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Malaria prevalence, transmission potential and efficacy of artemisinin-based combination therapy in the Kenyan Central highlands: a zone previously characterized as malaria-free

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Abstract

Background The current study sought to re-evaluate malaria prevalence, susceptibility to artemisinin-based combination therapy (ACT), transmission patterns and the presence of malaria vectors in the Kikuyu area of the Kenyan Central highlands, a non-traditional/low risk malaria transmission zone where there have been anecdotal reports of emerging malaria infections.

Methods Sampling of adult mosquitoes was done indoors, while larvae were sampled outdoors in June 2019. The malaria clinical study was an open label non-randomized clinical trial where the efficacy of one ACT drug, was evaluated in two health facilities. Microscopy was used at the facility while nested 18 s rRNA subunit gene PCR amplification and *MSP-1* and *MSP-2* family alleles genotyping was done in the laboratory. Anti-malarial resistance gene markers *Pfk13* and *Pfmdr1* were profiled.

Results *Anopheles funestus* mosquitoes were the predominant vectors at 76.35% of all larvae collections (N = 148). Only two non-blood fed, parasites negative adult mosquitoes were collected from houses sampled. Parasitological analysis of the 838 patients screened resulted in 41 positives whose treatment outcome was 100% Adequate Clinical and Parasitological Response (ACPR). From the 35 positive samples genotyped, 29 (82.9%) were polyclonal. The overall mean MOI was 2.8 (95% CI 2.36–3.35). The MOI for *msp-1* and *msp-2* genes, was 2.02 (95% CI 0.72–2.27) and 2.9 (95% CI 2.22–3.55), and parasite strains range of 1–3 and 1–7, respectively. Polyclonal variation in the two genes was at 76.4% and 70.3%, respectively. The *Pfk13* gene revealed no single nucleotide polymorphisms (SNP) associated with suspected artemisinin resistance nor was there any *pfmdr1* N86 mutant allele detected.

Conclusion The *Plasmodium* infections positivity rate observed in the study site was very low but significant. A proportion of participants who tested positive did not report recent history of travel. This observation together with the finding of competent known vectors can probably suggest that several of the cases could have been acquired and transmitted locally. The observed genetic diversity and polyclonal variations was on the contrary and suggest that these are imported cases. This however does not rule out a likely changing malaria transmission scenario in this zone, thus the need for further investigations.

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Background

Emerging infectious diseases are infections that have recently appeared within a population or those whose incidence or geographic range is rapidly increasing or threatens to increase soon. Malaria is a life-threatening disease caused by the infection of red blood cells with protozoan parasites of the genus *Plasmodium*. Four species of *Plasmodium* (*Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale*) most commonly infect humans. *Plasmodium falciparum* and *P. vivax* are the most prevalent species with *P. falciparum* being the most lethal in terms of disease severity and fatality rates [1]. Other emerging species, for example, *Plasmodium knowlesi* (a species of *Plasmodium* that primarily infects non-human primates) are increasingly being reported in humans inhabiting forested regions of some countries of South-East Asia and the Western Pacific regions, and in particular on the island of Borneo [2]. Kenya is home to the first four species of *Plasmodium* parasites that infect humans. The *P. falciparum* parasite, which causes the most severe form of the disease, accounts for more than 99% of the infections [3]. There are an estimated 3.5 million new malaria cases annually, with 10,700 deaths and an estimated 25 million people at risk of contracting the disease in Kenya [3]. As per the Kenya National malaria treatment guidelines, those who test positive for malaria should be treatment using artemether-lumefantrine (AL) (first-line treatment) or, in the absence of AL, dihydroartemisinin-piperaquine (second-line treatment), at the correct weight-based dosage. This disease burden, coupled with the numerous reports of resistance to the current diagnostic [4] and treatment [5–7], interventions makes malaria a primary concern for medical research.

Malaria is transmitted by infected mosquitoes of the genus *Anopheles* during the process of feeding on the human host. Africa, especially sub-Saharan Africa harbors the deadly combination of the most efficient vectors of malaria and climatic conditions that promote the rapid development of both the mosquito vector and malaria parasites within the mosquito. Two members of the *Anopheles gambiae* species complex, *Anopheles gambiae* sensu stricto (*s.s.*) and *Anopheles. arabiensis* mosquitoes together with *Anopheles funestus s.s.*, a member of the *An. funestus* group of mosquitoes are amongst the most important vectors in sub–Saharan Africa, including Kenya [8, 9]. *Anopheles stephensi*, an invasive malaria vector that is endemic to south Asia and the Arabian Peninsula, has also recently been reported in Kenya [10].

In Africa, highland zones are defined as those with an elevation higher than 1500 m above sea level. These zones are also characterized by low mean temperatures, generally below 20^{0}C and have been for a long time considered to have little or no malaria [11, 12]. The low malaria transmission in these zones is associated with poor survivorship of *Anopheles* mosquitoes and the fact that the low temperatures do not allow for the completion of the sporogonic cycle of the malaria parasite in the vector to allow the mosquitoes to become infective [13]. The relationship between ambient temperatures and the duration of the sporogonic cycle has previously been defined by Detinova [14]. Although temperatures in the highland do rise, the temperatures are on average not high enough to sustain a stable transmission cycle. With global warming, however, it is possible that malaria transmission may become a reality in such highlands since slight increases in temperature have been shown to have an exponential effect on mosquito survivorship and malaria transmission dynamics. Based on increasingly sophisticated climate modeling approaches, climatologists forecast the observed trends in global warming will continue in the foreseeable future [15].

During the 1980s and 1990s, several outbreaks of malaria that resulted in high fatalities were reported in countries of the eastern African highlands and this was attributed to several factors, including climate anomalies [16, 17]. There are anecdotal reports of residents of the highlands west of Nairobi who have been treated for malaria but the possibility of an active transmission has often been repudiated and the explanation that any cases are due to importation by people returning from malaria endemic zones considered more plausible.

Vector control using insecticide-treated nets and indoor residual spraying and malaria case management, consisting of early accurate diagnosis and prompt effective anti-malarial drugs treatment remain to be the mainstay of the fight against malaria [18]. The current assignment sought to carry out definitive studies on malaria transmission in the highlands west of Nairobi by determining the presence and transmission dynamics of malaria disease, the burden, and the treatment efficacy in selected health facilities in the area. The goal of the research was to generate primary data that will inform policies on malaria control in such unconventional settings.

Methods

Study oversight

This study was part of the larger study entitled "*Mapping emerging infectious diseases in selected sites in Kenya within the context of climate change*" at Centre for Biotechnology Research and Development, Kenya Medical Research Institute (KEMRI/CBRD). The study was approved and monitored by the KEMRI Scientific and Ethics Review Unit (SERU) (Study approval No. KEMRI/SERU/CBRD/3561.

Study area, study design and sampling

This study was carried out in Kikuyu sub-county of Kiambu County, Kenya (Fig. 1). Kikuyu sub-county is located about 20 km Southwest of Nairobi and is part of the Kenyan Central highlands with a population of 187,122 people as per the 2019 national census [19]. The area is generally cold and wet with the temperatures ranging from 10.9 to 24.6 °C and monthly rainfall ranging from 20 to 125 mm experienced in a bimodal pattern of long rains occurring in April – June and the short rains in October – November [20].

