

# Malaria prevalence, transmission potential and efficacy of Artemisinin Combination Therapy in the Kenyan Central highlands - a zone previously characterized as malaria free

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# Abstract

**Introduction:** 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 in the near future. The current study sought to re-evaluate malaria prevalence, susceptibility to ACTs, 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 malaria cases. The potential role of climate factors was also evaluated. The aim of the study was to generate data to inform malaria treatment policy and practice in the study area and country.

**Methodology:** Sampling of adult mosquitoes was carried indoors by manual aspiration and using CDC light traps while mosquito larvae were sampled outdoors using larval dippers and reared to adults in the laboratory. Mosquitoes were identified by morphology and subsequently using PCR and the presence of malaria parasites in field sampled adult mosquitoes investigated using ELISA. The malaria clinical study was an open label nonrandomized clinical trial where the efficacy of one artemisinin-based antimalarial combination drug, Artemether Lumefantrine (AL) was evaluated. Two health facilities Lusigeti and Gikambura were identified for the study. Microscopy was used to identify positive cases at the health facility and nested PCR amplification targeting subunit 18S rRNA gene used to confirm positivity in the lab. *P. falciparum* isolates were genotyped using nested-PCR of *MSP-1* (block 2) and *MSP-2* (block 3) family alleles to determine the multiplicity of the infections (MOI) and characterize any subsequent infection. Antimalarial resistance gene markers *Pfk13* and *Pfmdr1* were analyzed. Climate data for the study area was obtained from Climate Engine (<http://climateengine.org>) and analyzed to understand long term trends.

**Results:** A rich repertoire of mosquito vector species was identified from the area, with the *Anopheles funestus* group being the predominant vector species and comprising 76.35% of all collections. Only two adult mosquitoes which were non-blood fed and negative for malaria parasites were collected. Of the 838 patients screened, 471, with a slide positivity rate of 2.1% (10) were from Lusigeti and 421, with a slide positivity rate of 7.4% (31) were from Gikambura. Parasitological analysis of microscopy outcome of the 41 cases revealed 100% (95% CI 1.96) as Adequate Clinical and Parasitological Response (ACPR). There was probable delayed parasite clearance (parasites present on Day 3) in 3(7.3%) of the cases, and no severe adverse reaction was observed. Analysis of the *Pfk13* gene in the positive *P. falciparum* cases from the study sites revealed no SNP associated with artemisinin resistance. The *pfmdr1* 86Y mutation was found in 0% (0/41) of the isolates while the N86 wild allele was detected in 100%(37/37). Analysis of long term climate data showed an increase of about 1.3°C in both the mean minimum and maximum temperatures consistent with forecasts from other sources.

**Conclusion:** The positivity rate observed in the study site was very low but the fact that 87% of participants who tested positive did not report recent history of travel from the area and the finding of highly competent known vectors of malaria suggest a changing malaria transmission scenario requiring

further investigations. That circulating parasite strains showed full sensitivity to the available treatment option indicating the absence of antimalarial drug resistance which is a positive finding.

## Introduction

Malaria is a life-threatening disease caused by the infection of red blood cells with protozoan parasites of the genus *Plasmodium* that are transmitted to people through the bites of infected female *Anopheles* mosquitoes. Four species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) most commonly infect humans. *P. falciparum* and *P. vivax* are the most prevalent species and *P. falciparum* is the most dangerous (Zekar, 2021). Other emerging species, for example, *P. 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 (Jeyaprakasam NK, 2020). 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 (DNMP, 2021).

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* s.s. Giles and *An. arabiensis* Patton mosquitoes together with *An. funestus* s.s. Giles, a member of the *An. funestus* group of mosquitoes are amongst the most important vectors in sub-Saharan Africa, including Kenya (Karungu et al. 2019; Doumbe-Belisse et al. 2021). *Anopheles stephensi*, an invasive malaria vector that is endemic to south Asia and the Arabian Peninsula, has also recently been reported in Kenya. (Eric O. Ochomo, 2023)

Globally, there were an estimated 247 million malaria cases in 2021, with 228 million (95%) in the African region according to the World Malaria Report, 2022. Malaria claimed the lives of over 619,000 in the same year, mostly in sub-Saharan Africa with children below the age of 5 years being especially vulnerable to the disease (WHO, 2022). Locally, 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 (DNMP, 2021). This disease burden, coupled with the numerous reports of resistance to the current medical interventions makes malaria a primary concern for medical research.

