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RESEARCH ARTICLE

REVISED Prevalence of asymptomatic *P. falciparum* gametocyte carriage among school children in Mbita, Western Kenya and assessment of the association between gametocyte density, multiplicity of infection and mosquito infection prevalence. [version 2; peer review: 1 approved, 2 approved with reservations, 1 not approved]

Previously titled: Prevalence of asymptomatic *P. falciparum* gametocyte carriage in schoolchildren and assessment of the association between gametocyte density, multiplicity of infection and mosquito infection prevalence

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Abstract

Background: Asymptomatic *Plasmodium falciparum* gametocyte carriers are reservoirs for sustaining transmission in malaria endemic regions. Gametocyte presence in the host peripheral blood is a predictor of capacity to transmit malaria. However, it does not always directly translate to mosquito infectivity. Factors that affect mosquito infectivity include, gametocyte sex-ratio and density, multiplicity of infection (MOI), and host and vector anti-parasite immunity. We assess the prevalence of gametocyte carriage and some of its associated risk factors among asymptomatic schoolchildren in Western Kenya and to further analyse the association between gametocyte density, multiplicity of infection (MOI) and mosquito infection prevalence.

Open Peer Review

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	Invited Reviewers			
	1	**2**	**3**	**4**
version 2 (revision) 13 Apr 2021	? report	✓ report	? report	✗ report
version 1 29 Oct 2020	? report	? report	? report	✗ report

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Methods: *P. falciparum* parasite infections were detected by RDT (Rapid Diagnostic Test) and microscopy among schoolchildren (5-15 years old). Blood from 37 microscopy positive gametocyte carriers offered to laboratory reared *An. gambiae s.l.* mosquitoes. A total of 3395 fully fed mosquitoes were screened for *Plasmodium* sporozoites by ELISA. *P. falciparum* was genotyped using 10 polymorphic microsatellite markers. The association between MOI and gametocyte density and mosquito infection prevalence was investigated.
Results: A significantly higher prevalence of *P. falciparum* infection was found in males 31.54% (764/2422) (p-value < 0.001) compared to females 26.72% (657/2459). The microscopic gametocyte prevalence among the study population was 2% (84/4881). Children aged 5-9 years have a higher prevalence of gametocyte carriage (odds ratios = 2.1 [95% CI = 1.3–3.4], P = 0.002) as compared to children aged 10-15 years. After offering gametocyte positive blood to *An. gambiae s.l.* by membrane feeding assay, our results indicated that 68.1% of the variation in mosquito infection prevalence was accounted for by gametocyte density and MOI (R-SQR. = 0.681, p < 0.001).
Conclusions: We observed a higher risk of gametocyte carriage among the younger children (5-9 years). Gametocyte density and MOI significantly predicted mosquito infection prevalence.

Keywords
P. falciparum, asymptomatic, gametocyte density, MOI, mosquito infection prevalence, Mbita

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REVISED **Amendments from Version 1**

The first version of this manuscript was revised based on the reviewers' comments and recommendations. The title of the first manuscript was revised to reflect the study site in the title (Mbita, Western Kenya). In the Introduction, we have revised the content and added details about gametocyte density, MOI and mosquito infectivity. Additional references are also added.

The methodology section was also revised by adding additional details about the study site. Information about the economic activities, types of housing and family structures in the study area were included in the study site section. Also, the type of study "cross sectional study" was indicated and the number of schools where the study participants were recruited is also added in the version two.

The mode of transforming gametocytes counts to gametocyte density was added to provide more details "gametocyte counts were counted against 1000 white blood cells and the counts converted to parasites/μL assuming a density of 8000 WBCs/μL. 100 μL of blood samples were also collected on filter papers (two spots per paper)". The version two of the manuscript also includes the type of anticoagulant used during sample collection. The protocol for experimental feeding of mosquitoes used in the study is revised and shortened. However, the main details about the feeding assays are still presented.

The results section is also revised by adding some detailed statistically analysis and revised figures. The table 1 in the first version is now divided into two (Table 1 and Table 2) for clarity. Table 2 in the first version is omitted and the remaining tables are renamed accordingly. The figures are now presented in terms of gender and age groups. These changes are also reflected in the discussion section of version two as well. A section about the limitations of the study is included.

Any further responses from the reviewers can be found at the end of the article

Introduction

The intensification of global and local malaria control measures has led to marked reduction in disease burden in many regions including sub-Saharan Africa. The incidence of *Plasmodium falciparum* clinical cases and prevalence have declined by 40% and 50%, respectively, within the African continent between 2000 and 2015[1]. However, recent data indicates this trend might be reversing, with an estimated 213 million malaria cases and 380,700 related deaths in the World Health Organisation (WHO) African Region between 2017 and 2018, an increase relative to previous years[2]. Clearly, malaria continues to be a very serious public health problem on the African continent, threatening the lives of many people, particularly children and pregnant women. In Kenya, like many other African countries, *P. falciparum* is the dominant parasite species with about 70.2% of the population at risk of the disease[3]. Malaria is one of the leading causes of hospital admissions and death in the country, accounting for about 30% and 19% outpatient and inpatient cases, respectively, with an estimated inpatient death of 3–5%[2,4].

The Kenyan government, through the implementation of a national strategic malaria control plan, and subsequently by the launching of the next iteration of its national malaria strategy (KMS) 2019—2023, has intensified its fight against the disease in a bid to attain a **"**malaria free Kenya**"**. This involved the introduction and scaling up of interventions such as long-lasting insecticide nets (LLINs), rapid diagnostic tests (RDTs), and artemisinin-based combination therapy (ACT)[5,6]. The implementation of these interventions has resulted in a decline in malaria transmission in many parts of the country[7]. Nevertheless, the coastal part of the country and areas along the shores of Lake Victoria continue to face very high malaria transmission[8].

Malaria parasite transmission from humans to the mosquito vectors requires the presence of infectious mature gametocytes in the peripheral blood of the human host[9,10]. Based on the central role of gametocytes in propagating and sustaining malaria transmission, the prevalence of gametocytes and their densities are often used as surrogate indicators for the disease transmission potential[11,12]. The advent of highly sensitive molecular tools has enabled us to understand that every malaria positive individual is a current or prospective gametocyte producer and therefore, has some transmission potential. Studies in malaria endemic and high transmission areas have reported higher asexual parasite and gametocyte prevalence and densities in children relative to adults[13,14]. In high malaria transmission settings, due to repeated parasite exposure, older children and adults develop immunity against the parasite[15,16]. As a result, these age groups are most likely to experience asymptomatic infections harboring gametocytes at microscopic and sub-microscopic densities, thereby serving as efficient parasite reservoirs for sustaining malaria transmission[12,14,17]. Reports about high prevalence of asymptomatic infections and gametocyte densities in schoolchildren have been documented in some malaria endemic areas[17,18]. Asymptomatic malaria infections in schoolchildren mostly remain undiagnosed and are not treated due to the lack of clinical manifestation. Therefore, this group of people are largely neglected by most of the currently implemented malaria interventions and control programs[17,18]. In addition, following the decline in malaria burden in many endemic areas, information on the prevalence of asymptomatic *P. falciparum* infections and gametocyte carriage in schoolchildren, particularly in remote settings in sub-Saharan Africa, remains patchy[19]. Since asymptomatic infections and prevalence of gametocyte carriage in schoolchildren may significantly hamper the attainment of malaria control and elimination goals in sub-Saharan Africa[18,20], it will be important to further investigate dynamics and infectivity of asymptomatic carriers.

The presence of gametocytes in the peripheral blood of the human host does not necessarily translate into mosquito infectivity[9,21]. Some of the major factors that influence the successful transmission of *P. falciparum* gametocytes to the mosquito vectors include, human attractiveness and exposure to the mosquito vectors, host and vector immune responses, seasonality, gametocyte maturity and densities, and multiplicity of infection (MOI)[21,22]. MOI is the number of distinct parasite clones concurrently infecting a host. The link between MOI and gametocytemia of *P. falciparum* is still not fully elucidated[21]; however, some studies have reported a positive

association between MOI and gametocyte carriage[14,23]. The presence of multiple genetically diverse *P. falciparum* clones is reported to increase the chances of some parasite clones to evade the host anti-parasite immune responses, thereby promoting gametocyte development and persistence[14,24]. Studies have reported that mosquito infectivity is positively correlated to gametocyte density and primarily determined by female gametocyte density, however, transmission at low gametocyte densities can be limited by density of male gametocytes[25–28]. However, the proportion of variation in mosquito infection prevalence that can be explained by gametocyte density and MOI has not been fully elucidated.

In malaria endemic settings, asymptomatic infections characterized by high rates of polyclonal infections and variations in gametocyte carriage among different age categories is not uncommon[15,29,30]. Variations in gametocyte densities among the different age categories can be partly explained by the age-related anti-parasite immunity due to repeated exposure among the elderly children and adults age groups[21]. In order to accelerate malaria elimination , interventions geared towards interrupting the parasite transmission through efficient and effective identification and treatment of both asymptomatic and symptomatic parasite carriers will be of immense importance[14,15]. Understanding the association between gametocyte density, MOI and mosquito infectivity will enhance proper identification of parasite reservoirs responsible for sustaining the ongoing malaria transmission in the region[14]. Here, we report on the prevalence of gametocyte carriage and some of its associated risk factors among asymptomatic schoolchildren (age 5–15 years) in western Kenya and further assesses the association between gametocyte density, MOI and mosquito infection prevalence.

Methods

Ethics and consent

Parents or guardians of the children signed an informed consent form for participation in the study, having data analysed and publication of results. In addition, assent was obtained from older children between the ages of 12 and 15. The Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit (SERU) granted approval for the original study (KEMRI/RES/7/3/1). All experiments were performed in accordance with the relevant guidelines and regulations.

Study site

This cross sectional study was carried out in the Homa Bay County of Western Kenya formerly, the Nyanza province. Study participants were recruited from primary schools (41 primary schools) primarily within Mbita sub-county (within 50 km radius of Mbita town). The sub-county is situated on the shores of Lake Victoria and located between latitudes 0° 21’ and 0° 32’ South and longitudes 34° 04’ and 34° 24’ East. The area of the district is about 163.28 km^2 with a population of 124,938 (Figure 1). The compound is the main residential unit and mostly comprises

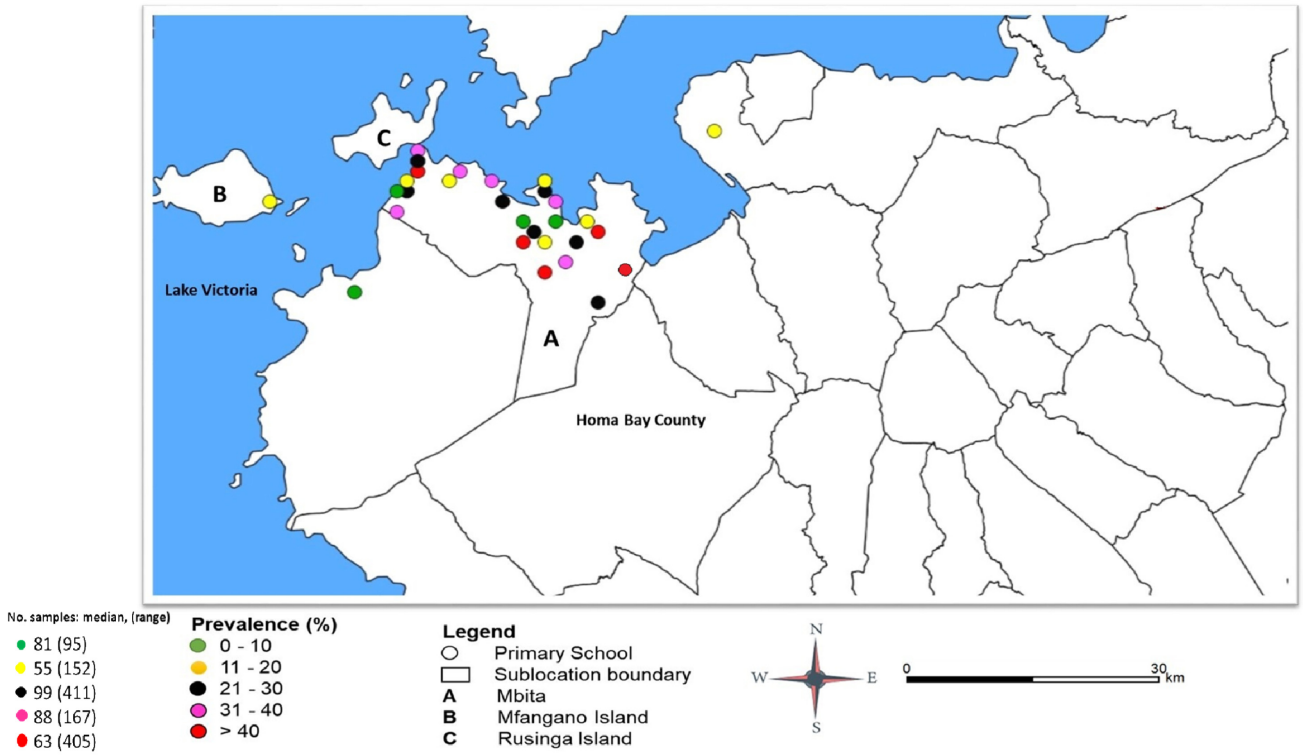

Figure 1. Map of Homa Bay County indicating the prevalence of *Plasmodium falciparum* infection among the schools in the study site. The site-specific prevalence (%) was calculated as the percentage of *P. falciparum* positive infections within each school.

of one or more households. The houses are a mix of traditional mud grass thatch huts and modern concrete and corrugated iron houses. Fishing, farming, and animal rearing are the major economic activities in the region. Perennial malaria transmission is reported in the region. The peak transmission occurs in July and relatively lower transmission levels are reported from November to January[31].

