from *P. falciparum*-infected participants gave rise to at least one mosquito infection, (i.e., infectious participants), the percentage of mosquitoes that are infected with any number of oocysts (i.e., the mosquito infection rate), and the mean number of oocysts in a mosquito sample (i.e., the oocyst density). Gametocyte prevalence at specified time points (days 0, 7, 14, 21, and 28) was a secondary outcome. The study also investigated the relationship between gametocytaemia and mosquito infectivity. The day 7 endpoint was considered the period of greatest efficacy seen in earlier research, as well as PQ's relatively limited therapeutic range and short elimination half-life of 4 to 9 h [23, 24, 49–52]. It is consistent with the standardized endpoints proposed by the PQ in the Africa Discussion Group [53].

Data statistical analysis

Microscopy asexual parasite density was presented as geometric means per µl blood. Differences in baseline asexual parasite density and gametocyte prevalence were tested between the two treatment groups using t-test and χ^2-tests (or Fisher exact test if any outcomes with $n < 5$),

respectively. Gametocyte prevalence at day 7 post-treatment, human-to-mosquito infectious rate, and mosquito infection rate between the ACT alone and ACT + SLD-PQ groups was compared using logistic regression analysis adjusted for age, sex, gametocytaemia, and asexual parasite density at baseline. Both adjusted odds ratios (AORs, 95% CI) and crude odds ratios (ORs, 95% CI) were reported. A Wilcoxon rank-sum test was employed to compare the mean oocyst densities across treatment arms. The research arms' gametocyte prevalence on various visit days was compared using χ^2-tests or Fisher exact tests if any results were found with $n < 5$. SPSS 22.0 (IBM, Armonk, NY, USA) was used to analyse the data.

Ethics considerations

The Addis Ababa University College of Natural Science Institutional Review Board, Ethiopia, provided ethical clearance (CNSDO/201/11/2018). Adult patients provided written informed consent, while children's parents or legal guardians assented. during the 28-day follow-up period, research participants received reimbursement for their transportation expenses.

Results

A total of 304 patients with uncomplicated falciparum malaria were screened, and 192 patients were enroled in this study (Fig. 2). 86 and 106 participants received ACT alone and ACT + SLD-PQ treatment, respectively, for the 28-day cohort study. The median (IQR) age was 22.5 (17–28.75) years, with 129 (67.2%) being male. Following enrolment, 7 (6.4%) and 9 (6.5%) participants left the study before D7 in the ACT alone and ACT + SLD-PQ treatment arm respectively (Fig. 2). There were no significant statistical differences in the geometric means of asexual parasite densities between ACT alone and ACT + SLD-PQ arms at baseline (4009.75/µl vs. 5925.30/µl) (Table 1). Similarly, there was no significant difference in the baseline gametocyte prevalence in ACT alone (10/86; 11.6%) and ACT + SLD-PQ (15/106; 14.2) arm by RT-qPCR examinations. However, no microscopic gametocyte carriage in patients was seen at enrolment and on all follow-up visits.

Host infectivity to mosquitoes

On day 7 post-treatment, blood samples were collected from 101 randomly selected patients for mosquito membrane-feeding assays, and 96 subjects completed mosquito membrane-feeding assays, i.e., 38 for the ACT alone arm and 58 for the ACT + SLD-PQ arm. 7100 mosquitoes were allowed to feed, 2960 (41.7%) for ACT alone and 4140 (58.3%) for the ACT + SLD-PQ treated blood samples. The proportion of samples yielding at least one infected mosquito was higher in the ACT alone group (9/38, 23.7%) than in the ACT + SLD-PQ (2/58, 3.4%) treatment group. The logistic regression model demonstrated a reasonable fit to the data, with a Nagelkerke R2 of 0.376 after adjusting for key covariates, including gametocytaemia. SLD-PQ therapy was substantially linked to a 93% decrease in the likelihood of mosquito infection, regardless of gametocytaemia, as evidenced by the OR of 0.08 (95% CI: 0.01–0.47; $p = 0.006$). These results demonstrate the substantial transmission-blocking effect of the addition of SLD-PQ therapy (Table 2).