In Africa, highland zones are defined as those with an elevation higher than 1500m above sea level. These zones are also characterized by low mean temperatures, generally below 20°C and were for a long time considered to have little or no malaria (Lindsay, 1998) (Matson, 1957). 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 (Wanjala and Kweka 2016). The relationship between ambient temperatures and the duration of the sporogonic cycle has previously been defined by

Detinova (Detinova, 1962). 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 (Allen, 2001).

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 (Malakooti *et al.* 1998, Lindblade *et al.* 2000). 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 antimalarial drugs treatment still remain to be the mainstay of the fight against malaria (WHO, 2015). The current study 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 data to inform policies on malaria control in such settings.

## Materials And 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. Quality-assured drugs were provided by the National Malaria control Programme, (Coartem; Novartis Pharma- the company did not play a role in the study.)

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. Linking of personal information by means of a unique identification number was only possible for the principal investigator and co-investigators.

### Study area, study design and sampling

This study was carried out in Kikuyu sub-county of Kiambu County, Kenya (Figure 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 (KNBS, 2019). The area is generally cold and wet with the temperatures ranging from 10.9 to 24.6°C and monthly rainfall ranging from 20mm to 125mm experienced in a bimodal pattern of long rains occurring in April – June and the short rains in October – November (“Kikuyu Climate-Data.Org” n.d.).

### **Mosquito sampling, handling and analysis**

Mosquito sampling was carried out between 11<sup>th</sup> and 13<sup>th</sup> June 2019 in two swamps in Kikuyu sub-county, namely Karai Rurii (1°25'S; 36°35'E) and Ondiri Swamp (1°15'S; 36°40'E) (Figure 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 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 meter of the bed or sleeping area the evening before at 18:00h and collected at 06:00h 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 (Edwards 1941, Gillies and Coetzee 1987). 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.* (1987) and the PCR assays of Scott *et al.* (1993) and Koekemoer *et al.* (2002) used to distinguish between members of the *An. gambiae* s.l. and *An. funestus* species complexes, respectively. Adult mosquitoes sampled were tested individually for the presence of *Plasmodium falciparum* parasites. This was done using mosquito heads and thoraces and by the Enzyme-linked Immunosorbent Assay (ELISA) of Wirtz *et al.* (1987).

### **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 10km 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 22<sup>nd</sup> Dec 2019.

### **Patient screening, Recruitment and follow-up**

From all patients suspected with malaria infections and referred to the facility lab, a finger prick was performed to obtain blood for the preparation of thick and thin blood smears. Smears were processed and read by expert microscopists.

Patients of all ages with microscopically confirmed malaria infection 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.

### **Treatment, Clinical and Laboratory Procedures**

Eligible patients received supervised treatment with AL (Coartem; Novartis Pharma), administered as half 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 trial on a daily basis over the determined recruitment period.

Blood Samples were collected from patients before and after drug administration according to the WHO/MAL/2009 guidelines for clinical assessment of antimalarial drugs as modified in the study SOPs. The criteria for inclusion, and exclusion as well as safety assessment are contained therein. On treatment days, blood sample collection was done before treatment. Blood smears were prepared, dried filter paper blood spots, 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 physical examination, blood smears and DBS were also collected from finger pricks on follow up days

### **Parasite DNA extraction**

Parasite genomic DNA was extracted from the blood spots collected on filter papers or whole blood samples using Chelex-100<sup>®</sup> (Bio-Rad Laboratories CA) method as previously described (Plowe CV, 1985) 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. To elute the extracted DNA, 150 µL Bufer AE was added to each well and incubated for 1 min at room temperature. This setup was centrifuged to recover the DNA and stored as indicated.

### ACT Resistance markers

To evaluate the resistance by *P. falciparum* to antimalarial drugs in the study area, molecular surveillance targeting the *Pfk13* and *Pfmdr1* Molecular Markers of ACTs genes was conducted. The polymorphic characteristics and haplotype network of the *Pfk13* gene were evaluated. *K13-propeller* genes were amplified by the nested PCR protocol described and optimized previously (Musyoka K., 2020) by using the primers listed in Table below. 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 mM dNTP Mixture, PCR buffer, and 4 mM Mg<sup>2+</sup>), 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 H<sub>2</sub>O. 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 at 4 °C.