Study subjects and sample collection

Primary schoolchildren between the ages five and 15 years residing in Homa bay county, Western Kenya were recruited and screened for *P. falciparum* malaria infection using a rapid diagnostic test (RDT) (SD Bioline Malaria Ag P.f/Pan HRP-II/pLDH) (Standard Diagnostics Ref 05FK60, Inc; Suwon City, Republic of Korea) and microscopy. Schoolchildren from the various primary schools in Mbita subcounty and the neighbouring villages (within 50 Km) were enrolled in a study that commenced in December 2016 to evaluate the effects of symbiotic microbes and mosquito vector competence. The samples analysed in this study were collected from December 2016 to December 2018. The inclusion criteria used for the sampling included being at primary school in Mbita or any of the surrounding villages within 50 Km of Mbita between the ages of 5–15 years and not showing any of the symptoms of malaria during screening.

Blood samples were collected by a clinician from each participant in their various schools for RDT and 10% Giemsa stained thin and thick blood films preparation for microscopy diagnosis of *P. falciparum* malaria infection. Microscopy was carried out *in-situ* and all the stained slides were then well packaged and transported to *icipe* TOC Mbita campus for storage. Gametocytes were counted against 1000 white blood cells and the counts converted to parasites/µL assuming a density of 8000 WBCs/µL. In addition, 100 µL of blood was collected on filter papers (two spots per paper) (Whatman 3MM; Whatman, Maidstone, United Kingdom) for DNA extraction. The filter paper dried blood spots (DBS) were stored at -20°C. Participants who were found by microscopy to carry *P. falciparum* gametocytes were contacted by the clinician through their parents/guardians for further sample collection on the same day. An additional 4 mL of venous blood was collected from participants by the clinician for use in the membrane feeding assays. A total of 4881 participants were screened in this study. This sample size was obtained based on the number of study participants within the designated study area that consented to partake in the study over the study period. The samples included in this study were originally collected to investigate differences in levels of infectivity between laboratory colony mosquitoes and the progeny of wild caught mosquitoes, genetic diversity, and multiplicity of infection[32], this study is a sub-study that was concurrently undertaken using the same samples.

Experimental infection of mosquitoes

A modified version of the standard membrane feeding assay protocol published elsewhere was used in this study[9]. In brief, larvae (G_1) from *icipe*-TOC insectary colonies of *An. gambiae s.s.* (G_0) were reared at 30.5°C (+/- 2°C) and 30% humidity at the insectary of *icipe* TOC Mbita campus. Venous blood samples (4 mL) collected in heparin tubes from each *P. falciparum* gametocyte positive individual were immediately fed to the mosquitoes. Experimental feeds were carried out in batches of 100 female *An. gambiae s.s.* mosquitoes (3–5 day-old) per feeding cup.The mosquitoes were fed on a total volume of 500 µl of blood via an artificial membrane attached to a 14 mm water-jacketed glass feeder (Chemglass, USA) maintained at 37°C. All the membrane feeding assays were carried out at the *icipe* Mbita TOC campus. A total of 37 gametocyte-positive venous blood samples collected from different individuals (only those that consented to provide the extra 4 mL of blood) were used to feed the mosquitoes. After 15–20 minutes, fully fed mosquitoes are selected and kept on glucose for seven days at 27°C – 29°C. On the tenth day post-infection, the mosquitoes that were alive from each feeding experiment were then collected and stored at -20°C in Eppendorf tubes and were later individually tested for *P. falciparum* carriage using ELISA. The proportion of infected mosquitoes was determined by detecting the *P. falciparum* circumsporozoite protein (CSP) in the stored mosquito samples using a modified CSP ELISA protocol adapted from[33]. In brief, mosquitoes stored in 1.5 mL Eppendorf tubes were incubated in 50 µL grinding buffer followed by homogenization in the buffer solution by thorough grinding using a pestle. The homogenized samples were transferred to plates (Corning Cat. No. 2797) and stored at -20°C overnight. Each of the ELISA plates was coated with 50 µL MAb capture antibodies (0.5 mg/mL Capture Monoclonal Ab Pf2A10-CDC, CAT #: MRA-890, MR4/ATCC, Virginia, USA). Dilution specifications for *P. falciparum* used here is the same as previously published and were incubated overnight at room temperature[33,34]. 200 µL of the blocking buffer was added to each well after removing the Mab then incubated for one hour at room temperature 50 µL of the mosquito homogenates was added to each well . The positive controls (Pf-PC, BEI resources, Virginia, USA). were serially diluted using blocking buffer because they are free of *Plasmodium* parasites. Negative controls were insectary-reared male mosquitoes ground up in blocking buffer. A two-hour incubation of the plates was carried out at room temperature after which, the plates were washed four times with 200 µL PBS-Tween using ELx50 ELISA washer (BioTek Instruments, Winooski, Vermont, U.S.A). ABTS Substrate Component containing solutions A and B were mixed in a 1:1 ratio to a final volume of 20 ml/plate (SeraCare 5120-0032). The monoclonal antibody (MAb) peroxidase conjugate 27 (0.5 mg/mL Peroxidase Labelled Mouse Monoclonal Ab Pf2A10-CDC, CAT #: MRA-890, MR4/ATCC, Virginia, USA) was prepared in specified concentrations for *Plasmodium falciparum* at a volume of 40 µL in 10 mL blocking buffer for each plate. 100 µL of the MAb peroxidase conjugate was added to each well after drying the plates, wrapped in an aluminium foil then incubated in a dark place at room temperature. After which the plates were washed four times using PBS-Tween, dried and 50 µL substrate solution added to each well followed by 30 minutes incubation at room temperature The plates wereread on ELx808 ELISA reader (BioTek Instruments, Winooski, Vermont, U.S.A) using Gen 5 3.0 Software (BioTek Instruments, Winooski,

Vermont, U.S.A) at a wavelength of 405 nm to determine optical density values of the samples. An optical density (OD) cut-off values for CSP positivity were computed by the addition of three standard deviations to the mean OD value of the CSP-negative distribution from each plate[34]. OD values of each plate were adjusted by pooling the negative controls together, then the pooled negative mean OD value determined and subtraction of this pooled mean OD value from the mean negative OD value per plate to obtain the specific correction value per plate. The unique correction value was then added/subtracted from all OD readings in each respective plate to normalize readings across plates. Standard curves of absorbance against sporozoite concentration were generated for each plate using the serial diluted positive controls. Quantification of samples was computed using the equation generated from the standard curve and their corresponding absorbance values.

Microsatellite genotyping

Genomic DNA (gDNA) was extracted from the DBS samples using the QIAamp DNA Mini Kit (Cat # 51304, QIAGEN, Hilden, Germany) based on the manufacturer's protocol. gDNA quality and concentration of each sample was determined using a Nanodrop 2000C (Thermo Fisher Scientific, Waltham, MA, USA) and samples were stored at -20°C until used. The microsatellite amplification, fragment analysis and MOI determination method is based on a previous study[35]. In brief, genomic DNA (gDNA) was extracted from filter paper dried blood spots samples using QIAamp DNA Mini Kit (CAT #: 51304, QIAGEN, Hilden, Germany). gDNA samples were genotyped using primer sets (See Table S1, *Extended data*[36]) targeting 10 polymorphic microsatellite markers via a hemi nested PCR protocol using 5X FIREPol Master Mix (Solis BioDyne, Estonia) in a SimpliAmp Thermal Cycler (Applied Biosystems, Loughborough, UK). A total reaction volume of 20 µL was prepared for the hemi one PCR and the components are as follows; 1X FIREPol Master Mix (CAT #: 04-11-00115, Solis BioDyne, Estonia), 0.3 µM forward primer, 0.3 µM reverse primer (Macrogen, South Korea) and 10 ng/µL of the template DNA. The hemi one PCR conditions include a 2 min initial denaturation at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 42°C, 30 sec at 40°C and 30 sec at 65°C; then a 5 min final elongation at 65°C. The hemi two reaction was also run in a 20 µL total reaction volume containing 1X FIREPol Master Mix (CAT #: 04-11-00115, Solis BioDyne, Estonia), 0.4 µM of each primer and 5 µL of hemi one amplicons and the reaction condition includes; 2 min initial denaturation at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 45°C and 30 sec at 65°C and 5 min final elongation at 65°C. ABI 3730XL (Applied Biosystems) was used for the separation of hemi 2 PCR products using GeneScan 400HD ROX Size Standard (Applied Biosystems, Foster City, CA). GeneMarker V3.0.1 software (SoftGenetics, LLC) was used for scoring and quantification of allele sizes and peak heights, respectively[37]. The samples analysed here are part of those used in our previous study[32]. These are filter paper dried blood spots collected from the study participants as described above. A total of 37 samples (samples used for the membrane feeding assays) were genotyped for this analysis.

Data storage and analysis

Age, gender, weight and *Plasmodium* parasitemia of each study participant together with mosquito infection prevalence and microsatellite genotyping data were obtained. Descriptive statistics and Pearson Chi-Square test for significance between groups were determined. Risk factors analysis was done using a binary logistic regression model and multiple correlation and regression analysis was used to determine the regression coefficients, statistical significance of regression model (*t* value), and proportion of mosquito infection prevalence (dependent) contributed by independent variables (gametocyte density and MOI) derived from the multiple coefficient of determination (R^2). The mosquito infection prevalence was determined as the percentage of mosquitoes infected with *P. falciparum* parasite after successfully feeding on the naturally infected human blood. Statistical analyses were conducted in IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, N.Y., USA). Schools were mapped using geographical information system (GIS) and the map generated using QGIS software version 2.4.0. Rainfall data for Mbita (0° 25' 0" South, 34° 12' 0" East) were obtained from Climate Engine, Desert Research Institute and University of Idaho, accessed on 08/04/2020[38].

Results

Demographic and parasitological characteristics of the study participants

In this study, a total of 4881 schoolchildren (age 5–15 years) were screened using RDT and the parasite status confirmed by microscopy. The total number of female and male participants were 2459 and 2422, respectively. Regarding the parasitological characteristics of the study participants, significant differences were observed among males and females, with higher *P. falciparum* prevalence among the males [male: 53.76% (764/1421); female: 46.24% (657/1421); *p*-value < 0.001]. However, considering the age versus sex distribution of gametocyte carriage, no significant difference was found by comparing the male (5-9 years) [53.65% (382/712)] to female (5–9 years) [46.35% (330/712)] and male (10-15 years) [53.88% (382/709)] to female (10-15 years) [46.12% (327/709)] (*p*-value = 0.958). There was also no statistically significant difference in *P. falciparum* parasite carriage between the age groups [5–9 yrs.: 50.10% (712/1421); 10–15 yrs.: 49.89% (709/1421); *p*-value = 0.072] (Table 1). The total number of mixed infections (*P. falciparum* plus *P. ovale* and/or *P. malariae*) detected in the study population was 204, with a non-significant difference between the age groups [5–9 years: 15.73% (112/712); 10–15 years: 12.98% (92/709); *p*-value = 0.139], while there were 1217 single infections (*P. falciparum* only). Most of the mixed infections were found in females compared to males [females: 16.74% (110/204); males: 12.30% (94/764); *p*-value = 0.017] (Table 2).

The population *P. falciparum* prevalence in this study calculated as the percentage of *P. falciparum* infections within the study sample was 29.11% (1421/4881). The level of *P. falciparum* carriage varies among study sites (range: 0–100%, *p*-value < 0.001) and across sampling periods (range: 11–78.4%, *p*-value < 0.001, Figure 1 and Figure 2).

Table 1. Parasitological characteristics of the study participants.

Variables	Age group (years)		Gender	
	5 – 9	10 – 15	Female	Male
Positive	50.10% (712/1421)	49.89% (709/1421)	46.24% (657/1421)	53.76% (764/1421)
Negative	52.98% (1833/3460)	47.02% (1627/3460)	52.08% (1802/3460)	47.92% (1658/3460)
χ2 (p-value)	3.328 (0.072)		13.770 (< 0.001)*	

Percentage of *P. falciparum* positive and negative infections among the study participants. χ2 = Pearson's chi-squared test and (*) indicates statistical significance.

Table 2. Characteristics of the positive infections.

