

Gametocyte Carriages of *Plasmodium falciparum* (pfs25) and *Plasmodium vivax* (pvs25) during Mass Screening and Treatment in West Timor, Indonesia a Longitudinal Prospective Study

Aylen Alicia Kosasih

University of Indonesia: Universitas Indonesia <https://orcid.org/0000-0001-6470-1808>

Cristian Koepfli

Notre Dame University

M. Sopiudin Dahlan

PT Epidemiologi Indonesia

William A. Hawley

Centers for Disease Control

J. Kevin Baird

Oxford University: University of Oxford

Ivo Mueller

Walter and Eliza Hall Institute of Medical Research

Neil F. Lobo

Notre Dame University

Inge Sutanto (✉ sutanto.inge@yahoo.com)

Universitas Indonesia <https://orcid.org/0000-0003-2488-5133>

Research

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Abstract

Background:

Three rounds of mass screening and treatment (MST) demonstrated no effect on *Plasmodium falciparum* and *P. vivax* incidence in West Timor, Indonesia. This study nested within that trial evaluated the effect of MST on gametocyte carriage.

Methods:

Microscopy and PCR diagnostics were applied to study subjects through 3 months of MST involving dihydroartemisinin-piperaquine (DHP) and primaquine based on infecting *Plasmodium* species. RT-qPCR targeting the *pfs25* and *pvs25* sequences was conducted to detect and quantify gametocytes in blood samples of *P. falciparum* and *P. vivax*-infected subjects. Data from the baseline and endpoint were compared ($p < 0.05$ as the significance threshold).

Results:

Between baseline and endpoint, prevalence remained unchanged for *P. falciparum* (6%=52/811 versus 7%=50/740, $p=0.838$) and decreased slightly for *P. vivax* (24%=192/811 versus 19%=142/740, $p=0.035$). No significant difference was observed in gametocyte carriage rates for either *P. falciparum* (43%=19/44 versus 59%=23/39, OR & 95%CI=1.89, 0.72-4.97) or *P. vivax* (39%=49/125 versus 37%=39/106, OR & 95%CI=0.90, 0.51-1.59). Despite the insignificant differences between the two time points, the majority of positive subjects at the endpoint were newly parasitemic individuals (new infections) (Pf:92%=46/50, Pv: 65%=93/142). This result was similarly demonstrated for the transmissible stage - where new gametocyte carriers dominated endpoint positive subjects (Pf: 96%=22/23, Pv: 95%=37/39).

Conclusion:

Although three rounds of MST decreased individual parasitemia and gametocytemia based on diagnosis and treatment, this failed to impact malaria prevalence at a population level due to the development of new infected individuals that can sustain transmission. These observations in part explain why MST failed to impact transmission of malaria in this transmission context.

Background

Global malaria elimination by 2030 will require dramatic progress spurred by innovative approaches aimed at doing so [1]. One of the greatest obstacles may be the existence of large reservoirs of infection beyond the reach of conventional methods of malaria diagnosis.

Human-to-mosquito transmission requires the uptake of male and female gametocytes (sexual stages) by the mosquito [6]. A growing number of studies are being conducted towards the identification of super-

spreader individuals that carry gametocytes and may contribute substantially to transmission in an area – pointing to the importance of treatment of the individual [7–10].

Gametocytes originate from the asexual cycle in humans. In an infected individual, a small proportion of the total parasites differentiate into gametocytes [6]. *Plasmodium falciparum* immature gametocytes sequester in the capillaries of inner organ until they mature, typically taking 10–14 days [6]. Since blood schizonticides do not kill mature gametocytes - they may be present 2–4 weeks post treatment [11–13]. Conversely, *P. vivax* development occurs concurrent to that of asexual parasites with treatment rapidly removing them from circulation [6, 14].