Mosquito sampling, handling and analysis

Mosquito sampling was carried out between 11 and 13th June 2019 in two swamps in Kikuyu sub-county, namely Karai Rurii (1^{0}25'S; 36^{0}35'E) and Ondiri Swamp (1°15'S; 36°40'E) (Fig. 2), and in villages within 5 km of the swamps. Purposive sampling was carried out whereby heads of households within this range were approached and consented to allow for the mosquito sampling, starting with households closest to the swamps. Ondiri Swamp and Karai Rurii are in close proximity to each other and are located about 4.5 km apart. Ondiri swamp has water throughout the year while Karai may dry out during the very dry season, although the black cotton

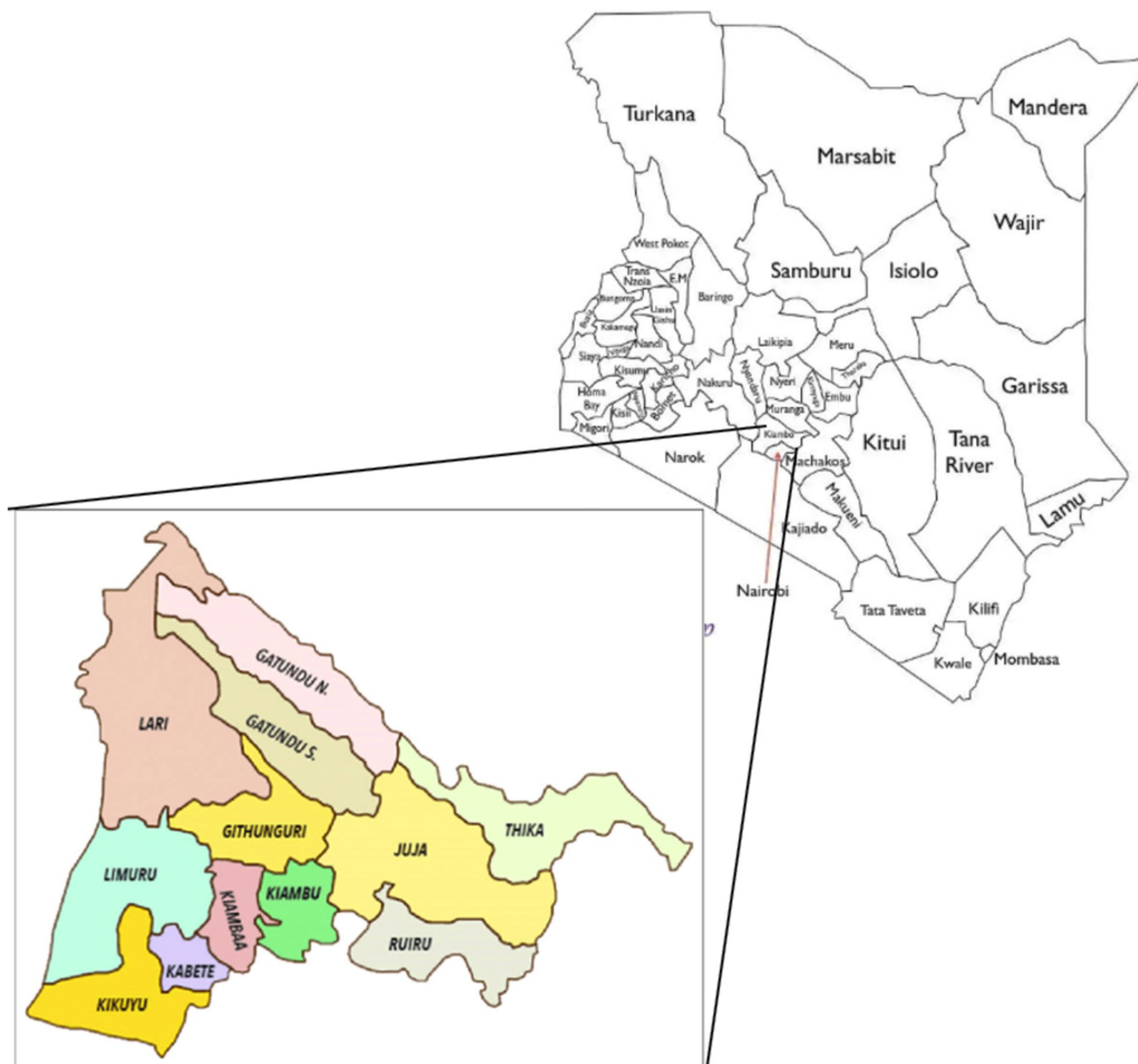

Fig. 1 Map of Kiambu county showing Kikuyu sub-county (bottom left) [Source: https://kiambu.go.ke/political-units]

Fig. 2 A view of Karai Rurii swamp (left) and Ondiri swamp (right) in Kikuyu Kenya where mosquito larval sampling was carried out

soil enables the retention of water long after the rains have ceased. Larval mosquitoes were sampled from the swamps using standard dippers or droppers while adult mosquitoes were sampled inside human dwelling by manual aspiration followed by the CDC light traps which were mounted within 1 m of the bed or sleeping area the evening before at 18:00 h and collected at 06:00 h the following morning. Larval sampling targeted *Anopheles* mosquitoes identified by morphology. Adult mosquito sampling was carried out in Kanyethi, Nderi and Gathima villages with 10 houses being sampled in each of the villages. The number of people who spent the night before in the houses where mosquito sampling was carried out was noted and recorded. Global Positioning System (GPS) coordinates of the houses were also noted. Mosquitoes sampled were transported to KEMRI/CBRD and characterized into the different *Anopheles* species based on morphological characteristics; larval mosquitoes were reared to adults before identification [21, 22]. For mosquitoes collected as adults, information on whether they were blood fed or not, or gravid was noted following observation under a dissecting microscope.

Members of the different *Anopheles* species complexes that were not distinguishable based on morphological characteristics were further identified using PCR; DNA was extracted from mosquito abdomens using the alcohol precipitation method of Collins et al. [23] and the PCR assays of Scott et al. [24] and Koekemoer et al. [25] used to distinguish between members of the *An. gambiae* sensu lato (*s.l.*) and *An. funestus* species complexes, respectively. Adult mosquitoes sampled were tested individually for the presence of *P. falciparum* parasites. This was done using mosquito heads and thoraces and by the Enzyme-linked Immunosorbent Assay (ELISA) of Wirtz et al. [26].

Plasmodium parasites infection and drug efficacy studies

For this an open label non-randomized 28 days, uncontrolled clinical follow up with one treatment arm to assess the efficacy of artemether-lumefantrine (AL) the first-line drug in the treatment of malaria in Kenya, was conducted. Two hospitals namely Gikambura Health Centre and Lusigeti Sub-County Hospital both located within Kikuyu sub-county of Kiambu County and within 10 km of the mosquito sampling sites were involved in this study. These health facilities are public institutions and patients visiting the facilities receive services at government subsidized rates. Initiation of the study and patient screening began in July, 2019 and proceeded with recruitment, treatment and post treatment follow up with the last patient recruited on the study completing on 22nd Dec 2019.

Quality-assured drugs were provided by the National Malaria Control Programme. Patients were enrolled in the study according to common KEMRI guidelines, which follows the Helsinki declaration and the ICH-GCP guidelines. The purpose of the study was explained in the local language, and an informed consent obtained from the patient, parent(s) or guardian(s) if willing to participate in the study. Confidentiality was strictly enforced with linking of patient's information using the unique identification number being only possible for the principal investigator and co-investigators.