Variables	Age group (years)		Gender	
	5 – 9	10 – 15	Female	Male
Mixed infection	15.73% (112/712)	12.98% (92/709)	16.74% (110/657)	12.30% (94/764)
Single infection (*P.falciparum* only)	84.27% (600/712)	87.02% (617/709)	83.26% (547/657)	87.70% (670/764)
χ2 (p-value)	2.192 (0.139)		5.661 (0.017)*	
Asexual	48.99% (655/1337)	51.01% (682/1337)	46.75% (625/1337)	53.25% (712/1337)
Gametocyte	67.86% (57/84)	32.14% (27/84)	38.10% (32/84)	61.90% (52/84)
χ2 (p-value)	11.253 (0.001)*		2.380 (0.123)	
Population gametocyte prevalence			2% (84/4881)	
Gametocyte prevalence (*P. falciparum* positives)			6% (84/1421)	

Population gametocyte prevalence is the percentage of gametocyte carriers among the total study population (*P. falciparum* positive and negative samples together), while the gametocyte prevalence among the *P. falciparum* positive samples is the percentage of gametocyte carriers among the *P. falciparum* positive samples only (excluding *P. falciparum* negatives). χ^2 = Pearson's chi-squared test and (*) indicates statistical significance.

Microscopy gametocyte p and associated risk factors in the study population

The total number of gametocyte carriers as detected using microscopy in the study was 84/4881, with 57 of the carriers found within the age group 5–9 years as compared to 27 in the age-group 10–15 years (*p*-value = 0.001, Table 2). The microscopy gametocyte prevalence among the *P. falciparum* malaria carriers (only *P. falciparum* positive individuals) was found to be 6% (84/1421). These represent the minimum gametocyte prevalence levels, due to the sensitivity limits of microscopy. The gametocyte carriage in females (38.10%, 32/84) and males (61.90%, 52/84) was not significantly different (*p*-value = 0.123). The *P. falciparum* infection rate and gametocyte positive rate both follow a gradual declining trend from 2016 to late 2018. However, a high *P. falciparum* infection rate does not always coincide with a high gametocyte positive rate, for example samples from June 2017 and April 2018. In addition, the *P. falciparum* infection rate does not appear to be heavily influenced by rainfall (Figure 2).

The analysis showed that risk of *P. falciparum* infection was highest among the males as compared to females [OR = 0.8 (95% CI = 0.7–0.9), $P < 0.001$] while the age of an individual was not an independent risk factor. However, children between the ages of 5–9 years have a higher risk of gametocyte carriage when infected with *P. falciparum* as compared to those between the ages 10–15 years [OR = 2.1 (95% CI = 1.3–3.4), $P = 0.002$].

Relationship between gametocyte density and multiplicity of *Plasmodium falciparum* infections (MOI) and mosquito infection prevalence

The total number of samples used in assessing the relationship between gametocyte density, MOI and mosquito infection prevalence was 37. However, 15 of the 37 samples failed to amplify during the microsatellite amplification PCR and are recorded as missing data. After the feeding experiments, 3395 mosquitoes were used in the CSP ELISA assay. All the blood samples offered to the mosquitoes have resulted in at least one infection. 463 mosquitoes were infected recording a mean infection rate of 12.71% (Median: 7.6, IQR: 10.97, SE: 2.63, SD: 16.1) and mean gametocyte density was 59.89 gametocytes µL -1 (Median: 24, IQR: 48, SE: 12.28, SD: 74.71), while the mean number of distinct alleles per isolate was 7.32 (Median: 6, IQR: 3, SE: 0.80, SD: 3.76) (see density and MOI data, *Underlying data*[36]). In this study, a significant positive correlation was found between

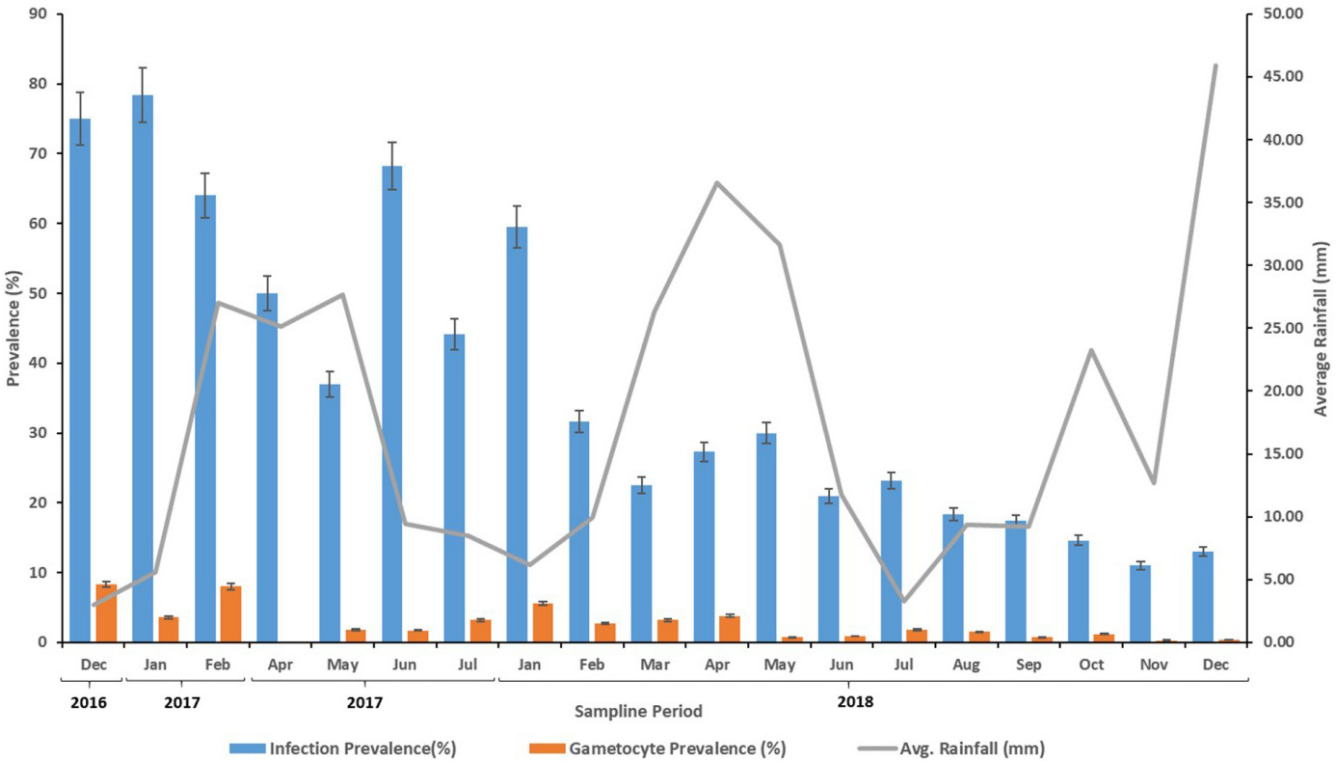

Figure 2. *P. falciparum infection* (blue) and gametocyte (brown) prevalence among the study participants and average rainfall (gray) during the various sampling periods.

P. falciparum gametocyte densities in the patient blood samples and mosquito infection prevalence (0.682, *p*-value < 0.0001). In addition, a positive correlation between multiplicity of *P. falciparum* infection (MOI) and mosquito infection prevalence was reported (0.451, *p*-value = 0.035). Notably, the correlation between MOI and gametocyte density was not statistically significant (0.167, *p*-value = 0.459). The mosquito infection prevalence is defined as the percentage of infected mosquitoes after day 10 of the membrane-feeding assay (Table 3 and Figure 3).

A multiple regression was run to predict mosquito infection prevalence from gametocyte density (gametocyte/µL) and MOI (Table 4). These variables statistically significantly predicted mosquito infection prevalence, $F(2, 19) = 20.235$, $p < 0.0001$, $R^2 = 0.681$ and both contributed significantly to the prediction, $p < 0.05$.

The multiple coefficient of determination (R-SQR. = 0.681) indicated that about 68.1% of the variation in mosquito infection prevalence is accounted for by the gametocyte density and MOI. Thus, the formulated equation for mosquito infection prevalence in this study is:

$$\hat{Y} = -6.644 + 0.151X_1 + 1.707\,X_2$$

Where $\hat{Y}$ is the expected mosquito infection prevalence, and X_1 and X_2 are the gametocyte density and MOI, respectively.

Discussion

We monitored the prevalence of gametocyte carriers and investigated risk factors among asymptomatic schoolchildren (age 5–15 years) in Western Kenya. An assessment of the relationship between gametocyte density, MOI and mosquito infection prevalence was also carried out. We found a moderate and declining rate of gametocyte prevalence in the study population, which is in agreement with the findings of other studies in the region[39,40]. Intensification of the fight against malaria in the region by the Kenyan government may be contributing to the decline in positivity rate and gametocyte carriage reported in our study[41]. Gametocyte prevalence was higher among the younger age groups (5–9 years), which accounted for 67.86% (57/84) of the total gametocyte carriers in the study population. Similar patterns of gametocyte carriage was reported by other studies[21,31]. This could be due to age-dependent development of anti-parasite immunity due to repeated exposure in endemic settings[21,31]. The high prevalence of gametocyte carriage among the younger age group (5–9 years) pinpoints the potential role of this age group in sustaining malaria transmission in the region. Children have been reported to be important contributors to the malaria infectious reservoir in many other settings[21]. Among the *P. falciparum* gametocyte positive individuals, males tended to be slightly overrepresented 61.9% (52/84) as compared to females 38.1% (32/84)]. However, this is not statistically significant. The *P. falciparum* prevalence was much lower in 2018 when compared to the 2017 season. This is likely due to

Table 3. Multiple correlation analysis of gametocyte density and multiplicity of *P. falciparum* infection (MOI) with the infection prevalence in the mosquitoes.

Parameters	Infection rate (*P*-value)	Gametocyte density (*P*-value)	MOI (*P*-value)
Infection prevalence	1	0.682 (< 0.0001)*	0.451 (0.035)*
Gametocyte density	0.682 (< 0.0001)*	1	0.167 (0.459)
MOI	0.451 (0.035)*	0.167 (0.459)	1

The dependent variable in this analysis is the infection prevalence. *Ref* represents the reference, (*) denotes statistical significance. Gametocyte density (Gam/µL) is presented without adjustment and the MOI is presented as a continuous variable.

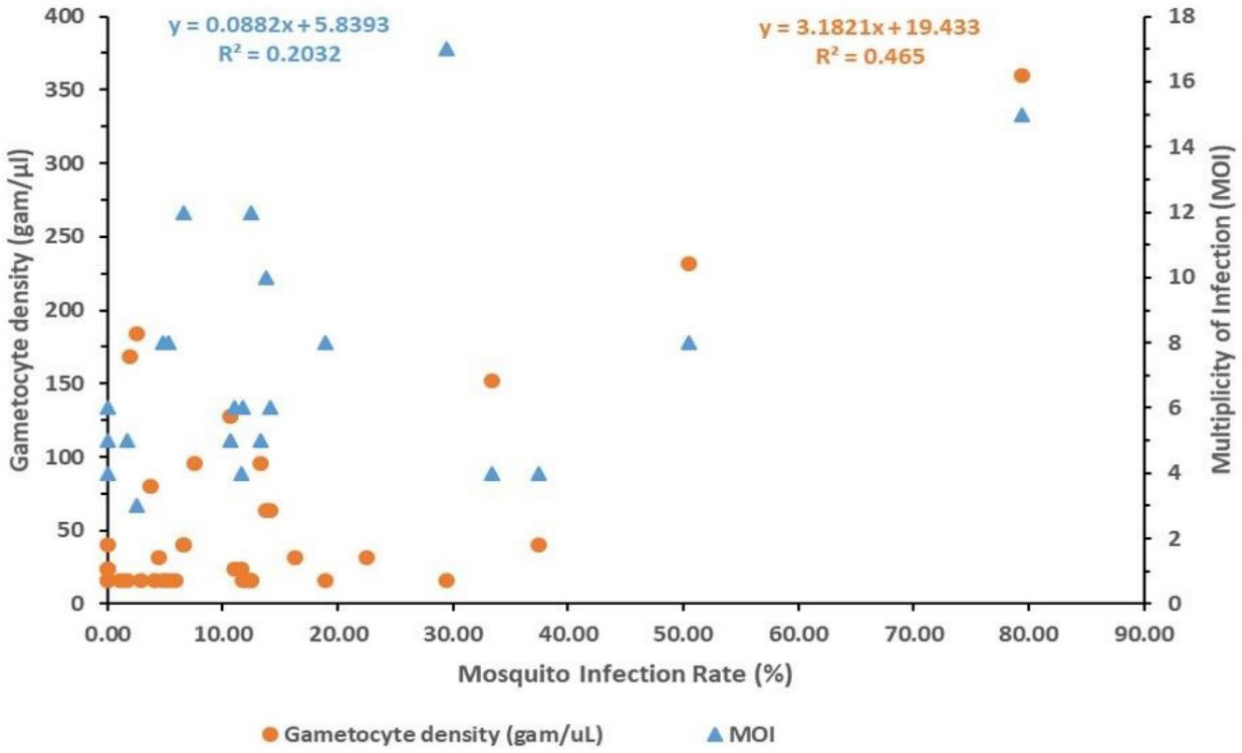

Figure 3. Relationship between gametocyte density (gametocyte/µL) and multiplicity of infection (MOI) with mosquito infection rate.

Table 4. Parameter of multiple linear regressions analysis.