Asymptomatic infections of *P. falciparum* and *P. vivax* are common in varied transmission settings [15–17]. These infections are often of low density and escape detection with microscopy or rapid diagnostic test (RDT) [15, 17]. As gametocytes commonly constitute only 1–5% of all parasites in the blood [18], molecular methods, such as reverse-transcriptase qPCR (RT-qPCR) of gametocyte-specific transcripts, are increasingly used to detect and quantify this stage [7–10, 19].

Though frequently reported to be less infective compared to higher density symptomatic infections [20–22], these asymptomatic infections may contribute substantially to transmission due to their high prevalence in communities [23]. It has been estimated that asymptomatic individuals are the source of up to 50% of transmission [15], thus, transmission stemming from asymptomatic infections may represent an important problem in control and elimination of malaria [24].

Mass drug administration (MDA) and mass screen and treat (MST) strategies have been developed and implemented as means of attacking that asymptomatic reservoir [2–5]. In MDA, treatment is given to all residents of a prescribed area without regard to infection status, whereas in MST treatment is only given to residents who test positive for infection.

We reported that three rounds of MST administered in cluster randomized fashion at sites in endemic West Timor in eastern Indonesia failed to diminish the incidence of malaria [5]. In order to better understand that finding, we specifically examined gametocyte carriage among residents living in those clusters.

Methods

Study area

This study was conducted in Wewiku subdistrict, Belu district, East Nusa Tenggara province, in eastern Indonesia (June–September 2013). The study area was reported to have the highest malaria endemicity in the province. The annual parasite index (API) per 1,000 population was 72 and 124 in 2011 and 2012 respectively (Belu district health office, personal communication), whereas in the entire East Nusa Tenggara province it was 25.8 and 21.1 during the same period [25]. *Anopheles barbirostris* was reported

as the major malaria vector [5]. Other less dominant vectors included *An. subpictus* and *An. vagus*. A more detailed description on the study site has previously been reported [5].

Sample Collection

The original cluster-randomized trial evaluated the impact of MST conducted a) twice, and b) three times, both within 3 months [5]. Positivity for malaria parasites by microscopy was 8% in both MST at baseline, and 9% each at the last round of MST [5]. MST exerted no impact on measured incidence of diagnosed infections in both arms. In this analysis, round-1 of MST from both treatment groups were pooled and considered the 'baseline', while the last time-point from both groups was also pooled and considered the 'endpoint'.

Blood samples (n = 1551) were collected into EDTA tubes from 866 study subjects. All microscopy-positive subjects were treated with dihydroartemisinin-piperaquine as a blood schizonticide, and primaquine (*P. falciparum*: 0.75 mg/kgBW single dose, *P. vivax*: 0.25 mg/kgBW/day for 14 days). For RNA extractions, 50 μ L of blood was mixed with 250 μ L of RNAProtect (Qiagen) within 4 hours of collection, and stored at -80°C until processing.

Molecular Analysis

Microscopic and PCR screening results have been reported in the parent study [5]. DNA quantification was performed by species-specific qPCR as described previously with some modification [26]. Details on the laboratory procedures are described in the Additional file 1. All *P. falciparum* and a set of randomly selected *P. vivax* positive samples underwent RNA extraction (Fig. 1) using the Quick RNA Mini-prep kit (Zymo Research, USA) according to manufacturer's instruction. *P. falciparum* and *P. vivax* gametocytes were quantified by two-step RT-qPCR of the gametocyte markers *pfs25* and *pvs25* [27]. Transcriptor First Strand cDNA kit (Roche) was used to generate cDNAs for each sample in triplicates. During this first step, 4 μ L RNA was mixed with 2 μ L random hexamer, 1 μ L anchored oligo (dT) primers, 4 μ L RT buffer, 2 μ L dNTP, 0.5 μ L RNase inhibitor, and 0.5 μ L Reverse Transcriptase enzyme for a final volume of 20 μ L. The incubation temperature was 10 min at 25°C, followed by 30 min at 55°C, then 5 min at 85°C. The tube was then immediately put on ice. The presence of mRNA transcript was verified using RT-qPCR targeting the 18S rRNA [28]. Only samples positive for 18S were analyzed for *pfs25* and *pvs25*. The *pfs25* and *pvs25* qPCR was conducted in 12 μ L total volume containing 6 μ L FastStart Essential DNA SYBR Green master (Roche), 4 μ L cDNA, and 0.417 μ M each of previously published primers [29]. The cycle conditions were as follows: 10 min at 95°C, followed by 45 cycles of 15 min at 95°C and 1 min at 58°C. T_M for *P. falciparum* was 74-75°C, and 79-80°C for *P. vivax* (Additional file 2). For quantification, series of plasmid harboring target sequence with concentration of 10^5 , 10^4 , 10^3 , 10^2 , 10, 5, and 1 copy per reaction were run in triplicate and a standard curve generated for each run. Negative (no template) controls were included in triplicates. The limit of detection (LOD) as assessed by running serial dilution of the plasmid in quintuples