Patient screening, recruitment and follow-up

This followed the WHO/MAL/2009 guidelines for clinical assessment of anti-malarial drugs as modified in the study SOPs. The criteria for inclusion, and exclusion as well as safety assessment are contained therein [27]. Briefly, all individuals presenting with suspected malaria (febrile illness) were screened using microscopy as potential participants in the study. Patients who were 6 months and older with microscopically confirmed uncomplicated *P. falciparum* mono infection (asexual parasitaemia of 1000–100,000/µl) and able to adhere to study treatment and follow up were recruited from the outpatient clinic in the hospitals. Eligible patients for whom (parental / guardian) informed consent (and the child's assent) was obtained were treated with artemether-lumefantrine as per the dosage schedule. Participants were required to report to the study clinic on post treatment day 1, 2, 3, 7, 14, 21 and 28 or at any other time if they felt unwell. Those who failed to keep the follow up appointments were visited by a community health worker and the study team for data and sample collection. Observations were recorded in the case record form and appropriate clinical care provided. In particular, efforts were made to explore any serious adverse effects due to the drug.

Laboratory, treatment and clinical procedures

From all the patients referred to the respective facility laboratory, a finger prick was done to obtain blood for the preparation of blood smears. Thick and thin Giemsa-stained blood slides were prepared before treatment and at every follow-up visit of days 1, 2, 3, 7, 14, 21, and 28. Slides were examined by two expert microscopists independently and considered negative if no parasites were seen after examination of 200 oil-immersion fields in a thick blood film. Parasite density was estimated by counting the number of asexual parasites in 200 white blood cells (WBC), taking a standard WBC count of 8,000 /µl. Species determination (conformation of mono-infection) was made based on assessment of thin films.

Eligible patients received supervised treatment with AL (Coartem®; Novartis Pharma), administered as a tablet (20 mg of artemether and 120 mg of lumefantrine) per 5 kg of body weight in a 6-dose regimen, new participants were enrolled into the study on a daily basis over the determined recruitment period. No food or supplementary drinks were provided before treatment.

Blood Samples were collected from patients before and after drug administration according to the WHO Guidelines for clinical assessment of antimalarial drugs and the study SOPs[27]. On treatment days, blood sample collection was done before treatment. Blood smears were prepared, dried filter paper blood spots on Whatman® 903, and approximately 50-µL whole blood samples were collected for microscopy and qPCR. These were stored in appropriate conditions until shipped to the malaria CBRD KEMRI lab. A standard clinical examination, blood smears and DBS were also collected from finger pricks on follow up days as per the guidelines.

Parasite DNA extraction

Parasite genomic DNA was extracted from the blood spots collected on Whatman® 903 filter papers or whole blood samples using Chelex-100® (Bio-Rad Laboratories CA) method as previously described [28], with a final volume of 200 µl for each sample and storage at − 20 °C until it was used for the amplification reaction. Briefly blood spotted filter papers were soaked for 24 h in 1 mL of 0.5% saponin-1 phosphate buffered saline. The mixture was washed in 1-mL PBS and boiled with Chelex −100® in PCR-grade water. This was then centrifuged and the supernatant containing the DNA recovered and stored as indicated.

ACT resistance markers

To evaluate for the indicators of resistance by *P. falciparum* to anti-malarial drugs in the study area, molecular surveillance targeting the *Pfk13* and *Pfmdr1* molecular markers of resistance to ACT and/or reduced susceptibility, was conducted. The polymorphic characteristics of the *Pfk13* gene were evaluated on the following SNPs M476I, Y493H, R539T, I543T, and C580Y. K13-propeller genes were amplified by the nested PCR protocol described and optimized previously [29], by using the primers listed in Table 1. For the first round of PCR, 0.5 µL DNA was amplified with 6 µL PCR Mix (1.25 U/mL, 0.2 µL Taq DNA Polymerase, 0.4 mMdNTP Mixture, PCR buffer, and 4 mM Mg2+), 0.6 µL forward primer (10 mM), 0.6 µL reverse primer (10 mM), and sterile ultrapure water to a final volume of 25 µL. For the second round of PCR, 1 µL primary PCR products were amplified with a 25 µL reaction system, including 6 µL PCR Mix, 0.6 µL forward primer (10 mM), 0.6 µL reverse primer (10 mM), and H2O. The amplification conditions were maintained at 95 °C for 3 min; followed by 35 cycles (94 °C for 30 s, 52.5 °C for 1 min 30 s, 72 °C for 1 min); 72 °C. For 10 min; then stored

Table 1 Pfk13 primer sets

	Primer Name	Primer sequence (5′ to 3′)
1	Pfk13_K1_F outer	CGGAGTGACCAAATCTGGGA
2	Pfk13_K4_R outer	GGGAATCTGGTGGTAACAGC
3	Pfk13_K2_F nested	GCCAAGCTGCCATTCATTTG
4	Pfk13_K3_R nested	TGCAGCAGGAAATACAACAGC

at 4 °C. The amplified PCR products were analysed in 1.5% agarose gel, purified using Exosap-it® (Affymetrix, Santa Clara, CA) as per the manufacturer's protocol) and then Sanger sequenced.

Restriction digestion of *Pfmdr* with *ApoI* and *Afl III*

Nested PCR, as reported previously, was performed to amplify codon 86 of *Pfmdr1* [28]. During nest1 reaction, primers P1- 5′ATGGGTAAAGAGCAGAAAGA3′ and P2-5′AACGCAAGTAATACATAAAGTCA3′ were used to amplify the region flanking codon 86. Nested primers P3 5′TGGTAACCTCAGTATCAAAGAA3′ and P4 5′ATAAACCTAAAAAGGAACTGG3′ were used to amplify the PCR product in nest 2 reaction. The finally amplified product was subjected to restriction digestion with *Afl III* (mutational allele) and *Apo I* (wild type allele) (New England Biolabs®, UK) by incubating at 37 °C for one hour with the one unit of each enzyme. The digests were resolved on 3% agarose gel, stained with ethidium bromide, and results were recorded on the gel documentation system.

Genotyping of the *msp-1 and msp-2* genes of *P. falciparum*

Nested PCR of the polymorphic regions of *msp-1* (block2) and *msp-2* (block 3), was performed using primers and methods as previously described [30–33]. Briefly, in the initial amplification, primer pairs corresponding to conserved sequences within the polymorphic regions of each gene were included in separate reactions. The product generated in the initial amplification was used as a template in six separate nested PCR reactions. In the nested reaction, separate primer pairs targeted the respective allelic types of msp-1 (K1, MAD20 and RO33), msp-2 (IC3D7 and FC27), with an amplification mixture containing 250 nM of each primer 2 mM of MgCl2 and 125 µM of each dNTPs and 0.4 units Taq DNA polymerase. The allelic specific positive control 3D7 and DNA free negative controls were included in each set of reactions [34]. Fragment analysis of *msp-1* and *msp-2* amplified products were then performed through electrophoresis on 2% agarose gels visualized under ultraviolet transillumination with light after staining with ethidium bromide. The size of DNA fragments was estimated by visual inspection using a 100 bp DNA ladder marker. The detection of a single PCR fragment for each locus was classified as an infection with one parasite genotype (monoclonal infection). Isolates with more than one genotype were considered as polyclonal infection. Alleles in each family were considered the same if fragment sizes were within 20 bp intervals for *msp-1* and *msp-2* genes [35–37].

Treatment outcome measures end points

The primary endpoints were PCR-corrected clinical and parasitological response (PCR corrected ACPR) at day 28. ACPR was defined as the absence of parasitaemia on day 28 irrespective of the temperature without previously meeting any of the criteria of early treatment failure or late clinical or parasitological failure. Patients with late asexual parasite reappearance were considered ACPR if the PCR analysis showed a new infection rather than a recrudescence (through PCR genotyping). The total treatment failure was defined according to the WHO criteria as the sum of early and late treatment failures. Secondary endpoints were; PCR uncorrected ACPR, Asexual parasite clearance time (PCT) (proportion of patients with remaining parasitaemia) defined as the time (in hours) from the start of a patient's treatment to 2 consecutive negative blood slides (collected at different days) [27]. Gametocyte carrier rates and geometric mean densities (excluding negatives) were compared on days 7, 14 and 28. and Adverse events, vital signs were monitored and changes assessed.