Parameters	Coefficients	Std. Error	t-statistic	*P*-value
(Constant)	-6.644	5.564	-1.194	**0.247**
Gametocyte density (gam/µL)	0.151	0.028	5.328	**< 0.001**
MOI	1.707	0.672	2.54	**0.020**

R = 0.825, R-SQR. = 0.681, Adj. R-SQR = 0.647, SE = 11.418. ***R*** is the multiple correlation coefficient, ***R-SQR.*** (R-square) is the multiple coefficient of determination, ***Adj. R-SQR*** represents the adjusted R-square, and ***SE*** is the standard error. MOI, multiplicity of infection. Gametocyte density (Gam/ µL) is presented without adjustment and the MOI is presented as a continuous variable.

an indoor residual spraying (IRS) campaign conducted by Africa Indoor Residual Spraying (AIRS) Kenya, in early 2018 in this region[42]. Nonetheless, gametocyte prevalence remained at moderate levels during all the sampling periods, indicating year-round gametocyte carriage in the study population irrespective of the rainfall levels and pattern. In malaria endemic settings, asymptomatic carriers are known to harbour gametocytes even during the non-transmission season and are reported to be responsible for the resurgence of malaria infections during the subsequent transmission season[38]. When combined with prevalent *Anopheles* mosquito vectors, asymptomatic *P. falciparum* gametocyte carriage can lead to perennial transmission of malaria in the region.

The only independent risk factor associated with *P. falciparum* infection found in this study was gender. Males have higher odds of *P. falciparum* infection in the study area as compared to females. Gender was reported as a risk factor in other studies in the region[31]. This finding is in line with the reports that female children are biologically less susceptible to infectious diseases as compared to male children[43]. Age was not found to be a risk factor for contracting *P. falciparum* malaria infection in this study but was linked with gametocyte carriage when infected with *P. falciparum.* Younger children (5–9 years) have a higher risk of gametocyte carriage when infected with *P. falciparum*. A study in Tanzania has also reported similar a association of age with increased gametocyte prevalence[44].

A significant positive association was found between gametocyte density and mosquito infection prevalence (correlation coefficient = 0.682, p-value < 0.001). Infection prevalence tends to be relatively higher among mosquitoes that fed on carriers with high gametocyte densities (> 20 Gam/uL). This result corroborates the findings of other studies[21,45]. In particular, it has been noted that over relatively low gametocyte densities, in the range observed in this study, an increase in gametocytaemia corresponds with a rapid increase in the proportion of infected mosquitoes[26,28]. This maybe a specific parasite strategy to maximize human-mosquito transmission (fertility assurance)[46,47].

The relationship between multiplicity of *P. falciparum* infection and mosquito infection prevalence is not well documented. We found that *P. falciparum* isolates harbouring multiple distinct clones positively influence the mosquito infection prevalence, since there was a significant positive correlation between MOI and mosquito infection prevalence (correlation coefficient = 0.451, p-value = 0.035). In contrast, a negative association between MOI and mosquito infection prevalence and intensity has been reported elsewhere[48]. In our study, the interaction between MOI and gametocyte density was not statistically significant, which is in line with other studies[48].

Although gametocyte density is clearly an important factor in predicting the success of *P. falciparum* transmission to the mosquito vector, gametocyte density alone in blood samples does not equate to their infectiousness to mosquitoes[49]. Therefore, understanding the association between gametocyte density and other parasite parameters like MOI with mosquito infection prevalence will improve our understanding of the dynamics of *P. falciparum* transmission. Our results indicate a significant and positive combined effect of the explanatory variables (gametocyte density and multiplicity of *P. falciparum* infection) on the mosquito infection prevalence [$F(2, 19) = 20.235$, $p < 0.0001$, $R^2 = 0.681$]. These results show that MOI and gametocyte density account for about 68.1% of the variation in mosquito infection prevalence. This may be linked to intense inter-strain competition due to increased investment in gametocytes and multiple clone infections (MOI) that may trigger the emergence of highly transmissible or virulent parasite strains thereby increase mosquito that may trigger the emergence of highly transmissible or virulent parasite strains thereby increase mosquito infectivity[50–52]. Another plausible explanation for the association between MOI, gametocyte density and mosquito infection prevalence found in this study may be due to the outcome of strategic balancing between in-host survival and between-host transmission[47,53–55]. At relatively low MOIs, the level of intra-host competition is relatively low and the *P. falciparum* parasites reduce conversion rates to enhance asexual replication and in-host survival through reproductive restraint. However, at high MOIs, the intra-host competition is too intense for reproductive restraint and the parasites tend to increase the conversion rate to facilitate between-host transmission[53,54]. The high mosquito infection prevalence observed at high MOIs can be explain by the maximised gametocyte production to increase the chances of between-host transmission.

Limitations of the study

The major limitation of the study is the small sample size used in determining the association between gametocyte density, MOI and mosquito infection prevalence. This was partly due to the limited number of study participants who consented to providing extra venous blood samples for the mosquito feeding assays. The asexual parasite density was not determined due to the study design centred on gametocyte density and MOI. The study used microscopy in determining gametocyte presence and density. However, microscopy is known to be of relatively low resolution, hence some gametocyte carriers might not be detected[56]. The membrane feeding assay might have low mosquito feeding efficiency when compared to direct skin feeding, however, ethical approval for this study required the use of membrane feeding assays[21].

Conclusions

Malaria prevalence and gametocyte carriage is high among asymptomatic schoolchildren, particularly the younger age group (5–9 years), in the region. The relatively stable and year-round prevalence of gametocyte carriage among the study participants in this study signals the role of schoolchildren in maintaining malaria transmission in the study area. The statistically significant and positive combined effect of the explanatory variables on the mosquito infection prevalence will help in determining the human infectious reservoirs in different malaria endemic settings. Malaria control interventions that are highly efficient in reducing multiple clone

parasite carriage and gametocyte density could aid in disrupting the transmission of the parasite, thereby facilitating the ultimate elimination of the disease in the region.

Data availability

Underlying data

Figshare: Data supporting a study of the prevalence of asymptomatic *P. falciparum* gametocyte carriage in schoolchildren and assessment of the association between gametocyte density, multiplicity of infection and mosquito infection prevalence. https://doi.org/10.6084/m9.figshare.13048088[36].

This project contains the following underlying data:

- Study participants screening data_1.xlsx (NB: Single = *P. falciparum* only, Mixed = *P. falciparum* plus either *P. ovale* or *P. malariae* or both)
- Gam density_MOI_Infection prevalence_3.xlsx.
- Sampling period_Infection and Gam prevalence_2.xlsx.
- Raw ELISA output data.xlsx
- Raw ELISA output data_OD values.xlsx
- Raw genotyping output data.xlsx

Extended data

Figshare: Data supporting a study of the prevalence of asymptomatic *P. falciparum* gametocyte carriage in schoolchildren and assessment of the association between gametocyte density, multiplicity of infection and mosquito infection prevalence. https://doi.org/10.6084/m9.figshare.13048088[36].

This project contains the following extended data:

- Supplementary file Table S1.docx.
- Sampling site and period analysed data.docx

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

Acknowledgements

We are indebted to all volunteers who partook in this study as well as their parents/guardians for granting consent. The authors are very much grateful to the entire research team of Dr. Jeremy K. Herren and the management of International Centre of Insect Physiology and Ecology (*icipe*) for permitting us to use their samples and giving us access to use their research facilities to conduct the study. Special thanks to Azumah Karim for his valuable assistance and advice in analysing the data.

References

1. Bhatt S, Weiss DJ, Cameron E, *et al.*: **The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015.** *Nature.* 2015; **526**(7572): 207–11.
PubMed Abstract | Publisher Full Text | Free Full Text
2. World Health Organization. **WHO. World Malar Rep.** 2019.
Reference Source
3. Kenya National Bureau of Statistics and ICF Macro: **Kenya Demographic and Health Survey 2008 – 2009.** Calverton: Kenya National Bureau of Statistics and ICF Macro. 2010.
Reference Source
4. Division of Malaria Control, Ministry of Public Health and Sanitation K: **Malaria Strategy.** 2009.
Reference Source
5. Division of Malaria Control, Ministry of Public Health K: **The National Malaria Strategy 2001–2010.** 2001.
Reference Source
6. Ministry of Health K: **Kenya Malaria Indicator Survey 2015.** Minist. Heal. Kenya. 2016.
Reference Source
7. Macharia PM, Giorgi E, Noor AM, *et al.*: **Spatio-temporal analysis of *Plasmodium falciparum* prevalence to understand the past and chart the future of malaria control in Kenya.** *Malar J.* 2018; **17**(1): 340.
PubMed Abstract | Publisher Full Text | Free Full Text
8. Noor AM, Gething PW, Alegana VA, *et al.*: **The risks of malaria infection in Kenya in 2009.** *BMC Infect Dis.* 2009; **9**: 180.
PubMed Abstract | Publisher Full Text | Free Full Text
9. Ouédraogo AL, Guelbéogo WM, Cohuet A, *et al.*: **A protocol for membrane feeding assays to determine the infectiousness of *P . falciparum.* naturally infected individuals to Anopheles gambiae.** *Malariaworld J.* 2013; **4**(16): 17–20.
Reference Source
10. Baker DA: **Malaria gametocytogenesis.** *Mol Biochem Parasitol.* 2010; **172**(2): 57–65.
PubMed Abstract | Publisher Full Text | Free Full Text
11. Wampfler R, Timinao L, Beck HP, *et al.*: **Novel genotyping tools for investigating transmission dynamics of *Plasmodium falciparum.*** *J Infect Dis.* 2014; **210**(8): 1188–97.
PubMed Abstract | Publisher Full Text | Free Full Text
12. Zeynudin A, Degefa T, Zeynudin A, *et al.*: **High Prevalence of Gametocyte Carriage among Individuals with Asymptomatic Malaria: Implications for Sustaining Malaria Control and Elimination Efforts in Ethiopia.** *Hum Parasit Dis.* 2015; **8**(2016): 17–25.
Publisher Full Text
13. Bousema JT, Gouagna LC, Drakeley CJ, *et al.*: ***Plasmodium falciparum* gametocyte carriage in asymptomatic children in western Kenya.** *Malar J.* 2004; **3**: 18.
PubMed Abstract | Publisher Full Text | Free Full Text
14. Lamptey H, Ofori MF, Kusi KA, *et al.*: **The prevalence of submicroscopic *Plasmodium falciparum* gametocyte carriage and multiplicity of infection in children, pregnant women and adults in a low malaria transmission area in Southern Ghana.** *Malar J.* 2018; **17**(1): 331.
PubMed Abstract | Publisher Full Text | Free Full Text
15. Lindblade KA, Steinhardt L, Samuels A, *et al.*: **The silent threat: Asymptomatic parasitemia and malaria transmission.** *Expert Rev Anti Infect Ther.* 2013; **11**(6): 623–39.
PubMed Abstract | Publisher Full Text
16. Stone WJR, Dantzler KW, Nilsson SK, *et al.*: **Naturally acquired immunity to sexual stage *P. falciparum* parasites.** *Parasitology.* 2016; **143**(2): 187–98.
PubMed Abstract | Publisher Full Text
17. Coalson JE, Walldorf JA, Cohee LM, *et al.*: **High prevalence of *Plasmodium falciparum* gametocyte infections in school-age children using molecular detection: patterns and predictors of risk from a cross-sectional study in southern Malawi.** *Malar J.* 2016; **15**(1): 527.
PubMed Abstract | Publisher Full Text | Free Full Text
18. Cohee L, Laufer M: **Tackling malaria transmission in sub-Saharan Africa.** *Lancet Glob Health.* 2018; **6**(6): e598–9.
PubMed Abstract | Publisher Full Text | Free Full Text
19. Brooker S, Clarke S, Snow RW, *et al.*: **Malaria in African schoolchildren:**

options for control. *Trans R Soc Trop Med Hyg.* 2008; **102**(4): 304–5.
PubMed Abstract | Publisher Full Text | Free Full Text

20. Kanwugu ON, Helegbe GK, Aryee PA, *et al.*: **Prevalence of Asymptomatic Malaria among Children in the Tamale Metropolis: How Does the PfHRP2 CareStart™ RDT Perform against Microscopy?** *J Trop Med.* 2019; **2019**: 6457628.
PubMed Abstract | Publisher Full Text | Free Full Text

21. Bousema T, Drakeley C: **Epidemiology and Infectivity of *Plasmodium falciparum* and *Plasmodium vivax* Gametocytes in Relation to Malaria Control and Elimination.** *Clin Microbiol Rev.* 2011; **24**(2): 377–410.
PubMed Abstract | Publisher Full Text | Free Full Text

22. Nilsson SK, Childs LM, Buckee C, *et al.*: **Targeting Human Transmission Biology for Malaria Elimination.** *PLoS Pathog.* 2015; **11**(6): e1004871.
PubMed Abstract | Publisher Full Text | Free Full Text

23. Nwakanma D, Kheir A, Sowa M, *et al.*: **High gametocyte complexity and mosquito infectivity of *Plasmodium falciparum* in the Gambia.** *Int J Parasitol.* 2008; **38**(2): 219–27.
PubMed Abstract | Publisher Full Text