Table 1 Baseline characteristics of the study participants (n = 192) at enrolment

Variable	Treatment arms	
	ACT alone (n = 86)	ACT + SLD-PQ (n = 106)
Sex, % male (n/N)	55.8 (48/86)	76.4 (81/106)
Age(years), median (IQR)	21 (15–30)	23 (17–27.25)
Age group		
< 5 years, no. (%)	6 (7)	2 (1.9)
5–15 years, no. (%)	17 (19.8)	13 (12.3)
> 15 years, no. (%)	63 (73.3)	91 (85.8)
Body temperature, mean (SD)	38.07 (0.59)	38.08 (0.58)
Geometric mean parasite density/µL	4009.75	5925.30
% Gametocytaemia (RT-qPCR)	10 (12.5)	15 (16.3)
% Gametocytaemia (microscopy)*	0.0	0.0
Hb concentration at enrolment (g/dl), mean (SD)	12.95 (1.6)	13.28 (1.2)

IQR interquartile range, *SD* standard deviation, *Hb* haemoglobin, *g/dl* gram per decilitre

Table 2 Percentages of infectious participants' blood were determined by membrane-feeding assays and treatment arm

Treatment	No. of infectious participants*		COR (95%CI)	*p* value	AOR (95%CI)	*p*-value
	Positive %(n)	Negative %(n)				
ACT alone	23.7 (9/38)	76.3 (29/38)	1			
ACT + SLD-PQ	3.4 (2/58)	96.6 (56/58)	0.12 (0.02–0.57)	0.008*	0.08 (0.01–0.47)	0.006*

Key: Proportion of individuals infecting at least one mosquito (i.e., the number of mosquitoes presenting at least one oocyst in the midgut out of the total number of dissected mosquitoes)

AOR = adjusted odds ratio; adjusted for age, sex, and gametocytaemia; CI = confidence interval

*Significant at p < 0.05

Mosquito infection rate

A total of 2844 blood-fed mosquitoes were dissected, 176 mosquitoes had oocysts, mosquito infection rate was 11-fold higher in the ACT alone treatment group (12.2%, 157/1287) compared to the ACT + SLD-PQ group (1.2%, 19/1557) (OR 11.2, 95%CI 6.9–18.2, $c^2 = 146.28$, d.f. = 1, $p < 0.0001$). Oocyst density was also significantly higher in mosquitoes infected by ACT alone treated patients (geometric mean 4.8(range 1–21) oocysts/mosquito) compared to ACT + SLD-PQ treated patients (3.3 (range 1–5) oocysts/mosquito) (t = 4.81, d.f. = 26, Wilcoxon W = 3.0, $p = 0.034$).

Gametocyte prevalence after treatment

*Pfs*25 qRT-PCR detected gametocytes in 13.0% (25/192) of individuals at baseline, whereas none of the enroled patients were positive microscopically at baseline. There was no significant difference in gametocyte prevalence at baseline between ACT alone (11.6%, 10/86) and ACT + SLD-PQ arm (14.2%, 15/106) (OR 1.25, 95%CI, 0.53–2.95, $p = 0.606$) (Table 3). By day 7 post-treatment, gametocyte prevalence was significantly lower in ACT + SLD-PQ treatment (2.8%, 3/106) compared to ACT alone treatment (11.6%, 10/86) (OR, 0.22, 95%CI, 0.06–0.83, $p = 0.026$) (Table 3). By day 14 post-treatment, no gametocyte had been detected in the ACT + SLD-PQ treatment group compared to 4.7% (4/86) gametocyte prevalence in the ACT alone treatment (OR 0.44, 95%CI 0.37–0.51, $p = 0.039$) (Table 3). No gametocyte was detected in both treatments after day 21 post-treatment. In the SLD-PQ arm, 93.3% (14/15) of the 15 participants who were sub-microscopically gametocytaemic on Day 0 cleared their gametocytes by Day 7. There was only one person (6.7%) who was still gametocytaemic. By Day 14, none of the 15 participants had any gametocytes.

