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Consistency and reproducibility of independent feedings using blood from two consecutive days at varying *Plasmodium falciparum* gametocyte densities based on both direct membrane feeding assay and direct skin feeding assay

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Abstract

Background New malaria control tools are needed to prevent the transmission of parasites from the host to the mosquito vector and vice versa. The infectiousness of *Plasmodium falciparum* gametocytes obtained from individuals to laboratory-reared mosquitoes should be quantified to employ easily applicable assays for evaluating transmission-blocking interventions. This study aimed to establish the relationship between parasite transmission from humans to mosquitoes both within a person and across persons by assessing the variation in the proportion of infected mosquitoes with at least one oocyst (oocyst prevalence) in a direct membrane feeding assay (DMFA) and direct skin landing feeding assay (DSFA) performed at two consecutive time points in the same human subject with *P. falciparum* gametocytaemia.

Methods A total of 400 adults without symptoms of malaria residing in Western Kenya were screened for the presence of *P. falciparum* gametocytes. Individuals who tested positive had DMFAs and DSFAs on two subsequent days of feeding, baseline and final visit, to compare mosquito infection rates between the two feeds.

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Results Blood samples from 42 individuals testing positive for gametocytes underwent mosquito infection assays. Survival rates of mosquitoes after feeding at baseline and final visit were 13.2 and 11.6 days for DMFA and 12.1 and 11.4 days for DSFA, respectively. The mean oocyst prevalence on feeding at baseline and final visit was 6.3% and 2.2% for DMFA and 5.2% and 2.3% for DSFA, respectively. A not significantly lower prevalence was not observed on at final visit (– 0.016% for DMFA, p = 0.795) and -0.025% for DSFA, p = 0.711 compared to feeding at baseline. The correlation of oocyst prevalence between feeding baseline and final visit for both was low (0.3 95%CI (0.15, 0.51). Exploratory analysis suggests a lower probability of infection on the second day, with lower oocyst density.

Conclusions These findings have implications for future studies and limit the utility of before-after designs in testing transmission-blocking interventions. The findings show comparable infection rates in both DMFA and DSFA, which allows use of less invasive membrane assays in future studies.

Trial registration NCT04666350.

Keywords Malaria, Gametocytes, Transmission blocking, Clinical trial design, Before-after

Background

In addition to the continued application of malaria preventive and control measures, many countries are embracing and committed to eradicating malaria in conjunction with burden reduction of clinical disease. New, safe, and effective vaccines, drugs, or biologics for blocking the transmission of parasites from the host to the mosquito vector and vice versa will help accomplish this goal. On the preclinical side, the recent innovation of ex vivo drug assay for assessing the transmission-blocking activity of compounds using clinical samples with naturally acquired gametocytes besides the classical in vitro culture of gametocytes has accelerated the discovery of such tools [1]. Optimization of experimental medicine platforms to efficiently and objectively estimate the effect of anti-malaria interventions on reducing mosquito-to-human parasite transmission is a high priority.

The formation of *Plasmodium* oocysts on the mosquito midgut wall after standard membrane feeding assay (SMFA), direct membrane feeding assay (DMFA), or direct skin feeding assay (DSFA) are commonly used as evidence for successful transmission [2] (Note: it is preferable not to hyperlink the references, as it interferes with the editing). In this study, we [PLEASE NOTE THAT MALARIA JOURNAL DOES NOT USE FIRST PERSON FORMAT; ADJUST ALL SENTENCES ACCORDINGLY] This study designed a platform for estimating the consistency and reproducibility of two consecutive DSFAs at 24-h intervals. Specifically, on screening day referred to herein as day of initial sample collection, participants were enrolled in to the study for the purpose of diagnosis for presence of gametocytes. By end of screening day, results from the research lab were reported to the clinician. The clinician used this result to contact the individuals who had tested positive for gametocyte lifecycle stage, requesting them to come and undergo mosquito infection assay. Those willing to participate further were then invited the following day, i.e. one day after diagnosis. This day was therefore referred to as Day 1 of the feeding. Day 2 was therefore the follow-up day for those who gave informed written consent to undergo mosquito infection. The initial sample collection, the day before day 1, is also defined by the Clinical Data Interchange Standards Consortium standards as day -1. The study also assessed the relationship between parasite transmission from humans to mosquitoes both within a person and across persons by assessing the variation in the proportion of infected mosquitoes with at least one oocyst (oocyst prevalence) in DMFA and DSFA performed at two consecutive time points in the same human subject with *Plasmodium falciparum* gametocytaemia. The results from this experimental medicine study provide additional considerations that can aid in the design of "before-after" clinical trials in which each human volunteer serves as their own internal control to be applied in follow-up Phase 2b efficacy trials.

Methods

Study area

The study was carried out in Kombewa sub-county, Kisumu County, Western Kenya; Latitude: 0° 6′ 12″ S; Longitude; 34° 31′ 4″ E. The region is classified based on malaria burden as Lake Endemic Zone, malaria holoendemic, experiencing malaria throughout the year with two peak seasons [3]. In 2016, malaria accounted for 29.9% of all outpatient visits and 36.9% of hospital admissions, with a 5.2% fatality rate in the sub-county, mostly among children younger than five years old [4]. Malaria burden appears to be driven by high entomological inoculation rate, representing a measure of the number of infectious bites per person per year, occurring at 5.12 infective bites per person per year with peaks in the rainy seasons [5, 6].

Study participants

Individuals, male and female, aged 18 to 55 years, residing within the Kombewa Health and Demographic Surveillance Systems study area, and in good general health as evidenced by medical history and clinical examination before entering the study were enrolled after providing written informed consent and agreeing to adherence to study procedures. Female participants agreed to the use appropriate contraceptive measures to prevent pregnancy for 30 days after receiving artemether and lumefantrine (Coartem®) and primaquine or were assessed as being non-childbearing (surgically sterilized or at least one-year post-menopausal). The male participants were required to endorse pregnancy risk reduction for at least three months after treatment with primaquine. All participants with positive PCR for *P. falciparum* gametocytes were included in this analysis.

Recruitment

The study experimental design is summarized in Fig. 1. To initiate the study, project staff convened community sensitization through public meetings ("barazas") prior to the human subject recruitment process. The community in which the study took place was informed about the nature and design of the study though community leaders (chiefs, local village elders, and opinion leaders) and local health authorities who formally briefed the potential participants in their own language(s).