24. Nassir E, Abdel-Muhsin AMA, Suliaman S, *et al.*: **Impact of genetic complexity on longevity and gametocytogenesis of *Plasmodium falciparum* during the dry and transmission-free season of eastern Sudan.** *Int J Parasitol.* 2005; **35**(1): 49–55.
PubMed Abstract | Publisher Full Text

25. Schneider P, Bousema T, Omar S, *et al.*: **(Sub)microscopic *Plasmodium falciparum* gametocytaemia in Kenyan children after treatment with sulphadoxine-pyrimethamine monotherapy or in combination with artesunate.** *Int J Parasitol.* 2006; **36**(4): 403–8.
PubMed Abstract | Publisher Full Text

26. Bradley J, Stone W, Da DF, *et al.*: **Predicting the likelihood and intensity of mosquito infection from sex specific *Plasmodium falciparum* gametocyte density.** *eLife.* 2018; **7**: e34463.
PubMed Abstract | Publisher Full Text | Free Full Text

27. Johnston GL, Smith DL, Fidock DA: **Malaria's missing number: calculating the human component of R0 by a within-host mechanistic model of *Plasmodium falciparum* infection and transmission.** *PLoS Comput Biol.* 2013; **9**(4): e1003025.
PubMed Abstract | Publisher Full Text | Free Full Text

28. Schneider P, Babiker HA, Gadalla AAH, *et al.*: **Evolutionary sex allocation theory explains sex ratios in natural *Plasmodium falciparum* infections.** *Int J Parasitol.* 2019; **49**(8): 601–4.
PubMed Abstract | Publisher Full Text

29. Grignard L, Gonçalves BP, Early AM, *et al.*: **Transmission of molecularly undetectable circulating parasite clones leads to high infection complexity in mosquitoes post feeding.** *Int J Parasitol.* 2018; **48**(8): 671–7.
PubMed Abstract | Publisher Full Text | Free Full Text

30. Greenwood BM: **Asymptomatic malaria infections--do they matter?** *Parasitol Today.* 1987; **3**(7): 206–14.
PubMed Abstract | Publisher Full Text

31. Olanga EA, Okombo L, Irungu LW, *et al.*: **Parasites and vectors of malaria on Rusinga Island, Western Kenya.** *Parasit Vectors.* 2015; **8**: 250.
PubMed Abstract | Publisher Full Text | Free Full Text

32. Touray AO, Mobegi VA, Wamunyokoli F, *et al.*: **Diversity and Multiplicity of *P. falciparum* infections among asymptomatic school children in Mbita, Western Kenya.** *Sci Rep.* 2020; **10**(1): 5924.
PubMed Abstract | Publisher Full Text | Free Full Text

33. Wirtz RA, Duncan JF, Njelesani EK, *et al.*: **ELISA method for detecting Plasmodium falciparum circumsporozoite antibody.** *Bull World Health Organ.* 1989; **67**(5): 535–42.
PubMed Abstract | Free Full Text

34. Wirtz R, Avery M, Benedict M, *et al.*: **Plasmodium falciparum Sporozoite ELISA.** In: Methods in Anopheles Research. 2010; 282–93.

35. Mobegi VA, Loua KM, Ahouidi AD, *et al.*: **Population genetic structure of *Plasmodium falciparum* across a region of diverse endemicity in West Africa.** *Malar J.* 2012; **11**: 223.
PubMed Abstract | Publisher Full Text | Free Full Text

36. Touray A, Mobegi VA, Wamunyokoli F, *et al.*: **Data supporting a study of the prevalence of asymptomatic P. falciparum gametocyte carriage in schoolchildren and assessment of the association between gametocyte density, multiplicity of infection and mosquito infection prevalence.** *figshare.* 2020.
http://www.doi.org/10.6084/m9.figshare.13048088

37. Beier JC, Asiago CM, Onyango FK, *et al.*: **ELISA absorbance cut-off method affects malaria sporozoite rate determination in wild Afrotropical Anopheles.** *Med Vet Entomol.* 1988; **2**(3): 259-64.
PubMed Abstract | Publisher Full Text

38. Huntington JL, Hegewisch KC, Daudert B, *et al.*: **Climate Engine: cloud computing and visualization of climate and remote sensing data for advanced natural resource monitoring and process understanding.** *Bull Am Meteorol Soc.* 2017; **98**(11): 2397–2410.
Publisher Full Text

39. Omondi P, Burugu M, Matoke-Muhia D, *et al.*: **Gametocyte clearance in children, from western Kenya, with uncomplicated *Plasmodium falciparum* malaria after artemether-lumefantrine or dihydroartemisinin-piperaquine treatment.** *Malar J.* 2019; **18**(1): 398.
PubMed Abstract | Publisher Full Text | Free Full Text

40. Idris ZM, Chan CW, Kongere J, *et al.*: **High and Heterogeneous Prevalence of Asymptomatic and Sub-microscopic Malaria Infections on Islands in Lake Victoria, Kenya.** *Sci Rep.* 2016; **6**: 36958.
PubMed Abstract | Publisher Full Text | Free Full Text

41. Muthui MK, Mogeni P, Mwai K, *et al.*: **Gametocyte carriage in an era of changing malaria epidemiology: A 19-year analysis of a malaria longitudinal cohort [version 2; peer review: 2 approved].** *Wellcome Open Res.* 2019; **4**: 66.
PubMed Abstract | Publisher Full Text | Free Full Text

42. **Kenya End of Spray Report 2018.** Rockville M. The PMI Africa IRS (AIRS) Project, Abt Associates Inc. 2018.

43. Gadalla AAH, Schneider P, Churcher TS, *et al.*: **Associations between season and gametocyte dynamics in chronic *Plasmodium falciparum* infections.** *PLoS One.* 2016; **11**(11): e0166699.
PubMed Abstract | Publisher Full Text | Free Full Text

44. Nyarko SH, Cobblah A: **Sociodemographic determinants of malaria among under-five children in Ghana.** *Malar Res Treat.* 2014; **2014**: 304361.
PubMed Abstract | Publisher Full Text | Free Full Text

45. Akim NI, Drakeley C, Kingo T, *et al.*: **Dynamics of *P. falciparum* gametocytemia in symptomatic patients in an area of intense perennial transmission in Tanzania.** *Am J Trop Med Hyg.* 2000; **63**(3–4): 199–203.
PubMed Abstract | Publisher Full Text

46. Reece SE, Drew DR, Gardner A: **Sex ratio adjustment and kin discrimination in malaria parasites.** *Nature.* 2008; **453**(7195): 609–14.
PubMed Abstract | Publisher Full Text | Free Full Text

47. Reece SE, Ramiro RS, Nussey DH: **Plastic parasites: Sophisticated strategies for survival and reproduction?** *Evol Appl.* 2009; **2**(1): 11–23.
PubMed Abstract | Publisher Full Text | Free Full Text

48. Morlais I, Nsango SE, Toussile W, *et al.*: ***Plasmodium falciparum* mating patterns and mosquito infectivity of natural isolates of gametocytes.** *PLoS One.* 2015; **10**(4): e0123777.
PubMed Abstract | Publisher Full Text | Free Full Text

49. Churcher TS, Bousema T, Walker M, *et al.*: **Predicting mosquito infection from *Plasmodium falciparum* gametocyte density and estimating the reservoir of infection.** *eLife.* 2013; **2**: e00626.
PubMed Abstract | Publisher Full Text | Free Full Text

50. Alizon S, de Roode JC, Michalakis Y: **Multiple infections and the evolution of virulence.** *Ecol Lett.* 2013; **16**(4): 556–67.
PubMed Abstract | Publisher Full Text

51. Wargo AR, de Roode JC, Huijben S, *et al.*: **Transmission stage investment of malaria parasites in response to in-host competition.** *Proc Biol Sci.* 2007; **274**(1625): 2629–38.
PubMed Abstract | Publisher Full Text | Free Full Text

52. Bose J, Kloesener MH, Schulte RD: **Multiple-genotype infections and their complex effect on virulence.** *Zoology (Jena).* 2016; **119**(4): 339–49.
PubMed Abstract | Publisher Full Text

53. Schneider P, Greischar MA, Birget PLG, *et al.*: **Adaptive plasticity in the gametocyte conversion rate of malaria parasites.** *PLoS Pathog.* 2018; **14**(11): e1007371.
PubMed Abstract | Publisher Full Text | Free Full Text

54. Carter LM, Kafsack BFC, Llinás M, *et al.*: **Stress and sex in malaria parasites: Why does commitment vary?** *Evol Med Public Health.* 2013; **2013**(1): 135–47.
PubMed Abstract | Publisher Full Text | Free Full Text

55. Koella JC, Antia R: **Optimal pattern of replication and transmission for parasites with two stages in their life cycle.** *Theor Popul Biol.* 1995; **47**(3): 277–91.
Publisher Full Text

56. Aydin-Schmidt B, Xu W, González IJ, *et al.*: **Loop Mediated Isothermal Amplification (LAMP) Accurately Detects Malaria DNA from Filter Paper Blood Samples of Low Density Parasitaemias.** *PLoS One.* 2014; **9**(8): e103905.
PubMed Abstract | Publisher Full Text | Free Full Text

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Version 2

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✗ **Lisa Ranford Cartwright**
Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

The authors have not responded to my review, and the revised manuscript does not address the main issues I raised. I cannot, therefore, change my original view. For example, the errors in the MOI table have not been explained or corrected (and the analysis then redone if necessary with the correct MOI numbers), and the additional data on mosquito numbers dissected have not been provided.

I also note that the revised Table 1 now does not make sense. For example, in the table, the number of 5-9 year olds who are parasite positive is given as 712/1421, but those who are negative is now 1833/3460. Why is the denominator different?

Also in the text, there is probably a new mistake in the text/table 1 between gametocyte positive and *P. falciparum* positive (unless all P.f positive are also gametocyte positive) - for example, the text says that the *P. falciparum* prevalence among the males is 764/1421, but the gametocyte carriage is given as 382/712 in the 5-9 year olds and 382/709 in the 10-15 year olds ie total 764 gametocyte positives, so all Pf positive are gametocyte positive?

These errors need to be corrected before indexing.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Genetics and transmission biology of P. falciparum.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 26 April 2021

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?

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The revised manuscript has addressed many of my comments. I have a few outstanding comments.

I still find figure 1 difficult to grasp. The prevalence estimates are difficult to interpret without exact confidence intervals or number of observations. The legend now gives prevalence and range but it would be more informative to let the size of the dot be informed by the number of observations, or some other way of presenting uncertainty in estimates.

The ELISA protocol is now better explained. I would still really like to see how convincing the positive mosquitoes were. Ten days after feeding is appropriate, by then positive mosquitoes should be highly positive. It is important to present (either in main text or supplemental info) the set threshold for positivity and the OD range of mosquitoes that are concluded to be positive. This is important to interpret indirect assessments of infection status.

Table 1 should be simplified. Male and females are mutually exclusive categories here. So percentage female is enough.

Table 2 should also be clarified. I am not sure what the X2 value relates to since it is presented under asexual parasites and gametocytes. The denominators also appear mixed. So each colum should have (roughly) the same denominator that is the percentage of individuals in a certain categorie (e.g. with a single clone infection (not single infection, which is a strange term) expressed as a percentage of the age group. Population gametocyte prevalence and gametocyte prevalence among Pf positives should not be in the table but in the main text.

Comparisons between age groups (e.g. 'The total number of gametocyte carriers as detected using microscopy in the study was 84/4881, with 57 of the carriers found within the age group 5–9 years as compared to 27 in the age-group 10–15') are sometimes difficult to follow. This specific example suggests a count analysis while I suspect prevalences are compared. It should be followed by a more in-depth analysis of gametocyte prevalence for a given parasite density. The currently phrasing suggests an effect of age on gametocyte production but this may of course be fully driven by age-dependent differences in parasite density.

The statement 'However, a high *P. falciparum* infection rate does not always coincide with a high gametocyte positive rate, for example samples from June 2017 and April 2018. In addition, the *P. falciparum* infection rate does not appear to be heavily influenced by rainfall', requires formal

analyses.

MOI and gametocyte density need to be incorporated in a multi-variate model. The current phrasing is not sufficient. Gametocyte density is positively associated, MOI is positively associated. The two are not statistically significantly associated but there is still a need for an appropriate multivariate model to interpret the findings.

Table 3 can be omitted.

Table 4 can be omitted.

The formula is probably too simplistic to do justice to the non-linear association between variables. The appropriate model is more complex than currently described (see Bradley *et al.*) and has DIC as indicator of fit to determine whether adding MOI will, for instance, improve model fit compared to gametocyte density alone. With uncertainties around gametocyte density and MOI and the limited number of observations, I feel the 68% is too precise and can be omitted.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Epidemiology, gametocyte biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 23 April 2021

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?

Linda E Amoah
Immunology Department, Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana

The responses to my first set of questions have led to a new set of questions/comments and suggestions.

Based on this quotation: 'The total number of samples used in assessing the relationship between gametocyte density, MOI and mosquito infection prevalence was 37.' I think it is inappropriate to use all the samples used in the original study in this paper. This paper should focus only on the total number that gave the 4 ml of venous blood or the gametocyte positive individuals. It is very misleading to report all the numbers when only a fraction were/are the focus of this report, so then you can only show a map of where the few samples used in this study were obtained from.