and determined as 10 copies/ μ L for *pfs25* and *pvs25*. Performance of the assay is described in Additional file 3. All triplicates of the cDNA were run and recorded as positive if a minimum of two of the three demonstrated a positive result. Quantities of the transcript were reported as the average transcript numbers of the replicates to the nearest CT values.

All laboratory analyses were performed blindly to subject identification (ID). Upon completion of all laboratory work, the results were linked to the subject ID, MST time point, and treatment status. All laboratory analyses were conducted at the Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, University of Indonesia, Jakarta.

Data analysis

Categorical variables were analyzed using chi square test. Numerical variables were analyzed using Student's t-test when the distribution was normal or Mann-Whitney when it was not normal. 18S rRNA gene copies and *pfs25/pvs25* transcripts were log transformed for these statistical analyses. Linear regression was conducted to investigate the correlation between 18S and *pfs25/pvs25* numbers. A p-value ≤ 0.05 was considered as statistically significant. All analyses were performed using SPSS version 23 (IBM, Armonk, New York).

Results

Characteristic of the study subjects

A total of 811 samples were collected at baseline and 740 at endpoint. These subjects represented 78% of the total parent study samples. Of the 102 *P. falciparum* positive samples (baseline = 50, endpoint = 52), 83 were available for RNA extraction (Fig. 1). Of the 334 *P. vivax* positive samples (baseline = 192, endpoint = 142), 231 samples were available for RNA extraction (Fig. 1). Comparable demographic characteristics were observed among study subjects between baseline and endpoint (Table 1). Furthermore, 685 (84%) subjects were sampled at both time points.

Table 1
Characteristic of the study subjects

	Baseline	Endpoint
Total subjects	811	740
Age		
< 5 y	90 (11.1%)	88 (11.9%)
5-15 y	177 (21.8%)	160 (21.6%)
> 15 y	543 (67.0%)	490 (66.2%)
Unknown	1 (0.1%)	2 (0.3%)
Male (%)	387 (47.7%)	337 (45.5%)

pfs25 / *pvs25* RT qPCR assay

A single peak was demonstrated in the melting analyses of both *pfs25* and *pvs25* RT qPCR. PCR efficiency for *pfs25* and *pvs25* were 94.7% and 88.1%, respectively. Both assays demonstrated r^2 of more than 0.98 (Pf: 0.986, Pv: 0.988) and LOD of 10 copies/ μ L (Supplementary file S3 Assay performance).

For both species, gametocyte transcript numbers were positively correlated with 18S gene copy numbers, with a stronger correlation observed for *P. vivax* than *P. falciparum* (Pf: $r = 0.321$, $p < 0.001$; Pv: $r = 0.778$, $p < 0.001$, Fig. 2A and B).

Parasitemia (microscopy and/or PCR) and gametocyte carriage between baseline and endpoint

Plasmodium falciparum: Between baseline and endpoint, no significant difference in prevalence was observed (6%=52/811 versus 7%=50/740, $p = 0.838$) (Table 2, Fig. 1). Furthermore, no significant difference was detected for the proportion of gametocyte positive infections (43%=19/44 versus 59%=23/39, $p = 0.189$) (Table 2, Fig. 1). The geometric mean *pfs25* transcript numbers did not show any significant difference either (baseline: 132.5 transcripts/uL, CI95 30.2-580.5, endpoint: 210.9 transcripts/uL, CI95 56.8-782.7, t-test, $p = 0.625$) (Table 2).