Table 1; *Pfk13* primer sets.

|   | Primer Name       | Primer sequence (5' to 3') | Position on the gene |
|---|-------------------|----------------------------|----------------------|
| 1 | Pfk13_K1_F outer  | CGGAGTGACCAAATCTGGGA       | 65-84                |
| 2 | Pfk13_K4_R outer  | GGGAATCTGGTGGTAACAGC       | 2161-2142            |
| 3 | Pfk13_K2_F nested | GCCAAGCTGCCATTTCATTTG      | 1279-1298            |
| 4 | Pfk13_K3_R nested | TGCAGCAGGAAATACAACAGC      | 2127-2108            |

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

### Restriction Digestion of *Pfmdr1* with *ApoI* and *Afl III*

Nested PCR, as reported previously, was performed to amplify codon 86 of *Pfmdr1* (Ranjitkar S, 2011). 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'ATAAACCTAAAAAGGAAGTGG3' were used to amplify the PCR product in nest2 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 (Snounou G., 2002). In brief, 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 MgCl<sub>2</sub> and 125 µM of each dNTPs and 0.4 units Taq DNA polymerase. The cyclic conditions in the thermocycler, for initial *msp-1* and *msp-2* PCR were as follows: 5 min at 95°C, followed by 30 cycles for 1 min at 94°C, 2 min at 58°C and 2 min at 72°C and final extension of 10 min at 72°C. For *msp-1* and *msp-2* nested PCR, conditions were as follows: 5 min at 95 °C, followed by 30 cycles for 1 min at 95°C, 2 min at 61°C and 2 min at 72°C and final extension of 5 min at 72°C. The allelic specific positive control 3D7 and DNA free negative controls were included in each set of reactions (Gosi P, 2013). Fragment analysis of *msp-1*, *msp-2* and *glurp* 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 (Kiwuwa MS, 2013). Alleles in each family were considered the same if fragment sizes were within 20 bp intervals for *msp-1* and *msp-2* genes (Mayengue PL, 2011),

## Treatment outcome measures end points

The primary endpoints were PCR-corrected 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 shows 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, Fever Clearance Time (FCT), defined as the time (hrs) from the start of a patient's treatment to the first consecutive axillary temp measurements below 37.5 for at least 48 hrs, 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). Gametocyte carrier rates and geometric mean densities (excluding negatives) were compared on days 7, 14 and 28. Changes of hemoglobin (Hb) concentration from day 0 to day 28, and Adverse events, vital signs were monitored and changes assessed.

## Climate data

Weather data for the study area was obtained from Climate Engine (<http://climateengine.org>).

**Data management and analysis**

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

In the lab, the K13 sequencing data was managed as we have previously described (Musyoka K., 2020) and this data shall be reported elsewhere. The *pfmdr* digests were reported as either wild type, mutant or mixed infection. The *msh-1*, *msh-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 *msh-1* or *msh-2*, by the number of samples in the same marker.

**Results**

**Mosquito presence, species distribution and parasite infection**

Only two adult Anopheles mosquitoes were collected indoors from all the 30 houses that were visited. These two mosquitoes were collected using CDC light traps from a single house in Kanyethi village and were non-blood fed. The mosquitoes were identified as belonging to the *Anopheles 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, a total of 148 female mosquitoes were successfully reared into adults and a rich repertoire of Anopheles species was identified by morphology (Figure 4). Of the 10 mosquitoes identified as *Anopheles gambiae* s.l. nine were *Anopheles arabiensis* while only one was *Anopheles gambiae* s.s. All 17 *An. funestus* group of mosquitoes successfully identified by PCR were *Anopheles funestus* s.s.

**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 positives consented to participate in the trial (**Figure 4**). All the recruited patients received at least a dose of the study drug and were thus included in the intention to treat analysis. In Lusegeti, data on history of travel was recorded with 5 (50%) of the patients reporting a recent travel to a malaria prone zone.

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

|   | ITT population            | PP population           |
|---|---------------------------|-------------------------|
| <b>Characteristic</b>                         | 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]        |
| Recruitment Temp, ± SD                        | 11.35189                  | 13.4091712              |
| Fever (recruitment temp >37.5 <sup>0</sup> c) | 26/41(63.41%)             | 16/28(57.14%)           |
| Parasite counts, median (IQR)[Q1,Q3]          | 8264.0(21912)[4870-26782] | 8272(18488)[3602-22090] |
| Gametocyte present, N (%)                     | 1/41(2.44%)               | 1/28(3.57%)             |
| <b>Sites</b>                                  |                           |                         |
| 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<br>N=10 | Gikambura<br>N=31 | Difference, %<br>(95% CI) | P-value |
| <b>Day 28</b>                                       |                  |                   |                           |         |
| Non-corrected ACPR                                  | 10(100%)         | 31(100%)          | 0                         | -       |
| PCR-corrected ACPR                                  | 10(100%)         | 31(100%)          | 0                         | -       |
| <b>Day 14</b>                                       |                  |                   |                           |         |
| Non-corrected ACPR                                  | 10(100%)         | 31(100%)          | 0                         | -       |
| PCR-corrected ACPR                                  | 10(100%)         | 31(100%)          | 0                         | -       |
| ACPR-adequate clinical and parasitological response |                  |                   |                           |         |