Infectiousness of patients with submicroscopic gametocytaemia

Mosquito infection was the outcome of 11.5% (11/96) of the 96 membrane-feeding assays that were performed. Mosquito infections were caused by submicroscopic gametocytaemia in 90% (9/10) of the ACT-alone group and 67% (2/3) of the ACT + SLD-PQ group. Both treatment arms showed a significant correlation between mosquito infection and submicroscopic gametocyte presence; Fisher's exact test revealed p < 0.001 for ACT alone and p = 0.002 for ACT + SLD-PQ (Table 4).

Table 3 Summary of gametocyte prevalence in different treatment groups during follow-up, measured by *Pfs25* PCR

Follow-up (days)	Treatment	Positive, % (n/N)	Negative, % (n/N)	COR	95%CI	*p*-value	AOR	95%CI	*p*-value
Day 0	ACT alone	11.6 (10/86)	88.4 (76/86)	1			1		
	ACT + SLD-PQ	14.2 (15/106)	85.8 (91/106)	1.253	0.53–2.95	0.606	1.202	0.50–2.90	0.683*
Day 7	ACT alone	11.6 (10/86)	88.4 (76/86)						
	ACT + SLD-PQ	2.8 (3/106)	97.2 (103/106)	0.221	0.06–0.83	0.026	0.215	0.06–0.84	0.027**
Day 14	ACT alone	4.7 (4/86)	95.3 (82/86)						
	ACT + SLD-PQ	0.0 (0/106)	100 (106/106)	0.436	0.37–0.51	0.039	0.436	0.37–0.51	0.039**

(n refers to the number of positive/negative gametocytes; N represents the total samples tested)

*Significant at p < 0.25

**Significant at p < 0.05

Table 4 Infectiousness to mosquitoes by treatment arm

Treatment			Mosquito infection		*p*-value
			Positive n (%)	Negative n (%)	
ACT alone	Day 7 pfs25 RT-PCR	Positive	9 (100%)	1 (3.4%)	
		Negative	0 (0.0%)	28 (96.6%)	p < 0.001*
ACT + SLD-PQ	Day 7 pfs25 RT-PCR	Positive	2 (100%)	1 (1.8%)	
		Negative	0 (0.0%)	55 (98.2%)	p = 0.002*

*Significant at p < 0.05

Discussion

This study evaluates the effectiveness of ACT therapy in reducing the spread of malaria from host to vector when combined with the WHO-recommended single low-dose PQ. A similar study by Dicko et al. [20] in Mali looked at individual infectivity to mosquitoes with similar regimens, however, these trials, carried out in a controlled environment with a small population of men and boys, limit the generalizability of the findings to real-world settings. This cohort study in Ethiopia aimed to evaluate the efficacy of SLD-PQ, addressing practical challenges such as implementation and adherence within standard healthcare systems.

This study adds value by detecting submicroscopic gametocytes using the pfs25 assay and microscopy in addition to the membrane feeding assay, which is the gold standard for mosquito infectivity. The study specifically examines the duration of *P. falciparum* gametocytes following treatment and its impact on transmission dynamics, including the proportion of infected individuals, the rate of mosquito infection following blood consumption from infected individuals, and the intensity of oocyst-stage infection in mosquitoes. These elements are critical to understanding and preventing the spread of malaria [14, 30, 54, 55].

This study demonstrates the effectiveness of both ACT-alone and ACT + SLD-PQ regimens, with high clinical and parasitological response rates by Day 28 and no parasitaemia on Day 7, indicating early clearance. However, between Days 21 and 28, a case of recurrent parasitaemia was observed in the ACT + SLD-PQ arm. Microscopically, the recurrence was verified in the patient who initially showed symptoms on Day 24 with a parasite density of 10,057/μL of blood. Since no drug concentration measurements were made at the time of the recurrence, it is impossible to determine whether the recurrence was caused by insufficient medication exposure or potential resistance. To better understand the recurrence process, future research should include pharmacokinetic studies and molecular analysis to distinguish recrudescence from reinfection and identify potential resistance markers.