Data management and analysis

At the Primary collection point, spread sheets were used to manage the entomological data. Data collected was checked in the field and at the end of each day cleaned to ensure completeness, consistency, credibility and eligibility. Statistical analyses were performed using Stata® v17.

Patients clinical data was double entered and managed on Open Clinica® database and summaries extracted on Stata®. For the parasitological analysis, the K13 sequencing data was managed as we have previously described [29] and this data shall be reported elsewhere. The *pfmdr* digests were reported as either wild type, mutant or mixed infection. The *msp-1, msp-2* allele frequencies were expressed as the proportion of samples containing an allelic family compared to the total number of samples that gene was detected in. Multiplicity of infection (MOI) was defined as the number of parasite genotypes per infection. Estimation of mean MOI was calculated by dividing the total number of fragments detected in *msp-1* or *msp-2*, by the number of samples in the same marker as previously defined and interpreted by different authors [31–33].

Results

Mosquito presence, species distribution and parasite infection

Only two adult *Anopheles* mosquitoes were collected indoors from all the 30 houses that were sampled over the 3 nights. These two mosquitoes were collected using CDC light traps from a single house in Kanyethi village

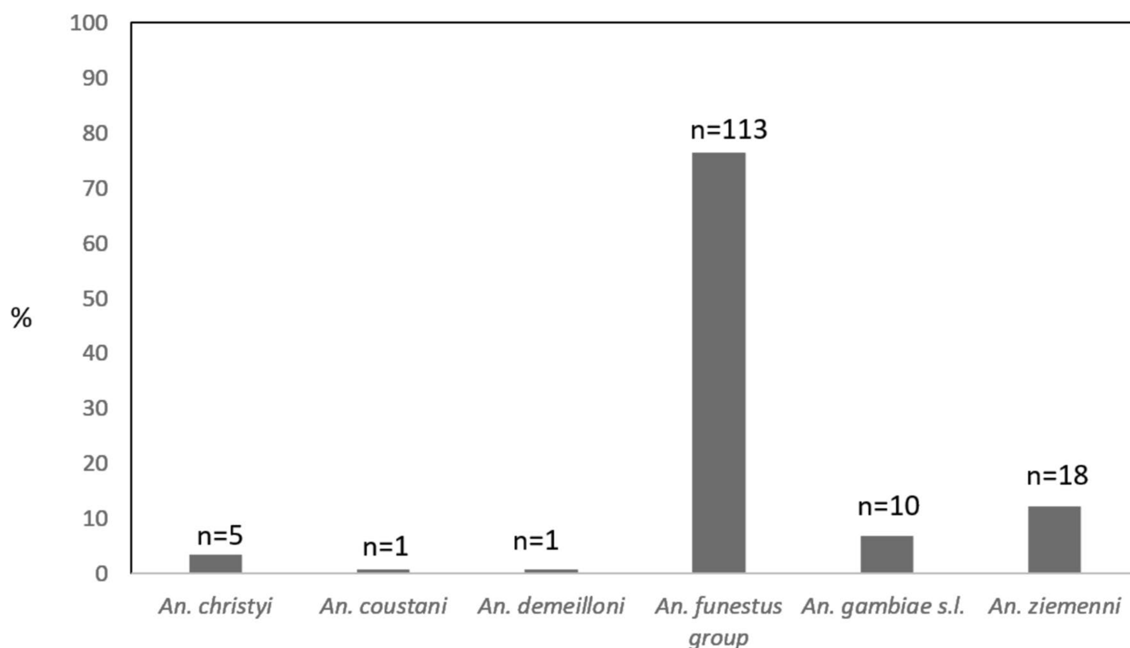

Fig. 3 Distribution of Anopheles mosquitoes collected from Kikuyu area, Kenya

and were non-blood fed. The mosquitoes were identified as belonging to the *An. funestus* group based on morphology but failed to be amplified by PCR despite numerous attempts and could, therefore, not be identified into the sibling species. Further analysis showed that these two specimens were negative for malaria parasites. From the mosquitoes collected as larvae over the 3 days, a total of 148 female mosquitoes were successfully reared into adults and a rich repertoire of Anopheles species was identified by morphology (Fig. 3). Of the 10 mosquitoes identified as *An. gambiae s.l.,* nine (9) were *An. arabiensis,* while only one was *An, gambiae s.s.* All the 17 mosquitoes of the *An. funestus* group were successfully identified by PCR as *An, funestus s.s.*

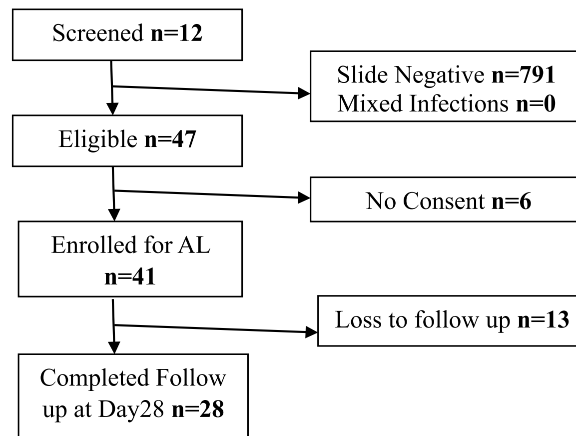

Fig. 4 Study participant recruitment and follow up flowchart

Demographic and parasitological characteristics of the study populations

From July to December 2019, a total of 838 patients attending outpatient clinics at Gikambura Health Centre and Lusigeti Sub-County Hospital with malaria symptoms were screened. Of the 838 patients suspected to be having malaria, 47(5.6%) had malaria slide positive results. Forty-one of the malaria slide-positive individuals consented to participate in the trial (Fig. 4). All the recruited patients received at least a dose of the study drug and were thus included in the intention to treat analysis (Table 2). The treatment outcomes are as summarized in Tables 3, 4. In Lusegeti, data on history of travel was recorded with five of the ten patients found to have malaria reporting a recent travel to a malaria-prone western or coastal endemic zones of Kenya, this data was however not captured at the Gikambura site.

Genetic analysis and molecular characteristics of the study populations

Allelic diversity of msp-1 and msp-2 genotypes

Successful amplification occurred in 87.5% (35/40) of samples for *msp-1* and *msp2* genotyping (27/31 in Gikambura and 8/9 in Lusigeti). One (1) sample from Lusigeti was not shipped to the KEMRI lab for analysis. Positivity based on 18sRNA also showed that 4 (10%)

Table 2 Baseline characteristics for both intention-to-treat and per-protocol population

	ITT population	**PP population**
Characteristic	N = 41	N = 28
Age in Years, median (IQR)[Q1,Q3]	24.0 (14.5) [19.5–34]	26 (18.5) [20.5–39]
Gender (female) – n/N (%)	17/37 (45.9)	11/28 (39.3)
Weight in Kgs, median (IQR)[Q1,Q3]	59.0 (18) [51.5–69.5]	58.5 (23) [47–70]
Height in Cms, median (IQR)	165.0 (15) [158.5–173.5]	168 (15) [159–174]
Fever (recruitment temp > 37.5^0c)	26/41 (63.41%)	16/28 (57.14%)
Parasite counts, median (IQR)[Q1,Q3] p/ul	8264.0 (21,912) [4870–26782]	8272 (18,488) [3602–22090]
Gametocyte present, N (%)	1/41(2.44%)	1/28(3.57%)
Sites		
Gikambura – N (%)	31(75.61%)	22(78.57%)
Lusegeti – N (%)	10(24.39%)	6(21.43%)

ITT Intention-to-treat and pp-per protocol

Table 3 Parasitological response (primary outcome)

	Intention to Treat Analysis (N = 41)			
	Lusigeti N = 10	**Gikambura N = 31**	**Difference, % (95% CI)**	**P-value**
Day 28				
Non-corrected ACPR	10(100%)	31(100%)	0	-
PCR-corrected ACPR	10(100%)	31(100%)	0	-
Day 14				
Non-corrected ACPR	10(100%)	31(100%)	0	-
PCR-corrected ACPR	10(100%)	31(100%)	0	-

ACPR adequate clinical and parasitological response

microscopy positive samples were negative, 1 in Lusigeti and 3 from Gikambura.