A higher number of *pfs25* transcripts were observed among the subjects taking drug compared to those without drug (with drugs: 182.8, CI95 29.1-1150.5, without drugs: 39.6, CI95 3.3-472.1, t-test, $p = 0.39$). Among gametocyte carriers, all children < 5 year of age ($n = 4$), 80% (4/5) of school-aged children (5–15 y), and 70% (7/10) adults (> 15 y) were positive by microscopy and thus given drugs.

Plasmodium vivax

Although the proportion of parasitemic subjects was significantly lower at the endpoint compared to baseline (23.7%=192/811 versus 19.2%=142/740, $p = 0.035$) (Table 2, Fig. 1), no significant difference was seen in the proportion of gametocyte positive infections (39%=49/125 versus 37%=39/106, $p =$

0.786) (Table 2, Fig. 1) as well as the geometric mean of transcript numbers (56.4, CI95 23.8–134.0 transcripts/uL and 65.6, CI95 26.5-162.2 transcripts/uL, p = 0.810) (Table 2).

Subjects identified for treatment harbored approximately 37-fold higher transcripts than the untreated carriers (geometric mean & 95%CI: 356.6, 97.2-1309.8 versus 9.6, 5.1–18.2, t-test, p < 0.001).

Table 2
Parasitemia and gametocyte carriages at baseline and endpoint by species

	Baseline	Endpoint	p value	OR (95%CI)
Total subjects	811	740		
<i>P. falciparum</i>				
Positive by LM and/or PCR	52 (6%)	50 (7%)	0.838	1.06 (0.69–1.61)
No. screened for <i>pfs25</i> ^a	44	39		
Gametocyte carriage (%)	19 (43%)	23 (59%)	0.189	1.89 (0.72–4.97)
Transcript numbers	132.5 (30.2-580.5)	210.9 (56.8-782.7)	0.625	0.6 (0.1–4.2) ^c
<i>P. vivax</i>				
Positive by LM and/or PCR	192 (24%)	142 (19%)	0.035*	0.77 (0.59–0.98)*
No. screened for <i>pvs25</i> ^b	125	106		
Gametocyte carriage (%)	49 (39%)	39 (37%)	0.786	0.90 (0.51–1.59)
Transcript numbers	56.4 (23.8–134.0)	65.6 (26.5-162.2)	0.810	0.9 (0.2-3.0) ^c
^a Based on RNA availability				
^b Based on random selection and RNA availability				
^c Mean ratio (95%CI)				
Abbreviation, LM: light microscopy				

Dynamic of parasitemic subjects and gametocyte carriers at baseline and final time point

Every parasitemic subject and gametocyte carrier was justified individually at each timepoint, and their status was followed or traced backwards. Furthermore, parasitemic/gametocyte status was linked to whether or not they took antimalarial drugs. The drugs were given under direct observation of the field study team.

Plasmodium falciparum

At baseline, 28 of 52 *P. falciparum* were microscopic positive and thus given drugs (Table 3). Of these subjects, 22 were followed at the endpoint and six subjects were lost to follow up. Of those 22, 20 were negative at endpoint while 2 remained positive (Fig. 3A). Among the 28 drug treated subjects, 15 were gametocyte carriers (Table 3). Ten of those 15 subjects were followed, and all were negative at the endpoint.

At the endpoint, on the other hand, there were 50 parasitemic subjects (Table 2). Of these subjects, 40 were negative at baseline, and 6 were not previously seen (Fig. 3A). The other four parasitemic consisted of two subjects with a previous drug treatment (originally from the 22 subjects taken drugs above), and two subjects without treatment (originally from 24 subjects without drugs above) (Fig. 3A, Table 3). Among the 50 parasitemic subjects, 23 were gametocyte carriers (Table 2). Of these carriers, 20 were negative at baseline, while the other 3 consisted of one carrier did not receive drugs and two subjects not seen at baseline (Fig. 3A).