According to the study's findings, gametocytes were highly prevalent at baseline, suggesting that even in low-transmission environments, people with symptomatic malaria can contribute significantly to the spread of the disease [11]. This is consistent with previous findings and highlights the insufficient sensitivity of microscopy in identifying potentially infectious individuals [56]. The presence of submicroscopic gametocytes highlights the critical requirement for focused intervention techniques to control and prevent transmission.

The results of this study revealed that single low-dose PQ treatment significantly reduced malaria transmission by lowering the proportion of patients with transmissible parasites, the proportion of mosquitoes infected, and the oocyst density in infected mosquitoes [20, 23]. These findings are in line with other research that demonstrated that combining ACT with a single lowest dosage of primaquine dramatically lowers the transmission of malaria seven days following therapy [22]. This is mostly because primaquine may sterilize *P. falciparum* gametocytes in less than a day, which reduces the infectious reservoir and stops transmission as soon as it is administered [15].

One interesting finding from this study was that, although gametocyte prevalence steadily declined following both treatments, gametocyte clearance was accelerated (before day 14) by ACT with single low-dose primaquine (SLD-PQ) as opposed to ACT treatment alone (before day 21). This finding is consistent with primaquine's well-known quick sterilizing effect, which neutralizes gametocytes within 24 h. Although primaquine completely blocks transmission and sterilizes the infection in 24 h, it may take several days to remove non-viable gametocytes [15]. The observed variations in gametocyte clearance between ACT + SLD-PQ and ACT alone most likely result from primaquine's quick sterilizing action on the gametocytes. Even though ACT alone can quickly eradicate asexual parasites and reduce clinical symptoms, there is still a chance of transmission because mature gametocytes can survive [57]. The addition of a single low-dose primaquine, however, attempts to close this gap by sterilizing gametocytes in a matter of hours and

preventing mosquitoes from acquiring them [5, 58, 59]. While ACT quickly diminishes asexual parasites, White et al. showed that they also indirectly lower gametocyte carriage by affecting the burden of asexual parasites. Though ACT does not directly target adult gametocytes, some people may still have them following treatment [29]. Furthermore, as ACT successfully eliminates immature gametocytes and asexual parasitaemias, gametocytes following treatment probably represent mature gametocytes that existed before ACT treatment. This result is also consistent with the growing body of evidence showing that single low-dose PQ is efficacious for reducing gametocyte carriage [17, 22, 50, 60], which emphasizes the necessity of adding gametocytocidal agents, such as PQ, to ACT regimens to achieve effective transmission interruption. It is also known that PQ clears gametocytes faster than ACT alone, however this usually happens for a week rather than instantly. This has been shown in various trials where PQ's ability to prevent transmission is evident within 24 h, although full gametocyte clearance may take several days [15, 17, 25, 61]. These results show that even while submicroscopic gametocytes are below the threshold for microscope detection, they are nonetheless infectious to mosquitoes and help sustain malaria transmission after therapy [62–64]. Although post-treatment transmissibility was the main focus of this work, it also shows that submicroscopic gametocytes can infect mosquitoes. These findings demonstrate how crucial it is to include both patent and submicroscopic gametocytes in plans to eliminate malaria.

Primaquine's unique action on mature gametocytes, which are necessary for the spread of malaria from humans to mosquitoes, makes it biologically successful in halting *P. falciparum* transmission [15]. Specifically targeting mature *P. falciparum* gametocytes, primaquine predominantly sterilizes them, decreasing their ability to infect mosquitoes during a blood meal [28, 65]. By interfering with this critical stage of the parasite's life cycle, PQ helps break the transmission cycle and reduce malaria transmission [66]. Although this approach does not directly benefit the dosed individual it makes a substantial contribution to efforts aimed at controlling and ultimately eliminating the disease [67].