After genotyping, the three allelic families (K1, MAD20 and RO33) of *msp1* gene and two (3D7 and FC27) of *msp*2 gene were observed in Kikuyu. A total of 148 allelic types were detected for the two genes in all localities: 69 for *msp1* (Fig. 5) and 79 for *msp2* (Fig. 6). The allele frequencies of *P. falciparum* in Kikuyu isolates among *msp-1* isolates, was 0.46 for K1 (180–300 bp), 0.32 for MAD20 (100–250 bp) and 0.22 for RO33 (100–230 bp). Among the multi allelic infections carrying two allelic family types, the proportions of samples with K1/MAD20, K1/RO33, and MAD20/RO33 was 31.4%, 11.4% and 5.7%, respectively. Infections with all three allelic types were detected in 25.7% of cases.

The allele frequencies of *P. falciparum* in Kikuyu isolates among *msp-2* isolates was 0.62 for FC27 (250–800 bp) and 0.3 for IC1/3D7 (100–600 bp) allelic families. The proportion of samples with only FC 27 and IC1/3D7 were 28.6% and 8.6%, respectively. The remaining 57.1% (20/35) were polyallelic FC27-IC1/3D7infections (Table 5).

Table 4 Secondary outcomes

	Intention to treat analysis (N = 41)			**Per protocol analysis (n = 28)**		
	Lusigeti N = 10	**Gikambura N = 31**	**Totals**	**Lusigeti N = 6**	**Gikambura N = 22**	**Totals**
Treatment failure -N (%)						
Early treatment failure	None			None		
Late treatment failure	None			None		
Slide positivity amongst patients seen on Day 3	0/10(0%)	3/31(9.68%)	3/41(7.32%)	0/6 (0%)	2/22 (9.1%)	2/28 (7.1%)
Recurrent parasitaemia – N (%)	None			None		
Adverse events – N (%)	None			None		
Adherence to study medication						
Completed study medication—N (%)	10(100)	31(100)		6(100)	22(100)	

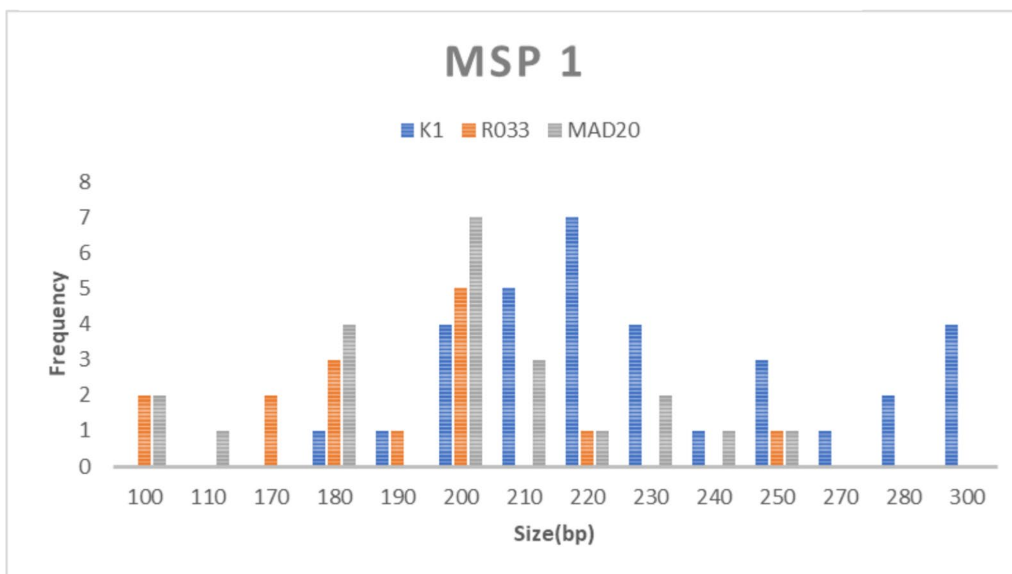

Fig. 5 Frequency distribution of msp-1 allelic families

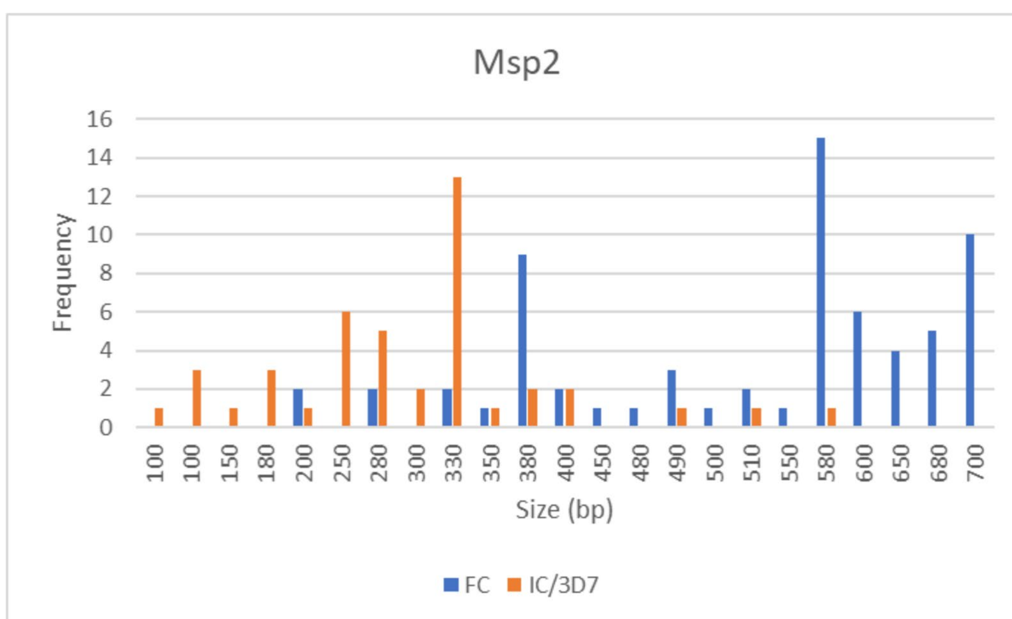

Fig. 6 Frequency distribution of msp-2 allelic families

Multiplicity of infection (MOI)

Using the previous description by Sondo et al. [33] for the determination of MOI and allelic frequency, from the 35 positive samples, 29 (82.9%) were polyclonal i.e., harboured more than one parasite genotype identified by the presence of two or more alleles of one or both genes with 17.1% being monoclonal. The overall mean MOI i.e., parasite clones per sample was 2.8 (95% CI 2.36–3.35). Calculating for msp-1 and msp-2 genes separately, the MOI was 2.02 (95% CI 0.72–2.27) and 2.9 (95% CI 2.22–3.55), and parasite strains range of 1–3 and 1–7, respectively. Polyclonal variation in the different genes was at 76.4% and 70.3% for *msp1* and *msp2*, respectively as summarized in Table 6 below. All the genotyped samples from Lusegeti, where patient travel

Table 5 Genetic diversity of *Plasmodium falciparum msp1* and *msp2*

Gene	Allele	Positive (%-n/N)	Fragment Size (bp)	Total no of alleles
*Msp1 *N = 35*	MAD20	22(62.8)	100–250	22
	K1	32(91.4)	180–300	32
	RO33	15(42.8)	100–250	15
	MAD20/K1	11(31.4)	–	–
	MAD20/RO33	2(5.7)	–	–
	K1/RO33	4(11.4)	–	–
	MAD20/K1/RO33	9(25.7)	–	–
Msp2	FC27	24(68.5)	250–800	49
	IC1/3D7	17(48.6)	100–600	30
	FC27/ IC1/3D7	14(40.0)	–	–

N number of total samples analyzed, n number of positive samples

history data was recorded, carried polyclonal infections irrespective of the travel history.