The discussion pertains mostly to the 84, I think this should reduce to the 37 whole blood donors. I also think that the title can remain if the prevalence of gametocytes is reported for the 37 individuals as opposed to the 2459 individuals.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: molecular biology, malaria transmission, diagnostics and vaccine design

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 23 April 2021

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Lynn Grignard
Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK

I have no further comments, thank you for incorporating reviewers' comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Malaria, molecular biology, transmission biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 06 April 2021

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Lisa Ranford Cartwright
Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

Summary:
The research aims were to investigate the prevalence of P. falciparum gametocyte carriage in asymptomatic schoolchildren in a region of Kenya and to investigate any association between the gametocyte density, the genetic complexity (of the total parasite population present in an individual) and their ability to infect mosquitoes following membrane feeding. The main findings of the paper are in agreement with previously published research, in that younger children had a higher likelihood of gametocyte carriage, that male children had a higher likelihood of being parasite positive than females, and that mosquito infection was positively correlated with gametocyte density. One finding of a positive association between genetic complexity of infection and mosquito infection prevalence was not in agreement with previous research.

Study design:
Asymptomatic individuals were identified by a screening of almost 5000 school children using an RDT, followed by microscopy to identify those with gametocytes present. The choice of RDT to screen for asymptomatic infections is problematic because such tests have a higher threshold for parasite detection - the specific RDT chosen seems to reliably detect parasite densities above 200 parasites per microlitre, but has a lower sensitivity below that (Djalle et al 2014 BMC Inf. Dis.). This makes it likely that individuals with lower level infections will be missed. The identification of gametocyte carriers using microscopy has also low sensitivity. The end result of the selection of these two methods is a likely underestimation of asymptomatic carriage of parasites and of gametocytes; only those individuals with higher asexual and gametocyte density will be included in the study. That risks bias in the analysis and conclusions since many asymptomatically-infected individuals would not be sampled. This issue needs to be discussed.

Sample size:
Perhaps as a result of the screening, which would miss lower level infections, there is an extremely small sample size. Of the 84 individuals carrying microscopically-detectable gametocytes, only 37 were used for mosquito infections. Were these 37 selected for the mosquito infection study based on any factor, or was it random? For example, did the 37 have the same age and gender split as the total gametocyte positive group? Of those 37, MOI data were only available for 22 individuals. The majority of the statistical analysis is therefore done on 22 data points only.

Data inconsistency:
There are some errors or inconsistencies in the data presented. The main one is in the MOI numbers that have been used for the analysis. I applaud the inclusion of all the raw data. However the calculations of MOI in the supplementary data (allele counts) do not match those used/ presented in the main paper in 11 of the 23 individuals e.g. Donor#3 has one allele listed for microsatellite TA60, but the MOI for that locus is given as 2. For unexplained reasons, the allele sizes for some microsatellites have been duplicated (so two alleles of the same size are listed and counted). Very high MOI values are not supported by the raw data given - for example, donor 3 has an MOI of 12 but the maximum number of alleles at any one locus is 10).

Incomplete data:
Only the percentage of mosquitoes infected is given, which does not give any idea about how

robust the differences are. The numbers infected/ dissected should be given, and statistical analysis would be better performed based on numbers rather than percentage infections, which would reflect the likely accuracy of the figures used.

Statistical analyses:
The statistical analyses are not well explained and could be improved. It is unclear why age and gender have been examined separately in the prevalence of infection and gametocyte carriage data. Although unlikely, there could be an imbalance of genders in the two age groups that would bias the results.
It is difficult to understand exactly how the infection data and correlations with MOI and gametocyte density have been carried out, and thus how robust the conclusions are given the very small sample size. For example, were the variables of gender and age also included in the regression analyses? The plot (Figure 3) is not convincing in supporting the conclusion that MOI is linked to infection prevalence (R^2 value of 0.2032), and the gametocyte density vs infection also has a low R^2 value, with the plot indicating the positive correlation is mainly due to three individuals with >30% infected mosquitoes, high gametocyte density and high MOI. The regression outputs show a somewhat surprising) significant association of gametocyte density and MOI on infection prevalence. However, it is likely that incorrect MOI values have been used. More information is needed on the modelling method used (linear regression? GLM?) and the factors included. The statistical analysis needs to be clarified and repeated using corrected data.

Overall:
The findings of the paper reinforce what has been previously reported from previous studies.

Minor points for clarification/correction:

1. Was each child only included once in the data set ie the same child was not sampled in both years?

2. The material used for positive controls/ to generate standard curves for the CSP ELISA is not sufficiently described. In addition, what is the threshold of this test for detection of parasites at the oocyst stage of infection?

3. The total number of female children examined/ positive is given in the text as 657/2457 but the total number of females examined is 2459 in the table.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Genetics and transmission biology of P. falciparum.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 17 February 2021

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?

Teun Bousema
Department of Medical Microbiology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

The authors present an analysis on infectivity from a limited number of gametocyte carriers from a narrow age range. Gametocyte quantification is done by microscopy, not ideal for this, but nevertheless very strongly associated with transmission risk. The data collection was, as indicated, for other studies. Nevertheless, the authors present one interesting finding. The article could be shortened considerably and can improve the presentation of the main finding of interest, the association between MOI and infectivity to mosquitoes. I have a large number of specific comments.

The authors report a higher parasite prevalence in males. This, if based on a good random selection of the population, is of interest and in line with recent findings from Uganda (Briggs eLIFE 2020). Any risk of bias in the source population should be described. What proportion of invited participants participated and is there any (self-) bias possible?

Prevalence of gametocytes can be presented for the entire population or from malaria-infected individuals. Both are of relevance, the former to estimate the age-structure in the 'infectious reservoir' and the latter highlighting differences in parasite density, gametocyte production or immunity. Both estimates should thus be presented. However, asexual parasite prevalence among parasite positive individuals is less interesting. All gametocyte carriers are asexual parasite carriers or very recently were.

Abstract:

- 'challenging An. gambiae s.l.' is an unusual choice of words. Allowing to feed or offering

blood to... is more conventional.

- The abstract would also benefit from a bit more detail on the predictors of mosquito infection rates. Now only the statistically significat predictors are mentioned but some narrative would help the readers.

- Also the (modest) sample size should be mentioned in the abstract. Both in terms of experiments and numbers of mosquitoes dissected.

Introduction:

- 'As a result, this category is most...' I assume age group is meant. This could be clarified.

- The phrasing 'Some studies have reported a positive association between mosquito infection rates of *P. falciparum* and gametocyte density, particularly at high gametocyte concentrations. However, at low gametocyte concentrations, a varying and less strong association is reported.' Is a bit misleading. As is quite clearly described in the literature (Bradley *et al.* eLIFE 2018 and Johnston PLoS Comp Biol 2012)[1,2], there is a clear positive association with sporadic infections at gametocyte densities below 10 gametocytes/uL.

- 'Two common characteristics of asymptomatic malaria infections in endemic settings are the prevalence of varying levels of gametocyte carriage among different age categories due to anti-parasite immunity and high rates of polyclonal infections' is a complicated sentence best broken up in 2 (or even 3).

- 'In order to ultimately eliminate malaria, interventions geared towards interrupting the disease transmission through efficient and effective identification and treatment of both asymptomatic and symptomatic parasite carriers will be of immense importance' is over-stating the evidence. Countries have eliminated malaria without a specific focus on asymptomatic infections. One can indeed expect that elimination would be accelerated by also targeting asymptomatic infections. I would propose parasite transmission rather than disease transmission, the symptoms are not transmitted by mosquitoes.

Methods:

- Figure 1, the study map, is interesting if all schools had a decent sample size. The number of observations (median, range) should be presented in the legend.

- 'This sample size was obtained based on the number of study participants within the designated study area that consented to partake in the study.' This is not very meaningful. How was the sample size decided upon? Did the authors aim to reach a certain sample size to address the current study questions or was the sample size decided upon to support other study questions?

- In the methods, please indicate what anticoagulant was used for phlebotomy and indicate the number of microscopy fields screened for gametocytes (or the number of white blood cells counted against) to give an idea of assay sensitivity.

- In the methods, please indicate the source of water-jacketed feeders and capacity (volume).

- It would be nice to understand why a qualitative assay (ELISA) was used as read-out and whether semi-quantitative read-out, as in Graumans MalJ 2017 was considered.[3]

- Please indicate what positive controls were used for the ELISA (source).

- Some details on the (minimum) number of dissected mosquitoes would be expected in the results section. In general, just present % infectious and % infected mosquitoes with more reference to denominators. The findings probably hold their value but at the moment it is unknown what feeding performance was and how mosquito survivorship may have affected the precision of outcomes.

- Any age-patterns, concluded to be non-exist here, are likely to be obscured by the small age range examined.

- It is unclear to me how mixed infections were examined. Is this by PCR or by microscopy?

- All references to gametocyte carriage should, at least early in the results and discussion section, be referred to as 'microscopy gametocyte prevalence' to make sure the reader understands the limitations of the diagnostic used.

- Comparisons of gametocyte prevalence by sex could be presented adjusted and unadjusted by total parasite density.

- Table 1 should be simplified. 'Positive' and 'negative' in the first rows are confusing and probably mutually exclusive.

- The presentation of asexual parasite prevalence and gametocyte prevalence (e.g. 99.99% vs 8% is not informative. Just present asexual prevalence and gametocyte prevalence by gender and age group.

- I would present for age and sex separately (so girls 5-9 vs boys 5-9 and girls 10-15 vs boys 10-15) even if only gender is statistically significantly associated with risk.

- Figure 2 can be omitted. It is not relevant to the current story.

- Table 2 can be omitted and captured in the text.

- Table 3 is confusing. It suggests that gametocyte density is the reference for gametocyte density. I understand that one cannot calculate a correlation coefficient here but that is not the same as calling it a reference category.

- Figure 3 suggests that MOI is a factor independent of gametocytes and is directly associated with infectivity. MOI can either be associated with higher gametocyte density (which appears not to be the case) or higher infectivity for a given gametocyte density. The latter would be better displayed if categories of gametocyte densities (e.g. <10, 10-20 and 20+, broadly tertiles) are defined as well as categories of MOI and for each gametocyte, class infectivity is given for low, intermediate and high MOI. That would allow an interpretation if, for each gametocyte class, MOI is associated with higher infectivity.

- Table 4 requires information about scale. Is gametocyte density included per uL with or without log10 adjustment? Is MOI included as a continuous variable? That would not be entirely intuitive and I would rather see MOI in categories since there is no reason I know of to assume that the difference between an MOI of 1 and 2 (single clone versus multiclone and thus potentially competition between clones) is the same as that between an MOI of 10 and 11.

Discussion:

- The statement 'Among the P. falciparum malaria positive individuals, males tended to be slightly overrepresented as both asexual 53.76% (764/1421) and gametocyte carriers 61.9% (52/84) as compared to females [asexual carriage; 46.23% (657/1421), gametocyte carriage; 38.1% (32/84)] is very confusing and should come with an estimate of statistical significance. There is no reason to add up asexuals and gametocytes to classify someone as parasite positive and then determine the proportion of these positives that is asexual positive. It just doesn't make much sense biologically or epidemiologically.

- The part on gametocyte sex ratio can be removed from the discussion. It is interesting but has no relevance to the current study that didn't assess sex ratio.

- The authors suggest competition between virulent and less virulent (defined as transmissible) strains as a mechanism underlying the association between MOI and mosquito infection rates. They cite references 49-51 but none of these, as far as I know, prove this association. They merely hint at inter-strain competition that is most likely to occur through increased investment in gametocytes or gametocytes of a certain sex.

References

1. Bradley J, Stone W, Da D, Morlais I, et al.: Predicting the likelihood and intensity of mosquito infection from sex specific Plasmodium falciparum gametocyte density. *eLife*. 2018; **7**. Publisher Full Text
2. Johnston GL, Smith DL, Fidock DA: Malaria's missing number: calculating the human component of R0 by a within-host mechanistic model of Plasmodium falciparum infection and transmission. *PLoS Comput Biol*. 2013; **9** (4): e1003025 PubMed Abstract | Publisher Full Text
3. Graumans W, Tadesse F, Andolina C, van Gemert G, et al.: Semi-high-throughput detection of Plasmodium falciparum and Plasmodium vivax oocysts in mosquitoes using bead-beating followed by circumsporozoite ELISA and quantitative PCR. *Malaria Journal*. 2017; **16** (1). Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Epidemiology, gametocyte biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 18 Mar 2021

Jeremy Herren, International Centre of Insect Physiology and Ecology (icipe), Nairobi, Kenya

The authors present an analysis on infectivity from a limited number of gametocyte carriers from a narrow age range. Gametocyte quantification is done by microscopy, not ideal for this, but nevertheless very strongly associated with transmission risk. The data collection was, as indicated, for other studies. Nevertheless, the authors present one interesting finding. The article could be shortened considerably and can improve the presentation of the main finding of interest, the association between MOI and infectivity to mosquitoes. I have a large number of specific comments.

Many thanks for these comments, we have done our best to address the issues raised.