Blood sample collection

Field worker teams (nurses and/or clinical officers with a field assistant) approached potential participants within their homesteads and briefed them on the study prior to obtaining written informed consent. The team collected demographic data and a brief malaria history to assess eligibility. A blood sample (2 mL) was obtained from all individuals meeting eligibility criteria at this stage at the participants' homestead and transferred to a tube containing ethylenediaminetetraacetic acid, placed in a sample transportation box at +4 to +8 °C, and sent to the central Malaria Drug Resistance Laboratory for PCR assays, first for diagnosis of presence of *Plasmodium* genus indicative of active infection and followed by detection of the *P. falciparum* gametocyte life-cycle stage which is infective to mosquitoes.

Diagnoses for all samples were complete and relayed back to clinical staff based in the field within 12 h after sample collection. This real-time relaying of results

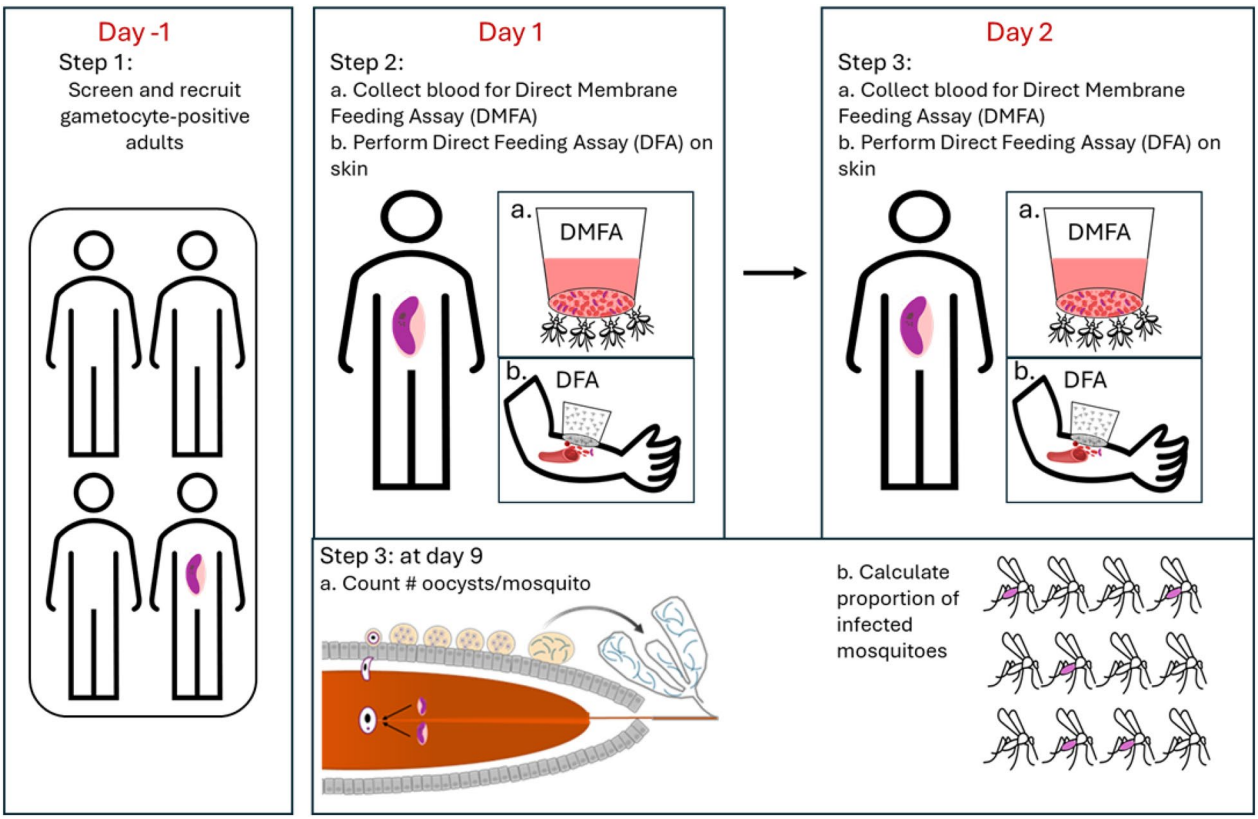

Fig. 1 Experimental design

enabled contacting of individuals who tested positive for gametocytes to be brought to the research clinic for onset of feeding baseline visit within 24 h of initial sample collection. For feeding day 1 – feeding baseline visit, the participants were hospitalized overnight for clinical care. Successive feeding day 2—feeding final visit was then conducted within 48 h of initial sample collection (within 24 h from feeding baseline visit), prior to initiation of standard of care case management for all participants.

Mosquito feeding assays

Mosquito feeding assays involved feeding unfed, sterile (pathogen-free), insectary-reared *Anopheles gambiae* mosquitoes on malaria-infected humans in order to obtain oocysts between day 8 and 9 and sporozoite counts on day 14 in the mosquito's midgut and salivary glands, respectively, thereby allowing an accurate evaluation of malaria transmission. Briefly, the Kenya Medical Research Institute (KEMRI)/ US Army Medical Research Directorate-Africa (USAMRD-A) entomology laboratory maintained a colony of *An. gambiae* sensu stricto (KISUMU strain) that was initially collected at Lwanda village, Siaya County, in western Kenya. Mosquitoes were given a 10% water-infused sucrose meal daily, maintained at 28 ± 2 degrees centigrade and 70–80% relative humidity similar to previously described ranges [7]. Cow's blood was used to maintain the colony, and only three-to-five-day old female mosquitoes were used for the feeding assays. This study conducted two types of mosquito-feeding assays using study participants concurrently: direct membrane feeding assay (DMFA) and direct skin feeding assay (DSFA).

DMFA was performed using the general procedures of Bousema et al. [8] and Da et al. [9] for membrane feeding, with the notable exception that serum in collected subject blood samples was not replaced with non-immune serum. Briefly, 0.12 mL of whole blood collected from study participants just prior to the feeding assays was transferred to 2 mL-capacity glass receptacles that comprise the artificial feeders warmed at 36 to 37 °C using a circulating water bath and provided to three- to five-day-old starved mosquitoes; n = 30 per cup; 2 cups per subject.