Although sensitive molecular gametocyte detection methods and mosquito infectivity assays were used in this study, the lack of baseline mosquito membrane-feeding tests, which are thought to be the best design for evaluating the transmission-blocking effect of antimalarial medications on *Plasmodium* gametocytes, is a limitation of this work. An in-depth examination of the medication effect across intervention groups, particularly concerning the decrease in the mean parasite count in mosquito samples, was not possible because these time points were excluded from the investigation. Among the limitations of this study are the inherent variability in mosquito feeding experiments, both within and between experiments, which may have complicated the estimation of the drug effect and should be taken into account in the analysis to better interpret the results. Additionally, because oocysts arising from gametocytes exposed to antimalarial drugs may not produce viable sporozoites, the reduction in sporozoite carriage should be the primary outcome of transmission-blocking assays; finally, the fact that sporozoite carriage was not directly assessed in this study may have limited the conclusions made about the effectiveness of the drug in preventing transmission. In addition, a week after receiving a single low-dose PQ treatment, a percentage of the gametocytes detected by molecular methods might not be infectious because it's unclear if non-viable gametocytes can produce Pfs25 mRNA, and some of the gametocytes identified might not have been infectious. The present study also uses the Pfs25 mRNA qRT-PCR, which identifies female *P. falciparum* gametocytes with specificity. As a result, male gametocytes were not assessed, which restricted the ability to fully comprehend gametocyte dynamics and their possible role in transmission. Another limitation of this study is the lack of molecular gametocyte quantification, which affects the ability to establish a clear relationship between low gametocyte densities and infectiousness. Therefore, future research examining the gametocytocidal effects of single low-dose PQ is preferable to incorporate mosquito-feeding assays periodically throughout the follow-up period.

Conclusion

The findings of this study support the WHO recommendation to use artemisinin-based combination therapy (ACT) in combination with a single low dosage of primaquine (0.25 mg/kg) to restrict the spread of *P. falciparum*. Despite transmissible submicroscopic gametocytes, post-treatment infectivity to mosquitoes was significantly reduced after the ACT and primaquine combination therapy. However, more research is needed to validate these findings across other transmission settings and demographic populations. Further research is required to assess this strategy's practical viability and efficacy under different conditions.

Abbreviations

ACT	Artemisinin-based combination therapy
AL	Artemether-lumefantrine
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
Cq	Quantification cycle

DNA	Deoxyribonucleic acid
ICEMR	International Center of Excellence for Malaria Research
IQR	Interquartile range
MFA	Membrane feeding assay
mRNA	Messenger RNA
PCR	Polymerase chain reaction
PQ	Primaquine
RH	Relative humidity
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SLD-PQ	Single low-dose primaquine
WHO	World Health Organization

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-025-05279-9.

Additional file 1

Additional file 2

Acknowledgements
We thank the Arjo sugar factory, and the staff of Arjo District Health Office for their support and cooperation; Tsehay Orlando (WHO certified microscopists at Adama Malaria Diagnostic Centre) for cross-checking malaria blood smears; attendants of the ICEMR project workers for their dedicated fieldwork and in the insectary, which is very much appreciated; and the Department of public health and occupational health, University of California, Irvine staff who supported this study; staff of Infectious diseases laboratory, UCI, and, the Department of Microbial, Cellular and Molecular Biology, Science faculty, Addis Ababa University, Addis Ababa, Ethiopia, and Tropical and Infectious Diseases Center (TIDRC), Jimma University, Jimma, Ethiopia.

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Funding
This work was supported by the National Institutes of Health (NIH) (D43 TW001505, R01 A1050243, and U19 AI129326). The study funders had no role in the study design, data collection, analysis, decision to publish, or manuscript preparation.

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

Declarations

Competing interests
The authors declare no competing interests.

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Received: 11 October 2024 Accepted: 1 February 2025
Published online: 17 March 2025

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