The authors report a higher parasite prevalence in males. This, if based on a good random selection of the population, is of interest and in line with recent findings from Uganda (Briggs eLIFE 2020). Any risk of bias in the source population should be described. What proportion of invited participants participated and is there any (self-) bias possible?

Unfortunately, we did not keep information on the proportion of invited participants that participated. The overwhelming majority of invited participants participated in the RDT test. Upon reflection and discussion we are not able to find anything that would have led to bias.

Prevalence of gametocytes can be presented for the entire population or from malaria-infected individuals. Both are of relevance, the former to estimate the age-structure in the 'infectious reservoir' and the latter highlighting differences in parasite density, gametocyte production or immunity. Both estimates should thus be presented. However, asexual

parasite prevalence among parasite positive individuals is less interesting. All gametocyte carriers are asexual parasite carriers or very recently were.

Abstract:

- 'challenging An. gambiae s.l.' is an unusual choice of words. Allowing to feed or offering blood to... is more conventional.

We have modified this to be, "After offering gametocyte positive blood to An. gambiae s.l. by membrane feeding assay, our results indicated that 68.1% of the variation in mosquito infection prevalence was accounted for by gametocyte density and MOI (R-SQR. = 0.681, p < 0.001)."

- The abstract would also benefit from a bit more detail on the predictors of mosquito infection rates. Now only the statistically significat predictors are mentioned but some narrative would help the readers.

We have revised as follows, "Gametocyte presence in the host peripheral blood is a significant factor of malaria parasite transmission. However, this does not translate to infectivity in the mosquito vector. Some of the predictors of mosquito infectivity include, the gametocyte sex-ratio and density, multiplicity of infection (MOI), and host and vector anti-parasite immunity"

- Also the (modest) sample size should be mentioned in the abstract. Both in terms of experiments and numbers of mosquitoes dissected.

We have revised as follows, "37 microscopy positive gametocyte carriers were selected to feed laboratory reared An. gambiae s.l. mosquitoes using membrane feeding method. 3395 fully fed mosquitoes were used to do ELISA for determining mosquito infection prevalence."

Introduction:

- 'As a result, this category is most...' I assume age group is meant. This could be clarified.

- The phrasing 'Some studies have reported a positive association between mosquito infection rates of *P. falciparum* and gametocyte density, particularly at high gametocyte concentrations. However, at low gametocyte concentrations, a varying and less strong association is reported.' Is a bit misleading. As is quite clearly described in the literature (Bradley *et al.* eLIFE 2018 and Johnston PLoS Comp Biol 2012)[1,2], there is a clear positive association with sporadic infections at gametocyte densities below 10 gametocytes/uL.

This has been revised and the stated references are added in the text.

- 'Two common characteristics of asymptomatic malaria infections in endemic settings are the prevalence of varying levels of gametocyte carriage among different age categories due to anti-parasite immunity and high rates of polyclonal infections' is a complicated sentence best broken up in 2 (or even 3).

We have revised as follows, "In malaria endemic settings, asymptomatic infections characterized by high rates of polyclonal infections and variations in gametocyte carriage among different age

categories is not uncommon [15, 28, 29]. Variations in gametocyte densities among the different age categories can be partly explained by the age-related anti-parasite immunity due to repeated exposure among the elderly children and adults age groups [21].”

- ‘In order to ultimately eliminate malaria, interventions geared towards interrupting the disease transmission through efficient and effective identification and treatment of both asymptomatic and symptomatic parasite carriers will be of immense importance’ is over-stating the evidence. Countries have eliminated malaria without a specific focus on asymptomatic infections. One can indeed expect that elimination would be accelerated by also targeting asymptomatic infections. I would propose parasite transmission rather than disease transmission, the symptoms are not transmitted by mosquitoes.

This section is revised in the introduction section in accordance with the comment above.

Methods:

- Figure 1, the study map, is interesting if all schools had a decent sample size. The number of observations (median, range) should be presented in the legend.

  This has been added to the map
- ‘This sample size was obtained based on the number of study participants within the designated study area that consented to partake in the study.’ This is not very meaningful. How was the sample size decided upon? Did the authors aim to reach a certain sample size to address the current study questions or was the sample size decided upon to support other study questions?

The samples included in this study were determined to answer other study questions. So this current study is a sub study that was concurrently undertaken using the same samples.

- In the methods, please indicate what anticoagulant was used for phlebotomy and indicate the number of microscopy fields screened for gametocytes (or the number of white blood cells counted against) to give an idea of assay sensitivity.

Heparin was used as anticoagulant, gametocytes were counted against 1000 white blood cells and the counts converted to parasites/µL assuming a density of 8000 WBCs/µL.

- In the methods, please indicate the source of water-jacketed feeders and capacity (volume).

Added to methods.

- It would be nice to understand why a qualitative assay (ELISA) was used as read-out and whether semi-quantitative read-out, as in Graumans MalJ 2017 was considered.[3]

We were not convinced that the ELISA test gave consistently accurate semi-quantitative read-outs on tests carried out with positive control dilutions.

- Please indicate what positive controls were used for the ELISA (source).

Pf-PC was used as a positive control for ELISA (BEI resources).

- Some details on the (minimum) number of dissected mosquitoes would be expected in the results section. In general, just present % infectious and % infected mosquitoes with more reference to denominators. The findings probably hold their value but at the moment it is unknown what feeding performance was and how mosquito survivorship may have affected the precision of outcomes.

We have amended the results based on this comment.

- Any age-patterns, concluded to be non-exist here, are likely to be obscured by the small age range examined.

We have added this in the discussion section.

- It is unclear to me how mixed infections were examined. Is this by PCR or by microscopy?

Mixed infections were detected using RDT test and microscopy examination.

- All references to gametocyte carriage should, at least early in the results and discussion section, be referred to as 'microscopy gametocyte prevalence' to make sure the reader understands the limitations of the diagnostic used.

We have addressed this by adding microscopy gametocyte prevalence for the purpose of clarity.

- Comparisons of gametocyte prevalence by sex could be presented adjusted and unadjusted by total parasite density.

They have now also been presented as unadjusted.

- Table 1 should be simplified. 'Positive' and 'negative' in the first rows are confusing and probably mutually exclusive.

These has been separated from the rest of the table.

- The presentation of asexual parasite prevalence and gametocyte prevalence (e.g. 99.99% vs 8% is not informative. Just present asexual prevalence and gametocyte prevalence by gender and age group.

All the presentations have been revised.

- I would present for age and sex separately (so girls 5-9 vs boys 5-9 and girls 10-15 vs boys 10-15) even if only gender is statistically significantly associated with risk.

We have revised as follows, "However, considering the age versus sex distribution of gametocyte carriage, no significant difference was found by comparing the male (5-9 years) [53.65% (382/712)] to female (5-9 years) [46.35% (330/712)] and male (10-15 years) [53.88% (382/709)] to female (10-15 years) [46.12% (327/709)] (p-value = 0.958)."

- Figure 2 can be omitted. It is not relevant to the current story.
  We did not omit this because Reviewer_1 was interested to see the infection pattern and recommended we add some details about figure two. This is the reason I did not omit it.

- Table 2 can be omitted and captured in the text.

Table 2 is omitted and captured in the text and the discussion section as well.

- Table 3 is confusing. It suggests that gametocyte density is the reference for gametocyte density. I understand that one cannot calculate a correlation coefficient here but that is not the same as calling it a reference category.

"Ref" were removed that was an oversight.

- Figure 3 suggests that MOI is a factor independent of gametocytes and is directly associated with infectivity. MOI can either be associated with higher gametocyte density (which appears not to be the case) or higher infectivity for a given gametocyte density. The latter would be better displayed if categories of gametocyte densities (e.g. <10, 10-20 and 20+, broadly tertiles) are defined as well as categories of MOI and for each gametocyte, class infectivity is given for low, intermediate and high MOI. That would allow an interpretation if, for each gametocyte class, MOI is associated with higher infectivity.

This was considered during the data analysis. However, we could not present the data as you have suggested due to the very small number of samples analyzed in this study. However, this will be considered in the subsequent studies.

- Table 4 requires information about scale. Is gametocyte density included per uL with or without log10 adjustment? Is MOI included as a continuous variable? That would not be entirely intuitive and I would rather see MOI in categories since there is no reason I know of to assume that the difference between an MOI of 1 and 2 (single clone versus multiclone and thus potentially

competition between clones) is the same as that between an MOI of 10 and 11.

This is an important point and will provide detailed information. However, considering the low sample size in this study we could not present the MOI as a category.

Discussion:

- The statement 'Among the P. falciparum malaria positive individuals, males tended to be slightly overrepresented as both asexual 53.76% (764/1421) and gametocyte carriers 61.9% (52/84) as compared to females [asexual carriage; 46.23% (657/1421), gametocyte carriage; 38.1% (32/84)] is very confusing and should come with an estimate of statistical significance. There is no reason to add up asexuals and gametocytes to classify someone as parasite positive and then determine the proportion of these positives that is asexual positive. It just doesn't make much sense biologically or epidemiologically.

We have revised as follows, "Among the P. falciparum gametocyte positive individuals, males tended to be slightly overrepresented 61.9% (52/84) as compared to females 38.1% (32/84). However, this is not statistically significant."

- The part on gametocyte sex ratio can be removed from the discussion. It is interesting but has no relevance to the current study that didn't assess sex ratio.

This part is revised and removed most of the content

- The authors suggest competition between virulent and less virulent (defined as transmissible) strains as a mechanism underlying the association between MOI and mosquito infection rates. They cite references 49-51 but none of these, as far as I know, prove this association. They merely hint at inter-strain competition that is most likely to occur through increased investment in gametocytes or gametocytes of a certain sex.

We have now indicated that this may be linked to intense inter-strain competition due to increased investment in gametocytes and multiple clone infections (MOI) that could favour emergence of highly transmissible or virulent parasite strains thereby increase mosquito infectivity [48, 49, 50].

Competing Interests: None.

Reviewer Report 01 February 2021

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?

Lynn Grignard
Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK

The authors present a well written manuscript titled "Prevalence of asymptomatic *P. falciparum* gametocyte
carriage in schoolchildren and assessment of the association between gametocyte density, multiplicity of infection and mosquito infection prevalence". The authors clearly define and outline their research question and background and give in depth details of the methods used in the study. The methods are described in great length, but there is the occasional omission of some detail. For example, in the section describing the membrane feeding assay, the authors should cite publications they have based their assay on (the protocol has not been newly developed by the authors in this publication) and state their modifications. Small details like the type of anticoagulant (heparin!) in the blood collection tubes is important for researchers to be able to replicate the experiment. The following section on ELISA contains too many details and could be streamlined i.e. you say you incubate "overnight" and then in the next sentence "the next morning" - as you mention the overnight incubation, there is no need to add "the next morning", you tell the reader that you equilibrate your samples to RT - and detail each step as you would in a lab protocol or SOP. These are just a couple of examples and the whole section needs shortening.
The results section is well structured and the data is adequately interpreted. I seem to be unable to find asexual parasite densities - overall but also for the subgroups.
Could the authors please explain/give more detail about the samples: There were 84 gametocyte

carriers - how many infected mosquitoes? There is feeding data for 37 individuals (is that because only 37/84 infected mosquitoes?). Of these 37 infectious individuals, only 22 have MOI data (again why is that?). Also, gametocyte densities might be better in median and IQR or geometric mean and 95% CI. Equally, MOI is more informative as a median and IQR compared to mean.
In the discussion, the authors say that "High infection prevalence was observed among mosquitoes that fed on carriers with high gametocyte densities." - this is true but looking at their data apart from 2/37 individuals all gametocyte carriers were below 200 gam/ul - and of the ones above 100 gam/ul only 4 infected more than the mean mosquito infection rate.
The manuscript could be improved as follows: The discussion needs a section on limitations. These should include and discuss (not exclusively): the use of microscopy to identify gametocyte carriers, the small number of MOI data points, the relatively small number of data points/category, membrane feeding versus skin feeding, MOI of asexuals vs gametocytes, relatively high MOI in light of transmission area.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Malaria, molecular biology, transmission biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 18 Mar 2021

Jeremy Herren, International Centre of Insect Physiology and Ecology (icipe), Nairobi, Kenya

The authors present a well written manuscript titled "Prevalence of asymptomatic *P. falciparum* gametocyte

carriage in schoolchildren and assessment of the association between gametocyte density, multiplicity of infection and mosquito infection prevalence". The authors clearly define and outline their research question and background and give in depth details of the methods used in the study. The methods are described in great length, but there is the occasional omission of some detail. For example, in the section describing the membrane feeding assay, the authors should cite publications they have based their assay on (the protocol has not been newly developed by the authors in this publication) and state their modifications. Small details like the type of anticoagulant (heparin!) in the blood collection tubes is important for researchers to be able to replicate the experiment.
The following section on ELISA contains too many details and could be streamlined i.e. you say you incubate "overnight" and then in the next sentence "the next morning" - as you mention the overnight incubation, there is no need to add "the next morning", you tell the reader that you equilibrate your samples to RT - and detail each step as you would in a lab protocol or SOP. These are just a couple of examples and the whole section needs shortening.