DSFA was performed using the protocol described by Bonnet et al. [10] and Bousema et al. [8]. Two cups, each containing 30 sterile three- to five-day-old *An. gambiae* sensu stricto (*s.s.)* female mosquitoes (KISUMU strain) were starved for five hours and transferred to a cardboard cup covered with netting before use. The mosquito-containing cup was placed on the calf or arm of each volunteer to feed for 15 min, after which antihistamine ointment was applied to the volunteer's skin.

Feeding baseline visit procedures

Consenting participants identified as gametocytaemic by RT-PCR were transported to the KEMRI/USAMRD-A laboratory in Kisian within 48 h. A blood sample (~ 5 mL) for DMFA was collected from each subject prior to the DSFA for use in parallel DMFA as well as molecular diagnosis for gametocytaemia. Upon completion of the first DSFA, the individual stayed overnight in the clinical trial center under staff supervision at the Kombewa Clinical Research Clinic-affiliated sub-county hospital (KCRC).

Feeding final visit procedures

Participants were transported to the KEMRI/USAMRD-A laboratory in Kisian for the second DMFA and DSFA assay within 24 h (± 3 h) of the first DMFA/DSFA. A different site of the skin was selected for DSFA on feeding final visit, as well as different cups of mosquitoes.

Participant management

Upon completion of feeding final visit of DSFA, after drawing blood, the attending clinician administered Coartem® tablets (Novartis; Basel, Switzerland), which contain artemether (20 mg) and lumefantrine (120 mg), in accordance with the Kenya Ministry of Health recommended case management guidelines for uncomplicated malaria. A dose of primaquine Advacare Pharma Mumbai, Maharashtra 400,072, India, was given to each participant to clear gametocytes. Each participant was provided with their remaining five doses (20 tablets) and advised to take the next dose after 8 h, followed by the remaining doses at 12-h intervals until completion of treatment. Participants were instructed to seek medical care should any symptoms occur.

Mosquito and insectary procedures

All fed (and potentially infectious) mosquitoes were held in locked environmental chambers located at the ACL-2 insectary at the USAMRD-A entomology facilities for both security and optimized conditions for maintenance of the mosquitoes. Any unfed mosquitoes were transferred to a second container and killed by placing in a − 20 °C freezer for 48 h prior to disposal. Out of the 30 mosquitoes in each cup, approximately 5% were not fed. Approximately 29 mosquitoes from one of the cups were held up to Day 9 or Day 10 post feed for oocyst count determination those in the remaining cup were held up to Day 14 or Day 15 post-feed for sporozoite count determination. The maximum anticipated mortality for mosquitoes on D9/D10 was approximately 15%, while 50% of the mosquitoes on D14/D15 died. The remaining live mosquitoes were available for oocyst counts and sporozoite counts.

Gametocyte detection

Molecular diagnosis for malaria was done by an initial round of PCR to amplify the conserved *Plasmodium* 18S ribosomal ribonucleic acid (rRNA) gene as described by Andagalu and coworkers [11, 12]. All samples that tested positive for *Plasmodium* species were diagnosed for the presence of gametocytes using a one-step RT-PCR that amplifies RNA without the need for conversion of the RNA into stable complementary DNA (cDNA) prior to detection of gametocyte-specific proteins Pfs16 and Pfs25. The primers designed for this step alongside the reaction conditions as previously described [13–16]. Based on this assay, The PCR cycle threshold value (cT) representing of the number of cycles needed to replicate enough nucleic acids detect gametocyte positive samples was set at below 31 cycles, equivalent to about < 1.28 gametocytes/μL. Samples amplifying for at least one or both Pfs16 and Pfs25 below the Ct proceeded for feeding.

Oocyst and sporozoites detection

Approximately half of fed mosquitoes were maintained for 9 to 10 days post-feeding for detection and quantification of oocysts in the midgut and previously described [11]. The midguts were dissected from mosquitoes, stained with 1% mercurochrome and examined by microscopy. Oocyst numbers in each midgut were recorded. Salivary glands from the remainder of fed mosquitoes were dissected 14 to 15 days post-feeding, submerged in phosphate-buffered saline to visualize and score motile sporozoites by microscopy.

Statistical methods

A descriptive analysis for each feeding visit and feeding method, the oocyst and sporozoite mean prevalence with its standard deviation. The Intra Class Coefficient was calculated as the overdispersion parameter of a maximum likelihood fitted beta-binomial distribution. The difference was estimated for each pair of entomological observations within each participant, and 95% CI was obtained using the Agresti Caffo method. To evaluate the hypothesis that the average mean difference in the prevalence between two assays within the same subject was zero, combined estimates (weighted mean and variance) were obtained using as weight the inverse of the variance of each paired difference obtained from the Agresti and Caffo method (see supplemental material for details on the calculation of the confidence intervals and the weights for the combined estimation). Differences between days and between assays in the number of oocysts were evaluated using zero-inflated Poisson regression, having as an outcome for each participant, feeding visit and feeding method, the number of oocysts, and as an offset the number of surviving mosquitoes. Correlation between prevalences by the same assay or by the same day were weighted by the number of mosquitoes in each observation. The analysis was made using SAS 9.4 (SAS Institute Inc Cary, NC) [17]. An exploratory analysis of the probability of infecting mosquitoes by feeding visit and feeding method, assuming that the probability of infection follows a beta-binomial distribution with a mean parameter following a linear combination of the feeding visit and method of feeding, and assuming a constant dispersion between days was estimated using a Bayesian model with non-informative priors in JAGS [18].

There is no prior knowledge of the oocyst prevalence or density on a repeated DMFA or DSFA in a human subject. As such, the sample size chosen for this study was primarily based on logistical and budgetary considerations that would estimate the variability in DSFA. Using simulations considering the number of positive mosquitos follows a beta-binomial distribution with parameters 1.6 and 6 (from a previous unpublished survey in the area) and considering 25 surviving mosquitoes for analysis after each feeding visit and assay, a study with 36 individuals had 90% power to reject the hypothesis that the difference in oocyst prevalence between consecutive feeding assays is greater than ± 7% for correlated data. However, the scenario has only 4% power, assuming independent data between days. The study targets the inclusion of 45 individuals after screening 400 for positive gametocytaemia at baseline.