Plasmodium vivax

At baseline, 54 of 192 parasitemic subjects were microscopic positive and thus given drugs (Table 3). Of these subjects, 50 were followed and included in the endpoint sample while four were lost to follow up. Of these 50, 44 were negative at endpoint while 6 were positive (Fig. 3B). Among the 54 subjects with drugs at baseline, 26 were gametocyte carriers (Table 3, Fig. 3B). Of these carriers, 23 were followed at endpoint and 22 were negative (Fig. 3B).

At endpoint, there were 142 parasitemic subjects (Table 2). Of these, 85 were negative at baseline, eight were newly diagnosed subjects, six were given drugs at baseline, and 43 were infected but not treated at baseline (diagnosed positive by PCR) (Fig. 3B). Among the 142 parasitemic subjects, 39 were gametocyte carriers (Table 2). Among these 39 carriers, 30 were negative at baseline, seven were newly diagnosed subjects, one was given drugs at baseline, one was without drug treatment (Fig. 3B).

Table 3
Subjects with and without drug
treatment at baseline

Description	
<i>Plasmodium falciparum</i>	52
Positive by LM (drug treated)	28
Positive by PCR (not treated)	24
Gametocyte carriage (<i>pfs25</i>)	19
Drug treated	15
Not treated	4
<i>Plasmodium vivax</i>	192
Positive by LM (drug treated)	54
Positive by PCR (not treated)	138
Gametocyte carriage (<i>pvs25</i>)	49
Drug treated	26
Not treated	23

Discussion

The current findings corroborate and help explain the observed failure of MST to impact malaria incidence. Despite delivery of effective therapy of patent malaria against all parasite stages, including gametocytes, insufficient asymptomatic reservoir clearance by this intervention resulted in new parasitemic and gametocyte carriers that appeared to sustain untrammled transmission within MST clusters. [5].

Towards understanding the gaps in protection with the MST intervention, it is particularly important to understand the origin of new parasitemic subjects and gametocyte carriers. There are two possibilities for the source of these infections. First, since a high proportion of them were negative at baseline, these infections may stem from newly infected individuals (Fig. 3). Being negative at baseline, most of these subjects (parasitemic and gametocytemic) were thus not given drugs at baseline. In addition, many infections were at too low densities to be detected by light microscopy and thus were also not treated. Several studies have demonstrated that the numbers of parasites and gametocytes may fluctuate in the blood [15, 29] presenting the second possibility that a proportion of carriers, may have escaped a positive diagnosis with levels below the detection limit, and remained not given drugs at baseline. This was partly due to the sequestering parasites which resulted in a negative diagnosis - but were present and detectable at the endpoint [30]. In addition, it is possible that gametocytes in peripheral blood may constitute brief

transience prior to more sustained sequestration in the shallow microvasculature of skin accessible to mosquitoes [31, 32]. We did not explore that possible anatomic compartment in the current study. Lastly, in *P. vivax* there may be a considerable proportion of the population that are bloodstage negative but may have dormant infections in their livers [33].

The actual impact MST has on transmission dynamics raises questions on its role in eliminating malaria. In this context, MDA complying with appropriate safety procedures may be considered over MST. The MDA approach would counter the gaps in protection seen with MST – here diagnostic limits of detection resulted in sustained transmission with a stable infectious reservoir of gametocytes. MDA studies have demonstrated that this approach may reduce incidence in low endemic malaria areas [2, 34]. However, combining this approach with vector control was shown to have bigger impact to lower the incidence [2].

The limitation of this study is the design which is nested within a community trial. The lack of randomization in the design of this study may possess confounding factors – even though multiple criteria, such as age and gender, were comparable between groups (Table 1).