The results section is well structured and the data is adequately interpreted. I seem to be unable to find asexual parasite densities - overall but also for the subgroups.

The asexual parasite densities were not included here because the study was centered on trying to find the relationship between gametocyte density, MOI and infectivity. So, we tend to only screen the potential asymptomatic carriers then if positive, we screen for gametocyte carriage and those that were positive for gametocyte carriage, the density of the gametocytes in the blood was determined.

Could the authors please explain/give more detail about the samples: There were 84 gametocyte carriers - how many infected mosquitoes?

This was stated in the method section. Each sample (37 in total) were fed to 100 mosquitoes. After feeding, the fully fed mosquitoes were kept for about 8 to 10 days. The number of mosquitoes infected was 463 mosquitoes.

There is feeding data for 37 individuals (is that because only 37/84 infected mosquitoes?).

This is because only 37 individuals consented to providing extra venous blood used during mosquito feeding and the same samples were used to extract genomic DNA for the microsatellite genotyping analysis.

Of these 37 infectious individuals, only 22 have MOI data (again why is that?).

Only those samples used for the mosquito feeding were used to extract genomic DNA for the microsatellite genotyping analysis. However, the 15 samples failed to amplify due to power failure and degradation of the stored DNA samples. Therefore, the MOI data was obtained for only 22 samples.

Also, gametocyte densities might be better in median and IQR or geometric mean and 95%

CI. Equally, MOI is more informative as a median and IQR compared to mean.

We have included this in the results sections

In the discussion, the authors say that "High infection prevalence was observed among mosquitoes that fed on carriers with high gametocyte densities." - this is true but looking at their data apart from 2/37 individuals all gametocyte carriers were below 200 gam/ul - and of the ones above 100 gam/ul only 4 infected more than the mean mosquito infection rate.

This statement was revised to match the stated comment. "Infection prevalence tends to be relatively higher among mosquitoes that fed on carriers with high gametocyte densities (> 20 Gam/uL."

The manuscript could be improved as follows:
The discussion needs a section on limitations. These should include and discuss (not exclusively):
the use of microscopy to identify gametocyte carriers,
the small number of MOI data points, the relatively small number of data points/category,
membrane feeding versus skin feeding,
MOI of asexuals vs gametocytes, relatively high MOI in light of transmission area.

We have tried to address these points by including the section on limitations covering all the major points stated above.

Competing Interests: None.

Reviewer Report 25 January 2021

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Linda E Amoah
Immunology Department, Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana

Title:

- The title must include 'Kenya' where the study was conducted.

Abstract:

- 'microscopy gametocyte' What does this mean?
- Some results are discussed as past tense and others as present. Consistency is required.

- 'MOI statistically, the 'statistically' can be removed.

Introduction:

- 'genetically diverse multiple' Should be multiple genetically diverse.
- The paragraph beginning 'it has also been.....' should be rephrased.

Methods:

- the study site description is very scanty.
- Was this a longitudinal study or cross sectional study?
- How many schools were used in the study?

Study subjects and sample collection:

- This section should be elaborated to include the number of samples collected for each aspect of the study.
- What exactly is KM? Is it Km?
- The paragraph beginning 'Blood samples... ' should be rephrased and include the volume of blood drawn.
- How much blood was spotted on the filter paper?
- No molecular determination of gametocyte prevalence was made. Is it a known fact that gametocyte prevalence and densities are not adequately captured by microscopy?

Experimental infection of mosquitoes:

- The description is very difficult to understand it should be rewritten to enhance clarity.
- The section begins with the statement 4 ml blood collected from ALL microscopy positive gametocyte carriers were immediately fed to mosquitoes (this number is 84 from the previous section). Then towards the middle, there is a statement that only 37 samples were fed to mosquitoes.
- Why were only 37 samples fed to mosquitoes?
- Then if female mosquitoes are used for the feeding experiment, why are male insectary reared mosquitoes used as negative controls?
- Concentration should not be written as volume 'a concentration of 40 ul.....'.
- Were individual mosquitoes tested in the ELISA? This should be clearly written,
- I am not sure how strong an analysis of 37 samples divided into subgroups would be.

Microsatellite genotyping:

- This section is poorly written. There is no consistency in reporting SI units. There are several instances where SI units are written directly after the number, without a space.
- Then there is a confusing statement: 'the samples analyzed here are part of those used in our previous study'? are these archived samples. are all the 4881 samples archived? or collected for this study?
- Why were only 37 samples analyzed?
- If 15 samples failed to amplify, that would leave only 22 samples.
- Is it possible to divide 22 into multiple groups and obtain statistically significant results?
- The map should be properly labelled so that all sublocations where samples were collected are labelled/named.
- There is no primary school located in 'C'.

Results:

- It would be very informative to separate the *P. malariae* infections from the *P. ovale* infections.
- Comparisons are being made but it seems the seasons were not considered in the analysis.
- The study spanned over 2 years and across a number of peak and off peak seasons and across communities with varying malaria parasite prevalence. The analysis should have taken all these into consideration.
- Figure 2 needs more information. % is indicated but a fraction should be either added as a foot note or supplementary file. this information is partially contained within the map but even with that, prevalence is grouped as a range. the exact prevalence per school per month should be provided. the supplementary table has the same % data as the figure.
- The major flaw in this study is the very small number of samples used for the assessment of the association between gametocyte density, MOI and infection prevalence. 22 samples are too small to use to identify significant associations and risk factors.

Discussions:

- It is difficult for me to comment on the discussion as I have major challenges with the results and the methods.

More details are required to support the interpretation of the supplementary tables. footnotes and description of what items ate in the columns and rows and what the numbers represent

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: molecular biology, malaria transmission, diagnostics and vaccine design

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 18 Mar 2021

Jeremy Herren, International Centre of Insect Physiology and Ecology (icipe), Nairobi, Kenya

Title:

- The title must include 'Kenya' where the study was conducted.

Title modified as follows:
Prevalence of asymptomatic *P. falciparum* gametocyte carriage among schoolchildren in Mbita, Western Kenya and assessment of the association between gametocyte density, multiplicity of infection and mosquito infection prevalence.

Abstract:

- 'microscopy gametocyte' What does this mean?

We have opted to use gametocyte prevalence as determined by microscopy to avoid any confusion.

- Some results are discussed as past tense and others as present. Consistency is required.

This have been addressed for the purpose of consistency

- 'MOI statistically, the 'statistically' can be removed.

The sentence is rephrased as "MOI significantly"

Introduction:

- 'genetically diverse multiple' Should be multiple genetically diverse.

The sentence is rephrased as "genetically diverse"

- The paragraph beginning 'it has also been.....' should be rephrased.

Rephrased as, "Parasite sex ratio (ratio of male and female gametocyte density) was reported to influence the transmission potential of P. falciparum parasite"

Methods:

- the study site description is very scanty.

Further information about the study site like the main economic activities, and housing systems were added.

- Was this a longitudinal study or cross sectional study?

This was a cross-sectional study employing participants from the primary schools within the study area.

- How many schools were used in the study?

Study participants were recruited from 41 primary schools including public and private schools.

Study subjects and sample collection:

- This section should be elaborated to include the number of samples collected for each aspect of the study.

We have included this information in each of the specific sections of the study for clarity.

- What exactly is KM? Is it Km?

Km for kilometer, we have now said "kilometer" to limit any confusion.

- The paragraph beginning 'Blood samples... ' should be rephrased and include the volume of blood drawn.

100 µL of blood samples were also collected on filter papers (two spots per paper)

- How much blood was spotted on the filter paper?

100 µL of blood samples were also collected on filter papers (two spots per paper) (Whatman 3 MM; Whatman, Maidstone, United Kingdom) for DNA extraction.

- No molecular determination of gametocyte prevalence was made. Is it a known fact that gametocyte prevalence and densities are not adequately captured by

microscopy?

Yes, this is a known fact from previous studies comparing molecular methods and microscopy and is based on the relatively low sensitivity of microscopy.

Experimental infection of mosquitoes:

- The description is very difficult to understand it should be rewritten to enhance clarity.

We have revised this section to ensure clarity but also using try to avoid missing out vital information for the purpose of reproducibility of the study.

- The section begins with the statement 4 ml blood collected from ALL microscopy positive gametocyte carriers were immediately fed to mosquitoes (this number is 84 from the previous section). Then towards the middle, there is a statement that only 37 samples were fed to mosquitoes.

The number of gametocyte positive individuals in the study population is 84. However, for the purpose of mosquito feeding, extra blood samples (4 mL) were collected only from those that have consented to that. Therefore, only 37 individuals have given consent to donate the extra 4 mL of venous blood to be used for the mosquito feeding hence the difference in the numbers.

- Why were only 37 samples fed to mosquitoes?

Those are the only individuals who consented to donate the 4 mL venous blood used for the mosquito feeding assay.

- Then if female mosquitoes are used for the feeding experiment, why are male insectary reared mosquitoes used as negative controls?

The male mosquitoes were used as negative controls because the male Anopheles mosquitoes do not feed on human blood and therefore are not infected with plasmodium parasite. Meaning they are clean and this a common practice and has been used in other studies.

- Concentration should not be written as volume 'a concentration of 40 ul.....'.

This was an error and has been corrected as " The monoclonal antibody (MAb) peroxidase conjugate 27 (0.5 mg/mL Peroxidase Labelled Mouse Monoclonal Ab Pf2A10-CDC, CAT #: MRA-890, MR4/ATCC, Virginia, USA) was prepared in specified concentrations for Plasmodium falciparum at a volume of 40 µL in 10 mL blocking buffer for each plate."

- Were individual mosquitoes tested in the ELISA? This should be clearly written,

Yes, and the information is added.

- I am not sure how strong an analysis of 37 samples divided into subgroups would be.

The sample size is relatively small due to some challenges. However, the statistical analysis employed in the study tends to have some level of strength though a more conclusive analysis

can be carried out using larger sample sizes.

Microsatellite genotyping:

- This section is poorly written. There is no consistency in reporting SI units. There are several instances where SI units are written directly after the number, without a space.

This has been corrected in the revised version.

- Then there is a confusing statement: 'the samples analyzed here are part of those used in our previous study'? are these archived samples. are all the 4881 samples archived? or collected for this study?

This statement is meant to show that we have published a first paper on genetic diversity and MOI in the study area before this one. That is what prompted this current publication and the samples used in the current study were all collected at the same time as those used in the previous publication. However, only the 37 samples used in the mosquito feeding were analyzed in this study.

- Why were only 37 samples analyzed?

This is because only 37 individuals consented to providing extra venous blood used during mosquito feeding and the same samples were used to extract genomic DNA for the microsatellite genotyping analysis.

- If 15 samples failed to amplify, that would leave only 22 samples.

Yes, and this was due to the degradation of those DNA samples.

- Is it possible to divide 22 into multiple groups and obtain statistically significant results?

From the analysis and even preliminary data analysis done prior to the final analysis, the statistical analysis was significant. The small sample sizes were also taken into strict consideration in selecting the test of significance methods used in the analysis.

- The map should be properly labelled so that all sublocations where samples were collected are labelled/named.

We have not labelled the names of schools on the map since we do not have express permission to reveal their locations.

- There is no primary school located in 'C'.

There is a primary school however, no participant was recruited from that school because they did not fall within the inclusion criteria.

Results:

- It would be very informative to separate the *P. malariae* infections from the *P. ovale* infections.

Yes, we were aware of this. However, the RDT used in the mass screening only separates P. falciparum from the rest of the other species and only indicated a band for mixed infection of P.

malariae and ovale as one. Since we are more interested about P. falciparum, we tend not to specifically screen for the other species.

- Comparisons are being made but it seems the seasons were not considered in the analysis.

While malaria is affected by seasons, this region is known to have high levels of infection year-round. Due to constraints regarding the number of samples, we believe it would not add much to the study to segregate samples by season.

- The study spanned over 2 years and across a number of peak and off peak seasons and across communities with varying malaria parasite prevalence. The analysis should have taken all these into consideration.

See above.

- Figure 2 needs more information. % is indicated but a fraction should be either added as a foot note or supplementary file. this information is partially contained within the map but even with that, prevalence is grouped as a range. the exact prevalence per school per month should be provided. the supplementary table has the same % data as the figure.

We have included this in the earlier version of the paper. However, based on other reviewers' comments, we have only indicated the % on the figures. The data presented in the paper and all other raw data are available and can be shared once requested by any interested party.
The major flaw in this study is the very small number of samples used for the assessment of the association between gametocyte density, MOI and infection prevalence. 22 samples are too small to use to identify significant associations and risk factors.
The relatively small sample size used in this study was largely due to major constraints like obtaining consent from the study participants particularly for membrane feeding assay, sample transportation and storage and power supply problems leading to such low sample number. However, we have tried so hard that to use appropriate statistical methods taking into consideration the sample sizes to minimize any serious error. We have recommended a further multi-center study using a larger sample sizes to validate the findings from this study.

Discussions:

- It is difficult for me to comment on the discussion as I have major challenges with the results and the methods.

More details are required to support the interpretation of the supplementary tables. footnotes and description of what items ate in the columns and rows and what the numbers represent

We hope these issues have been address in the significant changes to the revised version.

Competing Interests: None.