Results

A total of 400 volunteers signed informed consent and underwent screening procedures. Of them, 42 were gametocyte-positive by PCR at baseline and participated in the mosquito feeding assays on days 1 and 2. (See the Consort diagram in Fig. 2.) The mean age was 22.4 years (range: 20 to 52) and 52.5% were female. Approximately 73.8% of the participants indicated a recent episode of malaria, more than four weeks prior to baseline assessment, confirmed by an RDT test or other laboratory test, and were treated in an outpatient facility. Gametocytes quantified by qPCR at screening have PLU gen Ct median value of 24.6 (IQR 21.6, 26.9), PF16 gen Ct median value of 29.10 (IQR 26.9, 30.3) and PF25 gen Ct median value of 29.2 (IQR 26.9, 20.2). Not significant changes were found in the Ct levels at baseline of final visit (Kruskal–Wallis rank sum test $p = 0.08$ for PLU, 0.2 for PF25 and 0.09 for PF25). All but one participant remained positive for gametocyte PCR on feeding visit 2.

Table 1 shows the summary of the entomological results. In general, the number of surviving mosquitoes was low, with an average of 12 mosquitoes per subject/day/assay for the oocyst evaluation and 8.7 surviving mosquitoes per subject/day/assay for the sporozoite

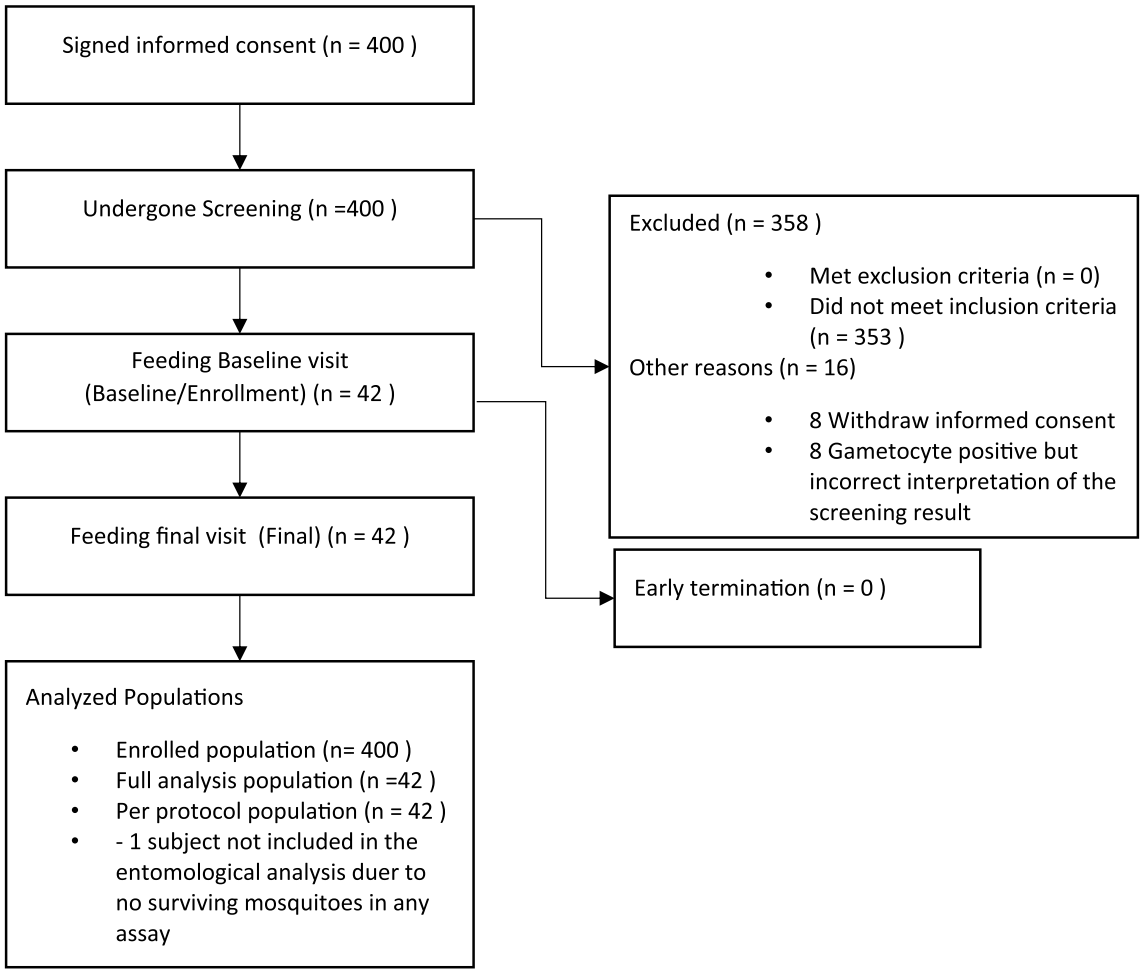

Fig. 2 Consort diagram

evaluation. The oocyst prevalence averages were 2.2% to 6.3% per subject/day/assay. Similarly, the prevalence of sporozoites had an average range from 1.8% to 5.1%. Figure 3 shows the scatter plot of the oocyst prevalence by feeding day. The overall correlation between feeding final visit and feeding baseline visit was 0.31 (95% CI 0.15 to 0.51).

Figures 4 and 5 show individual differences per subject in the oocyst prevalence on feeding final visit minus feeding baseline visit for DMFA and DSFA assay, respectively. The combined difference for the DMFA assay -1.6% (95% CI − 14% to 11%) and the DSFA assay was − 2.5% (95% CI − 16% to 11%). Table 2 shows, in addition, the differences between assays for each day. None of the comparisons was significant. Similar results are observed when the difference in the prevalence of sporozoites by feeding visit or by assay were evaluated (Table 3). Differences in oocyst density are presented in Table 4. There was no significant difference between the assays on the same day, but for both assays, the density was significantly lower on the feeding final visit when compared with feeding baseline visit. The exploratory analysis of mosquito infection probability using the beta-binomial Bayesian model estimates a baseline probability of infection of 6% (95% CI 4.4% to 8.25%). There was no significant difference between assays, but there was a significant difference between days, with the odds of infection on the feeding final visit 50% (95% CI 20% to 70%) lower than the feeding baseline visit (Table 5).