Determination of the existence of SNPs in *PfK13* and *Pfmdr*

PCR amplification and DNA Sanger sequencing to evaluate specific regions of K13 (codons 432–702), of the *Pfk13* gene to observe previously targeted SNPs associated with artemisinin resistance (M476I, Y493H, R539T, I543T, and C580Y) [29], revealed that all *P. falciparum* isolates carried the wildtype alleles found in the 3D7 reference genome.

All falciparum positive slides were examined for single nucleotide polymorphisms (SNP) at one positions of *pfmdr1* gene (N86Y). Restriction digests were successfully done on 28 and 8 samples from Gikambura and Lusegeti, respectively. All the sample had a wild type allele for the *pfmdr* N86 marker.

Discussion

Disease prevention, diagnosis and management efforts in resource-poor settings such as in Africa tend to focus on zones with clearly characterized disease transmission patterns. This paper sought to re-evaluation malaria transmission patterns in a non-traditional/ low risk malaria transmission zone where there have been anecdotal reports of malaria cases and presence of vectors of malaria in order to provide critical data on possible active transmission. Such information is crucial in guiding the policy on prevention, diagnosis and management that would contribute towards malaria elimination which has been a global goal for decades.

This is the first definitive report of the presence of vectors of malaria in Kikuyu highlands. Despite only two adult mosquitoes being sampled indoors, the presence of larvae stages of the malaria vectors in close proximity to human dwelling was recorded. As recently described by different authors, this may be potent for the establishment of an active malaria transmission cycle in the area in the face of climate changes being experienced compounded by the presence of clinical disease detected [38–41]. In the context of an emerging disease and the observed trends in global warming, this is bound to get out of control unless an intervention is put in place [42, 43].

For the duration of the study, 47 *Plasmodium* infection cases, representing a 5.6% infection rate among the suspected cases, were confirmed in the study area. This finding indicates that contrary to the general assumption, the study area is not a malaria free zone. A positivity rate of 5.6% even in a selected sub population cannot be ignored. The question that follows is whether the identified parasite population is locally established and spontaneous or imported. Of the 10 participants with *Plasmodium* infections in one site where travel history was captured, only 5 (representing 50%) reported a history of travel from a malaria endemic area within the previous 30 days before testing. This means that the remaining proportion could probably have acquired their infections locally. On the contrary, the observed genetic diversity and polyclonal variations with high MOI on the whole sample likely suggest that these are imported cases from areas with higher transmission rate and disease burden. These therefore

Table 6 Multiplicity of infections for *msp-1* and *msp-2* genes in study isolates

Gene	MOI	95% CI	SD (±)	Monoclonal infection % (n/N)	Polyclonal infection % (n/N)
Msp1	2.02	0.72–2.27	0.717(0.241)	23.5 (8/34)	76.4(26/34)
Msp2	2.9	2.22–3.55	1.761(0.664)	29.6 (8/27)	70.3 (19/27)
Overall	2.8	2.36–3.35	1.49(0.495)	17.1(6/35)	82.9(29/35)

gives two possible but conflicting answers to the question. The small sample size and short sampling duration limits the study in explaining the two conflicting observations. This can serve as an indicator and does not also rule out a likely changing malaria transmission scenario in this part of the country, previously stamped as malaria free, thus the need for further detailed investigations and possible re-classification.

The patients follow up after treatment was faced with a significant 'loss to follow up' (31.7%) which obviously affected the power of the study and thus limiting the data interpretation. Relocation at the time of follow up during the study was sighted as the cause in all the cases lost. This notwithstanding, the circulating parasite strains showed full sensitivity to the available treatment option with no observed treatment failures (Tables 3, 4). The few delayed parasite clearance, parasitaemia on Day 3 (n = 3), was attributed to drug availability variances since some of the patients may have been treated on empty stomachs a situation that is known to affect the absorption of AL. The 3 patients cleared their parasitemia by Day 4 of treatment initiation. Furthermore, the drug resistance markers profiled did not present any validated resistance marker in any of the samples. Thus, suggesting minimum risk of antimalarial drug resistance in the area.

Overall, the three allelic families (K1, MAD20 and RO33) of *msp1* gene and two (3D7 and FC27) of *msp*2 gene were observed in this study. A total of 148 allelic types were detected for the two genes in all localities: 69 for *msp1* (Fig. 5) and 79 for *msp2* (Fig. 6). Allele genotyping demonstrated the highly polymorphic nature of *P. falciparum* in Kikuyu isolates with respect to *msp-1* and *msp-2*. In this sample, 82.9% were polyclonal i.e. harboured more than one parasite genotype identified by the presence of two or more alleles of one or both genes with 17.1% being monoclonal. The overall mean MOI i.e., parasite clones per sample was 2.8. These findings suggested high complexity of *P. falciparum* population in the study area, and this could be pointing to multiple sources of these infections as opposed to a common origin. This can however only be true for those with travel history. There is a chance that the rest acquired an equally diverse population through local transmission. Again the few who indicated no recent travel history(n = 5) all had polyclonal infections further indicating the possibility of a diverse local transmission. The travel history data however was scanty and incomplete and thus limiting our conclusion.

Multiplicity of infection (MOI) is an indicator of malaria transmission level as it has been shown to be higher in high malaria transmission areas and decreases when transmission is low [36]. Seasonal weather variations have previously been shown to affect the MOI as well as host factors (age, immune status), epidemiological settings and parasite densities [33, 44–47] but these have all affirmed that MOI could be a good predictor of transmission intensity. In our current study MOI for *msp1* was 2.0 and for *msp2* was 2.9, giving an average MOI of 2.4 and an overall MOI of 2,8. The observed MOI were lower that recently reported values in Kenya of 4.8 for the malaria endemic Lake Victoria region, 4.4 for the epidemic prone Kisii Highland and 3.4 for the seasonal malaria Semi-Arid region [48]. Our MOI values were however higher than other previously reported studies in Africa, including three in Ethiopia (MOI: 1.8–2.6) between 2015 and 2018 [49–51], two in Congo (MOI: 1.99–2.4) [32, 34], in 2011, 2023 and two in Burkina Faso (MOI: 1.95–2.7) [33, 52]. These were again lower than what was reported elsewhere in Ethiopia (MOI: 3.2) in 2020 [31], Equatorial Guinea (MOI: 5.51) [53] in 2018 and Gabon (MOI: 4.0) [54] in 2018. Therefore, an average MOI of 2.4 and an overall MOI of 2.8 in our study site is an indication of a possible average to high transmission in this area whether local or otherwise.

The observed MOI, the presence of a competent vector in this area together with other emerging threats, such as *An. stephensi* that was recently identified for the first time in Kenya [10], infers the potential expansion of transmission zones. This could dramatically enhance the transmission of *Plasmodium* infections and reverse all the malaria control gains made over the years.