Nevertheless, this study demonstrated the presence of a substantial number of new parasitemic subjects and gametocyte carriers at the end of the MST trial – pointing to a limitation associated with the persistence of malaria transmission. Programs that consider MST as an intervention need to consider its mechanistic impact on malaria transmission, while also taking MDA into consideration in the drive towards malaria elimination.

Declarations

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Author's contribution

WAH, IM, NFL, IS designed the study. AK was involved in the collection of data, performed the laboratory work, analysed the data and drafted the manuscript. AK, CK, MSD, WAH, FHC, JKB, IM, NFL, IS contributed to the analysis and interpretation of the data. All the authors reviewed and approved the final manuscript.

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Availability of data and materials

The dataset generated by this study is available from the corresponding author upon request.

Ethics approval and consent to participate

This study has been approved by Ethical committee of Medical Faculty, University of Indonesia no. 37/H2.F1/ETIK/2013. Informed consent was obtained from all study subjects prior to samples taking.

Consent for publication

Not applicable

Competing interest

The author(s) declare(s) that they have no competing interests.

Author's details

¹PhD Program in Biomedical Sciences, Medical Faculty, Universitas Indonesia, Jakarta, Indonesia. ²Eijkman-Oxford Clinical Research Unit, Jakarta, Indonesia. ³Eck Institute for Global Health, University of Notre Dame, Notre Dame, IN, United States. ⁴PT Epidemiologi Indonesia, Jakarta, Indonesia. ⁵UNICEF, Jakarta, Indonesia. ⁶Center for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, United Kingdom. ⁷Infection & Immunity Division, Walter & Eliza Hall Institute, Melbourne, Australia. ⁸Department of Parasitology, Medical Faculty, Universitas Indonesia, Jakarta, Indonesia.