Discussion

This study investigated the consistency and reproducibility of DMFA and DSFA assays performed on the same subject in two consecutive days. The two feeding time points, 24 h apart, started among participants who tested positive for presence of gametocytes on the day of screening visit. Consequently, the first and second feeding occurred 24 and 48 h after detection of gametocytes, respectively. Studies have shown that gametocyte carriage status vary over time with or without intervention

Table 1 Summary of entomological results

	Outcome	Baseline-DSFA (41)	Final-DSFA (41)	Baseline-DMFA (41)	Final-DMFA (41)
Optical Microcopy	**Surviving Mosquitoes for Oocyst evaluation**				
	Mean (SD)	12.1 (7.5)	11.4 (6.5)	13.2 (8.2)	11.6 (6.9)
	Range	1; 27	2; 27	1; 27	1; 28
	Oocyst prevalence (%)				
	Mean (SD)	5.2 (7.8)	2.3 (4.9)	6.3 (16.2)	2.2 (4.4)
	Range	0; 33.3	0; 20	0; 100	0; 20
	Oocyst density				
	Mean (SD)	1.1 (1.8)	0.4 (0.8)	1.6 (4.1)	0.4 (0.8)
	Range	0; 9	0; 3	0; 23	0; 4
	Mosquitoes for Sporozoite evaluation				
	Mean (SD)	8.3 (4.7)	8.1 (4.8)	9.3 (5.8)	8.9 (5.5)
	Range	1; 18	1; 21	2; 23	1; 20
	Sporozoite prevalence (%)				
	Mean (SD)	5.1 (8.4)	1.8 (4.8)	4.5 (8)	2 (5.7)
	Range	0; 33.3	0; 16.7	0; 33.3	0; 25

SD: Standard deviation

[19] leading to modulation of infectiousness to mosquitoes [20]. Specifically, Infectiousness of an infection is reliant on the uptake of both male and female *P. falciparum* gametocytes. Additionally, a study by Bradley and coworkers observed that though mosquito infections may occur from blood with *P. falciparum* gametocyte densities including those below the microscopic threshold for detection [21, 22] and gametocyte density itself is an important determinant of sex ratio [23, 24] that is key in modulating infectiousness to mosquito [25]. These factors are transient in natural infections. Scheduling of the feeding time-points within 48 h after detection of gametocyte aimed to lower confounding factors that would reduce accuracy of estimating reproducibility of successive infections. Notably, all but one participant remained positive for gametocyte PCR on final visit, suggesting that utility this strategy of selecting these time-points for evaluating approaches and assays relying on temporal detectable gametocytes. It is worth noting that though participants did not receive any intervention between screening and final visit, The negative gametocyte results at final visit for the one participant underscores role of complex host-parasite factors in modulating gametocyte as previously observed [19] as a challenge to accurate evaluation of interventions.

The number of surviving mosquitoes for the oocyst and sporozoite evaluation was low. A study by Holmes and coworkers on bloodmeal regulation in mosquitoes has showed that dehydrating mosquitoes prior to feeding increase blood feeding propensity, improve retention, and decrease excretion of a post-dehydration bloodmeal. This study also associated alteration of these factors to changes in predicted vectoral capacity that is integral to mosquito infection [26]. This recent finding highlights need for additional attention to mosquito physiology by mosquito infection studies. Mosquito physiology was not measured throughout this study since the importance of such experiments had not been described at the time of designing of this study. It is therefore likely that these factors could have contributed to the high mosquito mortality in this study hence impacting the generalizability of the results.

A significant difference in mosquito feeding between days 1 and 2 with respect to the prevalence of oocyst density or percentage of infected mosquitoes was not seen, although the correlation between feeding days 1 and 2 was low. Oocyst density and the probability of infecting mosquitoes was marginally lower on final visit compared to baseline visit. Oocyst detection is a well-known challenge in malaria transmission research [7] and highlights the importance of regular PCR confirmation of (a subset of) infected guts [27]. Primarily, this study recruited and followed up participants at the two visits spanning 48 h across a calendar year's long and short-rainy seasons separated by dry spells. All the individuals were asymptomatic and generally in good health condition but were hospitalized at the study's hospital facility that was within ~ 25 kms radius of the participants' house hold for clinical monitoring. Though multiple factors influence gametocyte production [27], environmental cues could