Limitations of the study

Some limitations of this study include the narrow vector sampling window of only 3 nights, incomplete travel history data and the use of *msp* genotyping which, as others marker based on DNA fragment size, could reduce the genetic diversity evaluation of the parasites strains due to the small sample size. Also as previously observed, concluding a high transmission intensity from the presence of multi-clonal infections alone is limited by other variables including estimates of MOI varying by genotyping method used, potential impact from sampling seasonality and a non-linear relationship seen between MOI and transmission intensity [55]. Lastly there was a very high proportionate loss to follow up on the study patients and this further reduced the final cases in the per protocol analysis affecting the overall generalizability of the study conclusions.

Conclusion

Although the positivity rate observed in the study site was very low, this was not as previously defined. This presents a likely changing malaria transmission scenario in this part of the country, previously stamped as malaria free, requiring further investigations, possible re-classification and immediate interventions. Approximately half

of participants who tested positive in one of the study facilities did not report history of travel from the area but they had polyclonal infections with no difference in MOI when compared to those who had travelled. The genetically diverse parasite population coupled with the finding of highly competent known larval vectors of malaria in the area, the emerging changes in the global weather patterns and vector dynamics, suggests that there is a chance of the occurrence of a sustained natural transmission cycle in the study area. Further studies preferably encompassing longitudinal entomological sampling and detailed patient travel histories, will help better clarify the status of malaria transmission in the area. Additionally, comparative genetic analysis of the *Plasmodium* parasites from the study area and known malaria transmission zones will improve understanding of the origins of these infections and the gene flow patterns. The finding of full susceptibility by the parasites to available treatment options is good news from a malaria management perspective, an indication of the absence of antimalarial drug resistance is clinically reassuring but close monitoring will be needed.

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Author contributions
FK and LK designed the study and mobilized the funding. KT, MO, LW and MB were involved in field studies and all laboratory activities for the parasite arm. SK, SA and LW were involved in field studies and all laboratory activities for the vector arm. FK, LM and DM provided technical advice for genotyping, data interpretation, performed statistical analysis and wrote the first draft of the manuscript of the results. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was approved and monitored by the KEMRI Scientific and Ethics Review Unit (SERU) (Study approval No. KEMRI/SERU/CBRD/3561). An approval to use the public health facilities and visit the households was also granted by the health department in the County Government of Kiambu (KIAMBU/HRDU/AUTHO/2019/04/24/Kamau L). Patients were enrolled in the study according to common KEMRI guidelines, which follows the Helsinki declaration and the ICH-GCP guidelines. The purpose of the study was explained in the local language, and an informed consent obtained from the patient, parent(s) or guardian(s) to participate in the study. For household vector sampling, the unit heads were informed of the procedures to be carried out inside the house and the hazards, risks or benefits associated with these were explained clearly before they could consent to the study. Approval to publish the study outcome was granted by the Director General KEMRI.

Competing interests
The authors declare no competing interests.

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References

1. Zekar L, Sharman T. *Plasmodium falciparum* malaria. Treasure Island (FL): StatPearls Publishing; 2022. https://www.ncbi.nlm.nih.gov/books/NBK555962
2. Jeyaprakasam NK, Pramasivan S, Liew JWK, Van Low L, Wan-Sulaiman WY, Ngui R, et al. Evaluation of Mosquito Magnet and other collection tools for Anopheles mosquito vectors of simian malaria. Parasit Vectors. 2021;14:184.
3. Kenya Malaria Indicator Survey 2020. Ministry of Health, Government of Kenya. 2021. https://dhsprogram.com/pubs/pdf/MIS36/MIS36.pdf
4. Berhane A, Anderson K, Mihreteab S, Gresty K, Rogier E, Mohamed S, et al. Major threat to malaria control programs by *Plasmodium falciparum* lacking histidine-rich protein 2. Eritrea Emerg Infect Dis. 2018;24:462–70.
5. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM. Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med. 2008;359:2619–20.
6. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, et al. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med. 2009;361:455–67.
7. Phyo AP, Nkhoma S, Stepniewska K, Ashley EA, Nair S, McGready R, et al. Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. Lancet. 2012;379:1960–6.
8. Karungu S, Atoni E, Ogalo J, Mwaliko C, Agwanda B, Yuan Z, et al. Mosquitoes of etiological concern in Kenya and possible control strategies. Insects. 2019;10:173.
9. Doumbe-Belisse P, Kopya E, Ngadjeu CS, Sonhafouo-Chiana N, Talipouo A, Djamouko-Djonkam L, et al. Urban malaria in sub-Saharan Africa: dynamic of the vectorial system and the entomological inoculation rate. Malar J. 2021;20:364.
10. Ochomo EO, Milanoi S, Abong'o B, Onyango B, Muchoki M, Omoke D, et al. Detection of *Anopheles stephensi* mosquitoes by molecular surveillance. Kenya Emerg Infect Dis. 2023;29:2498–508.
11. Lindsay SW, Martens WJ. Malaria in the African highlands: past, present and future. Bull World Health Organ. 1998;76:33–45.
12. Matson AT. The history of malaria in Nandi. East Afr Med J. 1957;34:431–41.
13. Wanjala CL, Kweka EJ. Impact of highland topography changes on exposure to malaria vectors and immunity in Western Kenya. Front Public Health. 2016;4:227.
14. Detinova TS. Age-grouping methods in Diptera of medical importance: with special reference to some vectors of malaria. J Parasitol. 1962;48:456.
15. Allen MR, Frame DJ, Huntingford C, Jones CD, Lowe JA, Meinshausen M, et al. Warming caused by cumulative carbon emissions towards the trillionth tonne. Nature. 2009;458:1163–6.
16. Malakooti MA, Biomndo K. Shanks GD Reemergence of epidemic malaria in the highlands of Western Kenya. Emerg Infect Dis. 1998;4:671–6.
17. Lindblade KA, Walker ED, Onapa AW, Katungu J, Wilson ML. Land use change alters malaria transmission parameters by modifying temperature in a highland area of Uganda. Trop Med Int Health. 2000;5:263–74.
18. WHO. Guidelines for malaria. 3rd Edn. Geneva: World Health Organization, 2015.
19. Kenya National Bureau of Statistics - 2019 Kenya Population and Housing Census. Volume I: Population by County and Sub-County. 2022. https://www.knbs.or.ke/?wpdmpro=2019-kenya-population-and-housing-census-volume-i-population.
20. Kikuyu climate: Weather Kikuyu & temperature by month. Climate-data.org. 2021. https://en.climate-data.org/africa/kenya/kiambu/kikuyu-57885
21. Edwards FW. Mosquitoes of the Ethiopian Region. III. - Culicine Adults and Pupae. London: British Museum (Natural History); 1941.