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Figures

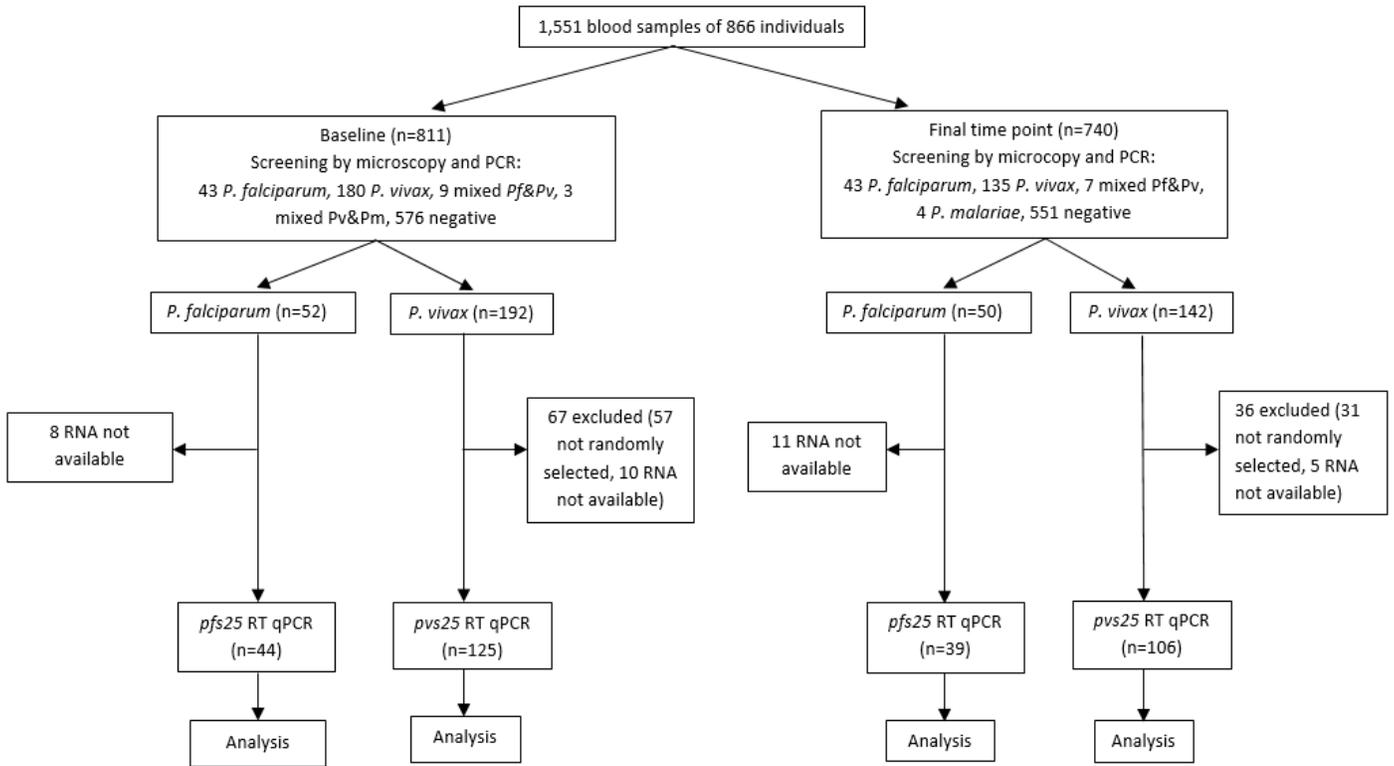
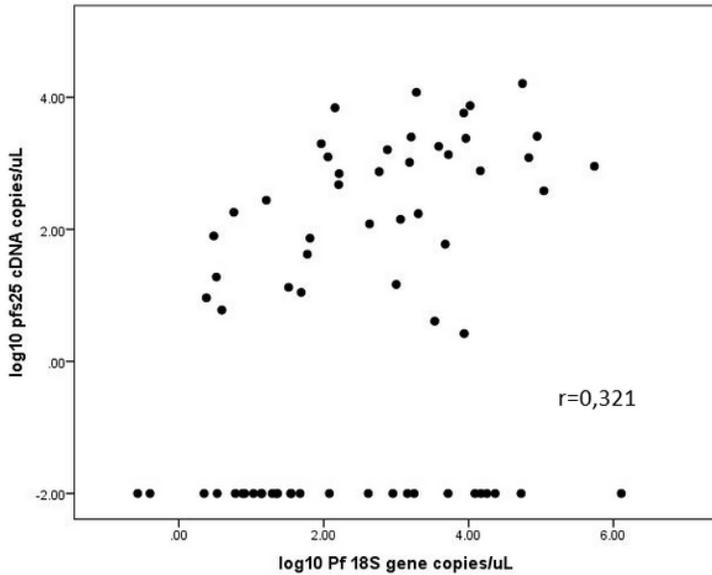


Figure 1

Study framework

A *P. falciparum*



B *P. vivax*

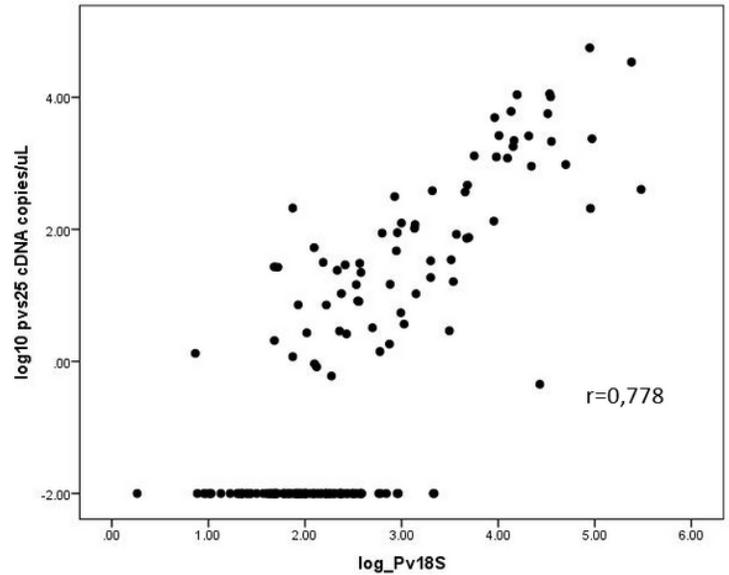


Figure 2

A positive correlation was demonstrated between 18S gene copy numbers/ μL and gametocyte transcript numbers/ μL for (A) *P. falciparum* (*pfs25*) as well as for (B) *P. vivax*/*pvs25*. Linear regression analyses

both demonstrated a significance correlation ($p < 0.001$).