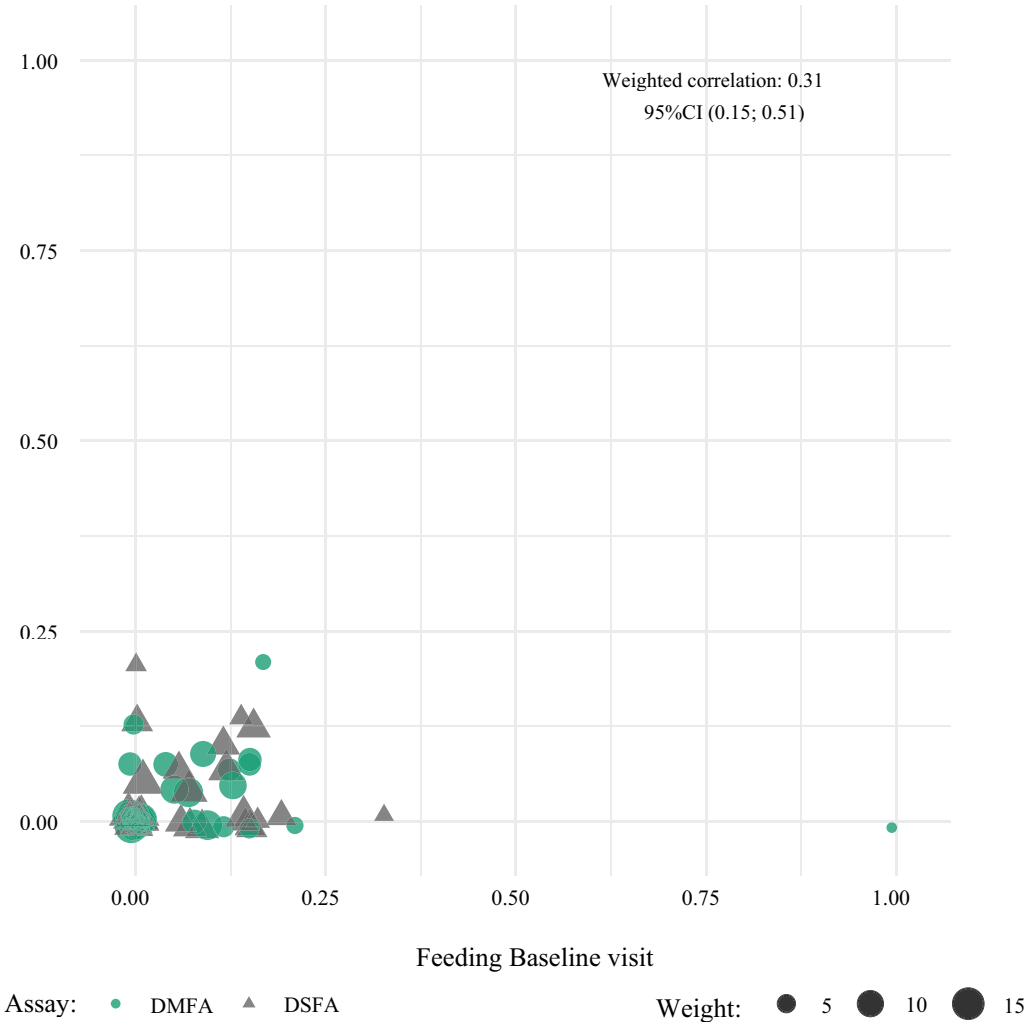

Fig. 3 Oocyst prevalence by feeding visit. Each dot represents the Oocyst prevalence by feeding (DMFA circles, DSFA, Triangles). The size of the dot I(Weight) is proportional to the number of alive mosquitoes for the prevalence estimation

also influence parasite transmission, as demonstrated for other vector-borne diseases [27, 28], are core in modulating release into the human blood circulation for effective infection. For this study that spanned across the entire year's seasons, subtle fluctuation in ambient conditions over the 24 h separating the independent infection assay time-points could be responsible for the observed differences between baseline visit and final visit based on oocyst density and the probability of infection. Moreover, these minor variations underscore the success of the study design in reducing biases that would be incurred with extended time interval of the two feeds relative to initial gametocyte detection. Broadly, the observation of comparable readouts between baseline visit and final visit given the burden of gametocytaemia appear to suggest the 24-h interval to be adequate for evaluating intervention effect. Additionally, 148/400 (37%) participants tested positive for *Plasmodium* genus with 50/400 (12.5%) of these further testing positive for gametocyte. This observation shows the burden of asymptomatic malaria in the region. Gametocytaemia rate is evocative of the contribution of asymptomatic infection to sustaining transmission and field expedience of transmission blocking for studies as basis for effective deployment once they are approved.

Assessment of individual differences per subject in the oocyst or sporozoite prevalence on feeding final visit minus feeding baseline visit based on DMFA and DSFA assay readouts, respectively did not show differences between assays. Further exploratory analysis of mosquito infection probability using the beta-binomial Bayesian model estimating a baseline probability of infection showed no significant difference between assays. Studies comparing DMFA and DSFA