22. Gillies MT, Coetzee M. A supplement to the Anophelinae of Africa south of the Sahara (Afrotropical Region). South African Institute for Medical Research. 1987;55.
23. Collins FH, Besansky NJ, Mendez MA, Rasmussen MO, Finnerty V, Mehaffey PC. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. Am J Trop Med Hyg. 1987;37:37–41.
24. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am J Trop Med Hyg. 1993;49:520–9.
25. Koekemoer LL, Kamau L, Coetzee M, Hunt RH. A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. Am J Trop Med Hyg. 2002;66:804–11.
26. Wirtz RA, Burkot TR, Graves PM, Andre RG. Field evaluation of enzyme-linked immunosorbent assays for *Plasmodium falciparum* and *Plasmodium vivax* sporozoites in mosquitoes (Diptera: Culicidae) from Papua New Guinea. J Med Entomol. 1987;24:433–7.
27. WHO. Method for surveillance of antimalarial drug efficacy. Geneva, World Health Organization, 2009. https://www.who.int/publications/i/item/9789241597531
28. Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am J Trop Med Hyg. 1995;52:565–8.
29. Musyoka KB, Kiiru JN, Aluvaala E, Omondi P, Chege WK, Judah T, et al. Prevalence of mutations in *Plasmodium falciparum* genes associated with resistance to different antimalarial drugs in Nyando, Kisumu County in Kenya. Infect Genet Evol. 2020;78:104–21.
30. Snounou G. Genotyping of *Plasmodium* spp.: nested PCR. Methods Mol Med. 2002;72:103–16.
31. Abamecha A, El-Abid H, Yilma D, Addisu W, Ibenthal A, Bayih AG, et al. Genetic diversity and genotype multiplicity of *Plasmodium falciparum* infection in patients with uncomplicated malaria in Chewaka distric,t Ethiopia. Malar J. 2020;19:203.
32. Simpson SV, Nundu SS, Arima H, Kaneko O, Mita T, Culleton R, et al. The diversity of *Plasmodium falciparum* isolates from asymptomatic and symptomatic school-age children in Kinshasa Province Democratic Republic of Congo. Malar J. 2023;22:102.
33. Sondo P, Derra K, Rouamba T, Diallo S, Taconet P, Kazienga A, et al. Determinants of *Plasmodium falciparum* multiplicity of infection and genetic diversity in Burkina Faso. Parasit Vectors. 2020;13:427.
34. Gosi P, Lanteri CA, Tyner SD, Se Y, Lon C, Spring M, et al. Evaluation of parasite subpopulations and genetic diversity of the *msp1*, *msp2* and *glurp* genes during and following artesunate monotherapy treatment of *Plasmodium falciparum* malaria in Western Cambodia. Malar J. 2013;12:403.
35. Mayengue P, Ndounga M, Malonga F, Bitemo M, Ntoumi F. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in *Plasmodium falciparum* isolates from Brazzaville Republic of Congo. Malar J. 2011;10:276.
36. Kiwuwa MS, Ribacke U, Moll K, Byarugaba J, Lundblom K, Färnert A, et al. Genetic diversity of *Plasmodium falciparum* infections in mild and severe malaria of children from Kampala, Uganda. Parasitol Res. 2013;112:1691–700.
37. Ndiaye T, Sy M, Gaye A, Ndiaye D. Genetic polymorphism of Merozoite Surface Protein 1 (msp1) and 2 (msp2) genes and multiplicity of *Plasmodium falciparum* infection across various endemic areas in Senegal. Afr Health Sci. 2019;19:2446–56.
38. Martens WJM, Niessen LW, Rotmans J, Jetten TH, McMichael AJ. Potential impact of global climate change on malaria risk. Environ Health Perspect. 1995;103:458–64.
39. Githeko AK, Lindsay SW, Confalonieri UE, Patz JA. Climate change and vector-borne diseases: a regional analysis. Bull World Health Organ. 2000;78:1136–47.
40. Lim AY, Cheong HK, Chung Y, Sim K, Kim JH. Mosquito abundance in relation to extremely high temperatures in urban and rural areas of Incheon Metropolitan City, South Korea from 2015 to 2020: an observational study. Parasit Vectors. 2021;14:559.
41. Nosrat C, Altamirano J, Anyamba A, Caldwell JM, Damoah R, Mutuku F, et al. Impact of recent climate extremes on mosquito-borne disease transmission in Kenya. PLoS Negl Trop Dis. 2021;15: e0009182.
42. McCarthy JJ, Canziani OF, Leary NA, Dokken DJ, White KS. Climate change 2001: impacts, adaptation, and vulnerability. Contribution of Working Group II to the third assessment report of the Intergovernmental Panel on Climate Change. Cambridge, UK; Cambridge University Press; 2001.
43. Watson RT, Zinyowera MC, Moss RH, Intergovernmental Panel On Climate Change. The Regional impacts of climate change: an assessment of vulnerability : a special report of IPCC Working Group II. Cambridge: Cambridge University Press, UK; 1998.
44. Smith T, Felger I, Tanner M, Beck HP. Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections. Trans R Soc Trop Med Hyg. 1999;93:59–64.
45. Konaté L, Zwetyenga J, Rogier C, Bischoff E, Fontenille D, Tall A, et al. Variation of *Plasmodium falciparum* msp1 block 2 and msp2 allele prevalence and of infection complexity in two neighboring Senegalese villages with different transmission conditions. Trans R Soc Trop Med Hyg. 1999;93:21–8.
46. Bendixen M, Msangeni HA, Pedersen BV, Shayo D, Bødker R. Diversity of *Plasmodium falciparum* populations and complexity of infections in relation to transmission intensity and host age: a study from the Usambara Mountains, Tanzania. Trans R Soc Trop Med Hyg. 2001;95:143–8.
47. Franks S, Koram KA, Wagner GE, Tetteh K, McGuinness D, Wheeler JG, et al. Frequent and persistent, asymptomatic *Plasmodium falciparum* infections in African infants, characterized by multi-locus genotyping. J Infect Dis. 2001;183:796–804.
48. Andika B, Mobegi V, Gathii K, Nyataya J, Maina N, Awinda G, et al. *Plasmodium falciparum* population structure inferred by msp1 amplicon sequencing of parasites collected from febrile patients in Kenya. Malar J. 2023;22:263.
49. Mohammed H, Mindaye T, Belayneh M, Kassa M, Assefa A, Tadesse M, et al. Genetic diversity of *Plasmodium falciparum* isolates based on msp-1 and msp-2 genes from Kolla-Shele area, Arbaminch Zuria District, southwest Ethiopia. Malar J. 2015;14:73.
50. Mohammed H, Kassa M, Assefa A, Tadesse M, Kebede A. Genetic polymorphism of Merozoite Surface Protein-2 (MSP-2) in *Plasmodium falciparum* isolates from Pawe District, North West Ethiopia. PLoS ONE. 2017;12: e0177559.
51. Mohammed H, Kassa M, Mekete K, Assefa A, Taye G, Commons RJ. Genetic diversity of the msp-1, msp-2, and glurp genes of *Plasmodium falciparum* isolates in Northwest Ethiopia. Malar J. 2018;17:386.
52. Somé AF, Bazié T, Zongo I, Yerbanga RS, Nikiéma F, Neya C, et al. *Plasmodium falciparum* msp1 and msp2 genetic diversity and allele frequencies in parasites isolated from symptomatic malaria patients in Bobo-Dioulasso, Burkina Faso. Parasit Vectors. 2018;11:323.
53. Chen JT, Li J, Zha GC, Huang G, Huang ZX, Xie DD, et al. Genetic diversity and allele frequencies of *Plasmodium falciparum msp1* and *msp2* in parasite isolates from Bioko Island, Equatorial Guinea. Malar J. 2018;17:458.
54. Ndong Ngomo JM, M'Bondoukwe NP, Yavo W, Bongho Mavoungou LC, Bouyou-Akotet MK, Mawili-Mboumba DP. Spatial and temporal distribution of PFMSP1 and PFMSP2 alleles and genetic profile change of *Plasmodium falciparum* populations in Gabon. Acta Trop. 2018;178:27–33.
55. Yavo W, Konaté A, Mawili-Mboumba DP, Kassi FK, Tshibola Mbuyi ML, Angora EK, et al. Genetic polymorphism of msp1 and msp2 in *Plasmodium falciparum* isolates from Côte d'Ivoire versus Gabon. J Parasitol Res. 2016;2016:3074803.

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