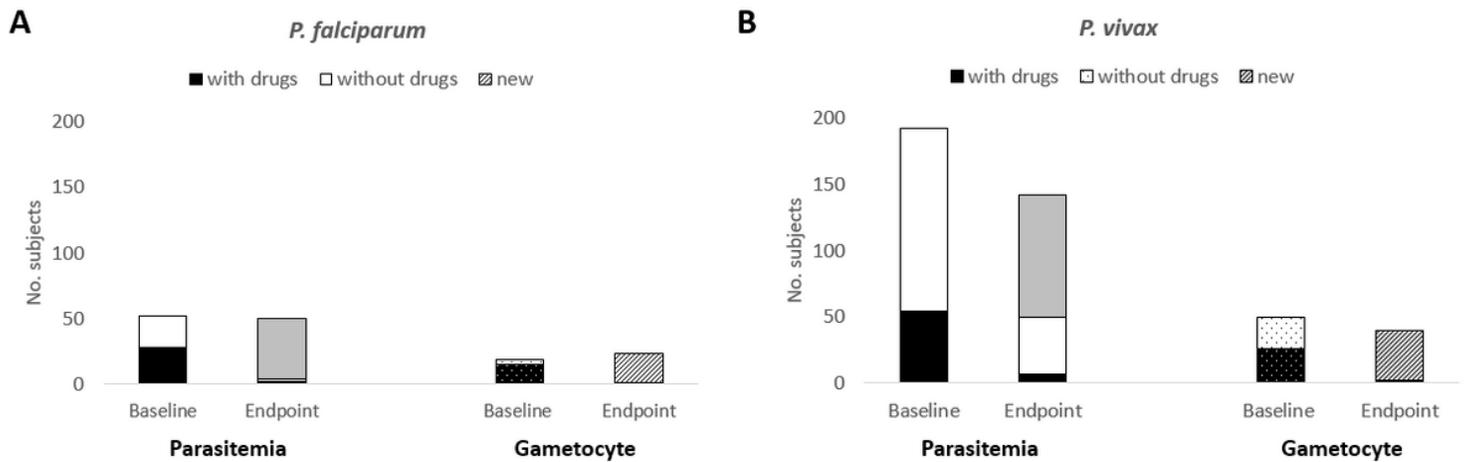


Figure 3

A. Number of *P. falciparum*-positive individuals and gametocyte carriers at baseline and endpoint. At baseline, 54% (28/52) parasitemic subjects were given drugs (), and 46% (24/52) were without drugs (). At the endpoint, only two of each subject with and without drugs remained positive. However, 46 new infected subjects () appeared at the endpoint. Furthermore, in gametocyte carriers, similar pattern was observed. Seventy-nine percent (15/19) carriers were given drugs (), and 21% (4/19) were without drugs (). At the endpoint, nearly all gametocyte carriers were new subjects (96%=22/23, grey bar=), and only one subject without drugs was diagnosed as positive. B. Number of *P. vivax*-positive subjects and gametocyte carriers at baseline and endpoint. At baseline, 28% (54/192) parasitemic subjects were given drugs (), and 72% (138/192) were not given drugs (). At the endpoint, six of those with drugs and 43 of those without drugs were found positive. In addition, 93 new infected subjects () appeared. A similar pattern was also observed in gametocyte carriers. Fifty-three percent (26/49) carriers were given drugs (), and 47% (23/49) were not given drugs (). At the endpoint, majority (95%=37/39) of gametocyte carriers were new (), while only two (one with drugs and one without drugs) remained as carriers.

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