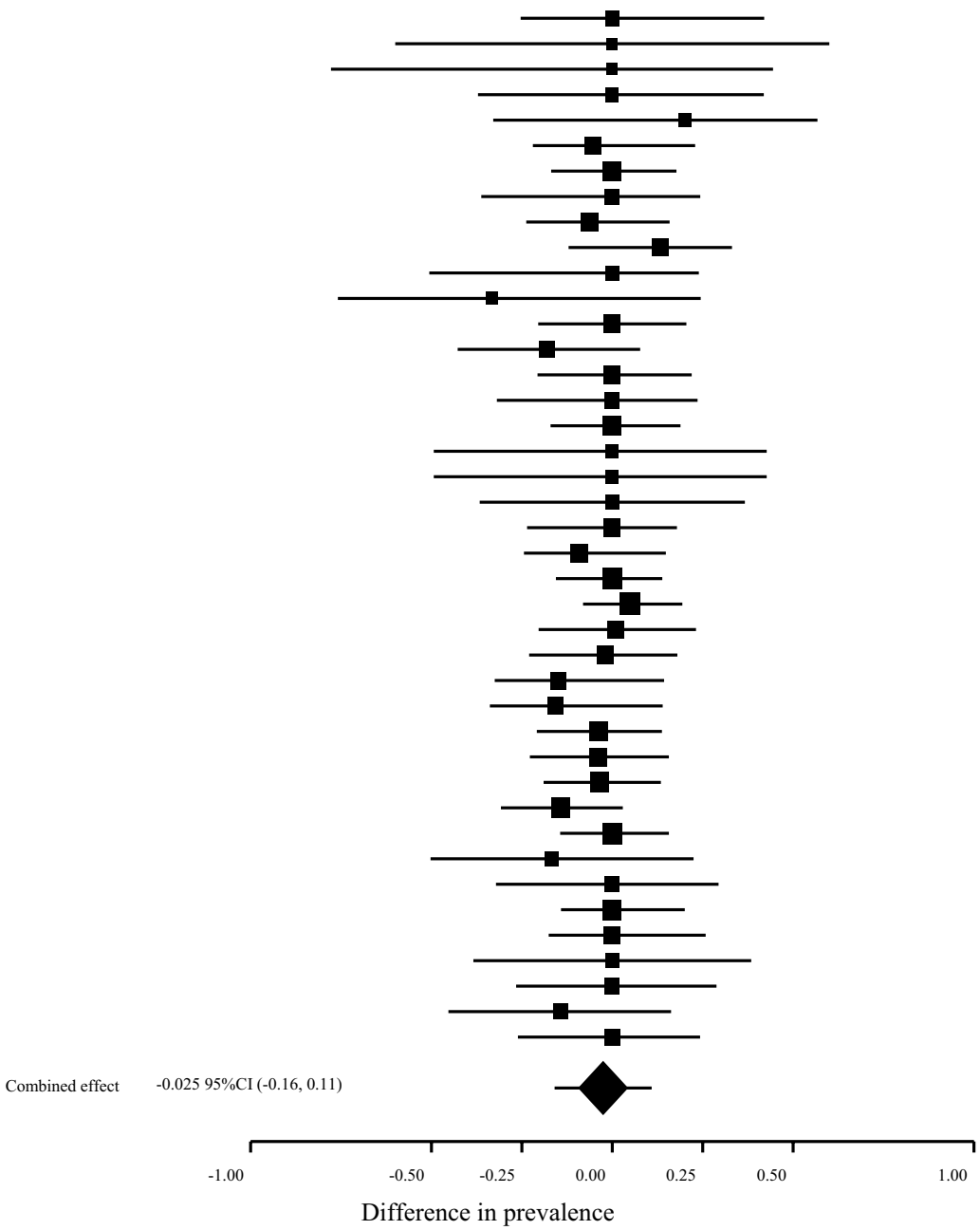

Fig. 4 Difference in oocyst prevalence between feeding final visit and feeding baseline visit by DSFA. Each line represents the estimated subject difference in prevalence between feeding final visit and feeding baseline visit (square) with the individual 95% confidence interval. The diamonds represent the combined estimate with its 95% CI by DSFA

assay readouts elsewhere show that membrane feeding accurately reflect those of natural biting [29–31]. However, low infection rates observed by this study could be associated to low gametocyte density as reported in previous studies [21, 27, 32–34]. A recent study by Ouattara and coworkers proposes enrichment of the infection with naïve serum [35] for enhancing infectious rates that has remained a major obstacle in objective evaluation of transmission-blocking.

These results may reflect the possible suboptimal conditions under which the entomological experiments were executed. The study provides several potential explanations that could inform how to optimize the conditions for a successful and interpretable trial aimed at

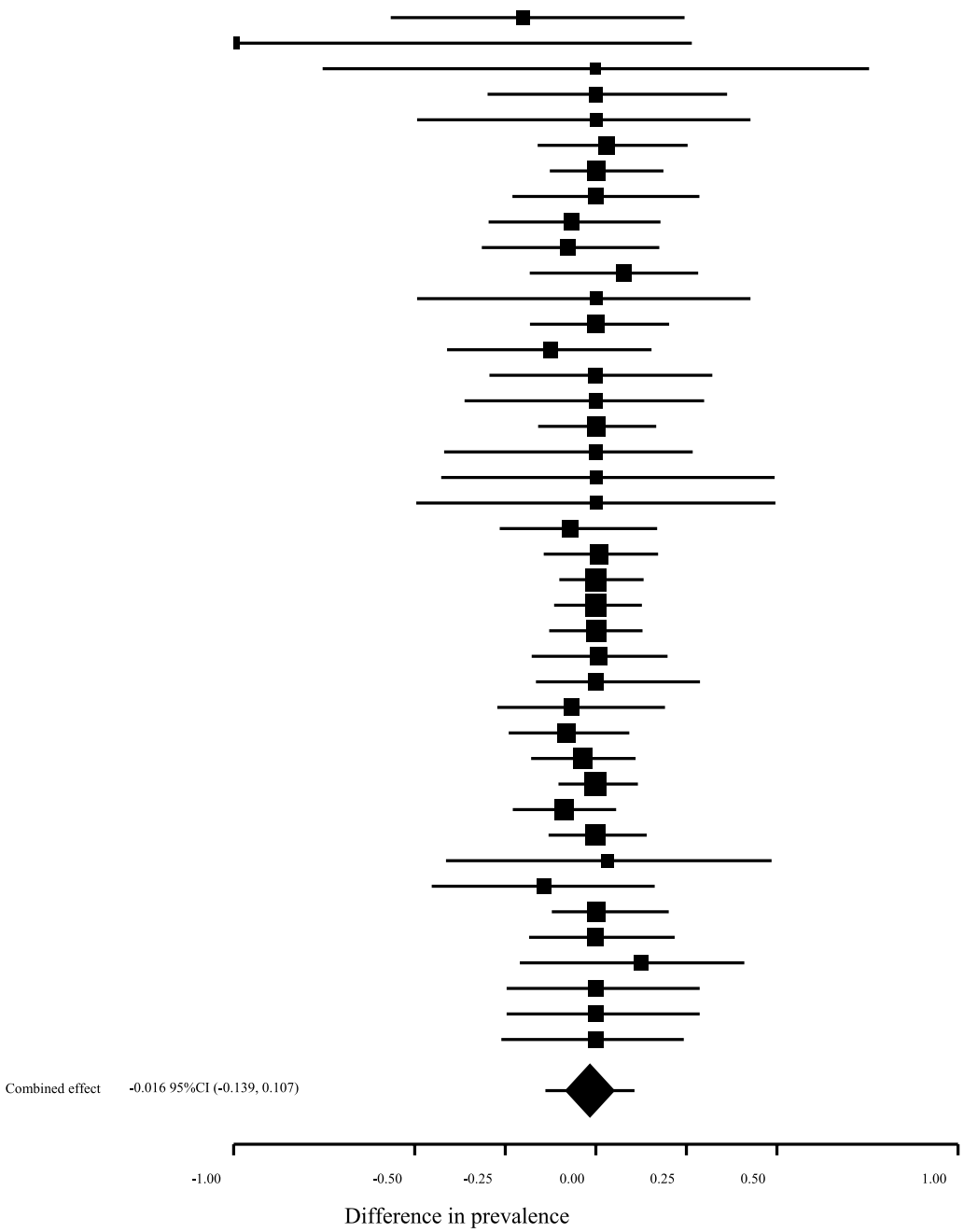

Fig. 5 Difference in oocyst prevalence between feeding final visit and feeding baseline visit by DMFA. Each line represents the estimated subject difference in prevalence between feeding final visit and feeding baseline visit (square) with the individual 95% confidence interval. The diamonds represent the combined estimate with its 95% CI by DMFA

testing potentially new and novel transmission-blocking or transmission-reducing tools under development. The study identified three critical bottlenecks.

First, the laboratory and insectary infrastructure required to isolate, propagate, and maintain large numbers of *An. gambiae* mosquito colonies requires a labour-intensive programme of skilled entomologists alongside a suitable environment. The longevity and reproducibility of mosquito colony propagation depends upon multiple factors that includes both temperature and humidity requirements, food and blood source, and a mosquito colony which has been adapted to survive in contained laboratory environments. This would entail state-of-art infrastructure to monitor mosquito physiology as based

Table 2 Difference in oocyst prevalence between feeding visits and between assays

Comparison	n	Combined estimate	LCI	UCI	p-value	I_2(%)	CV
DSFA final–baseline visit	41	− 0.025	− 0.160	0.109	0.711	0.0	2.70
DMFA final–baseline visit	41	− 0.016	− 0.139	0.107	0.795	0.0	3.85
Baseline visit DSFA–DMFA	41	0.012	− 0.136	0.160	0.874	0.0	6.29
Final visit DSFA vs DMFA	41	0.005	− 0.078	0.089	0.901	0.0	8.03

n: Number of observations included in the meta-analysis; LCI: Lower limit of the 95% confidence interval; UCI: Upper limit of the 95% confidence interval; p-value: For the test that the combined estimation is different from zero; I2: I square test; CV: Coefficient of variation

Table 3 Difference in sporozoite prevalence between feeding days and between assays

Comparison	n	Combined estimate	LCI	UCI	p-value	I_2(%)	CV
DSFA final–baseline visit	41	− 0.023	− 0.164	0.118	0.750	0.0	3.13
DMFA final–baseline visit	41	− 0.032	− 0.178	0.117	0.681	0.0	2.43
Baseline visit DSFA–DMFA	41	0.001	− 0.122	0.125	0.984	0.0	49.2
Final visit DSFA vs DMFA	41	0.019	− 0.102	0.123	0.855	0.0	5.47

n: Number of observations included in the meta-analysis; LCI: Lower limit of the 95% confidence interval; UCI: Upper limit of the 95% confidence interval; p-value: For the test that the combined estimation is different from zero; I2: I square test; CV: Coefficient of variation

Table 4 Oocyst density comparison by assay and day

Outcome[a]	Relative rate	LCI	UCI	p-value
Oocyst density				
Final/Baseline in DSFA assay	0.38	0.21	0.70	0.019
Final/Baseline in DMFA assay	0.23	0.11	0.45	0.003
DMFA/DSFA in baseline visit	0.97	0.53	1.77	0.925
DMFA/DSFA in final visit	0.96	0.46	2.01	0.912

LCI: Lower limit of the 95% confidence interval; UCI: Upper limit of the 95% confidence interval; p-value by the likelihood ratio test

[a] Each row is a separate Zero Inflated Poisson model adjusted by the number of mosquitoes and subject

on the emerging necessity in forestalling mortality. The number of such laboratories across sub-Saharan Africa is unfortunately limited, and standardization of methodologies and exchange of laboratory-adapted mosquitoes is recommended.

Second, the number of mosquitoes placed in each cup versus the number of cups required for each feed, especially for direct skin feeds needs to be optimized. This needs to be balanced against the ethical considerations associated with the level of discomfort that study participants are subjected to, considering that there are no direct benefits to these participants. The desire is to have as many mosquitoes feed on gametocytaemic blood as is possible. Overcrowding of cups leads to higher mosquito mortality.

Finally, the source and the quantity of gametocytes from the blood of a malaria-infected person used to feed and infect a large number of mosquitoes either through a membrane or directly on the person's skin along with the ethical imperatives to kill such parasites when identified is a key indicator of success.

Theoretically, a successful mosquito feeding assay needs at least 1 male and 1 female gametocyte in 1 to 2 µl of blood to infect a mosquito that can then develop

Table 5 Bayesian model to estimate the probability of mosquito infection by feeding visit and assay

Outcome	Effective sample	Mean	Median	Low	High
Probability of infection (Baseline visit, DMFA)	1338	6.0%	6.0%	4.4%	8.2%
Odds ratio for the probability of infection DSFA vs DMFA	1210	0.74	0.74	0.47	1.15
Odds ratio for the probability of infection Final vs Baseline	1222	0.50	0.50	0.30	0.80
Dispersion parameter	1592	16.67	15.81	4.11	32.87

LCI, UCI: 95% limits of the credible intervals

Effective sample out of 10^7 simulations

into oocysts and infectious sporozoites. Practically, this amounts to anywhere from 5 to 10 gametocytes/µl of blood for reproducible mosquito-feeding assays. The identification of such gametocyte carriers is typically done by detecting gametocytes either by microscopy on a dried blood film or by exquisitely sensitive molecular detection assays as used in this study. The determination on whether and when to treat participants infected with malaria is informed by whether a trial participant is asymptomatic or symptomatic, the density of parasites as detected by microscopy or PCR, and country-specific treatment guidelines and local and national ethical committees.

This study was limited (ethical committee and country guidelines on treating asymptomatic malaria carriers) by testing the before-after mosquito-feeding assay in asymptomatic adults with the provision that only PCR assay would be used to detect for the presence of *P. falciparum* gametocytes. Both the age of the participant (adults usually have much lower densities of gametocytes than adolescents and children) and the detection methodology (PCR is more sensitive than microscopy resulting in gametocytes densities < 2/µl blood) probably adversely affected the reproducibility of detecting parasites in mosquitoes over two consecutive days.

With these results, the study concludes that based on the pre-specified conditions and requirements in Western Kenya to conduct such a before-after assay on the same individual does not offer advantages over a parallel design where a randomized controlled trial of a transmission-interrupting tool is tested in an appropriately sized study against a control (placebo) group between individuals rather than within individual.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-025-05360-3.

Additional file 1.

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Author contributions
HMA, JA, BA, BO, LOT and CFO, study design, implementation, wrote the main manuscript text NKC, AR, JM, ECG, BRO, PS Study implementation, manuscript review MAO, IO, JH Study implementation, clinical oversight and initial draft of write-up sub-sections, manuscript review DWJ, RNM, ROO, JAJ, EWM, MAM, DOO, DA, FLE study implementation, lab oversight and initial draft of write-up sections, manuscript review LM, MR, VM, KI, YW Study implementation, clinical oversight and initial draft of write-up sub-sections, manuscript review.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study protocol was approved by the Ethical Review Committee of the Kenya Medical Research Institute (KEMRI SSC Protocol No: 4106) and the Walter Reed Army Institute of Research Institutional Review Board (WRAIR IRB Protocol No: WRAIR#2610). Written informed consent was obtained from all study participants prior to their enrollment in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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