Table 4: Secondary outcomes

|   | Intention to treat analysis (N=41) |                   |             | Per protocol analysis (n=28) |                   |                |
|---|------------------------------------|-------------------|-------------|------------------------------|-------------------|----------------|
|   | Lusigeti<br>N=10                   | Gikambura<br>N=31 | Totals      | Lusigeti<br>N=6              | Gikambura<br>N=22 | Totals         |
| <b>Treatment failure -N (%)</b>           |                                    |                   |             |                              |                   |                |
| Early treatment failure                   | 0(0%)                              | 0(0%)             | 0(0%)       | 0(0%)                        | 0(0%)             | 0(0%)          |
| Late treatment failure                    | 0(0%)                              | 0(0%)             | 0(0%)       | 0(0%)                        | 0(0%)             | 0(0%)          |
| Fever clearance, median time in hrs (IQR) | ND                                 | ND                | -           |                              |                   |                |
| All participants in the study             | 10/10                              | 31/31             | 41/41       | 6/6                          | 22/22             | 28/28          |
| Participants who cleared day 2 and beyond | 0/10(0%)                           | 3/31(9.68%)       | 3/41(7.32%) | 0/6                          | 2/22<br>(9.1%)    | 2/28<br>(7.1%) |
| Recurrent parasitaemia – N (%)            | 0                                  | 0                 | 0           | 0                            | 0                 | 0              |
| Adverse events – N (%)                    | 0                                  | 0                 | 0           | 0                            | 0                 | 0              |
| Adherence to study medication             |                                    |                   |             |                              |                   |                |
| Completed study medication - N (%)        | 10(100)                            | 31(100)           |             | 6(100)                       | 22(100)           |                |

## Genetic analysis and molecular characteristics of the study populations

### Allelic diversity of *msp-1*, *msp-2* the population

Successful amplification occurred in 90.0% (36/40) of samples for *msp-1* (28/31 in Gikambura and 8/9 in Lusigeti) and 87.5% (35/40) for *msp-2* (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%) microscopy positive samples were actually negative, 1 in Lusigeti and 3 from Gikambura.

Overall, the three allelic families (K1, MAD20 and RO33) of *Msp1* gene and two (3D7 and FC27) of *Msp2* gene were observed in this study. A total of forty nine alleles types were detected for the two genes in all localities: twenty seven for *Msp1* (Fig 1) and twenty two for *msp2* (Fig 3).

Allele genotyping demonstrated the highly polymorphic nature of *P. falciparum* in Kikuyu isolates with respect to *msp-1* and *msp-2*. Among *msp-1* isolates, eleven K1 (180–300bp), nine MAD20 (100–250bp) and seven RO33 (100–230bp) allelic families were noted. The frequency of samples with only K1, MAD20, and RO33 were 22.9, 0 and 0%, respectively. The remaining 77.1% (27/35) were polyclonal infections. Among polyclonal infections carrying two allelic types, the frequency 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.

Among *msp-2* isolates, eleven FC27 (250-800 bp) and eleven IC1/3D7 (100–600 bp) allelic families were noted. The frequency of samples with only FC 27 and IC1/3D7 were 28.6 and 8.6%, respectively. The remaining 57.1% (20/35) were polyclonal FC27-IC1/3D7infections. Table - below,

Table 4: Genetic diversity of *Plasmodium falciparum* *msp1* and *msp2*

| Gene              | Allele        | Positive (%-n/N) | Fragment Size (bp) | No of Alleles |
|-------------------|---------------|------------------|--------------------|---------------|
| <i>Msp1</i> *N=35 | MAD20         | 21(60)           | 100-250            | 9             |
|                   | K1            | 32(91.4)         | 180-300            | 11            |
|                   | RO33          | 14(40)           | 100-250            | 7             |
|                   | MAD20/K1      | 11(31.4)         | -                  | -             |
|                   | MAD20/RO33    | 2(5.7)           | -                  | -             |
|                   | K1/RO33       | 4(11.4)          | -                  | -             |
|                   | MAD20/K1/RO33 | 9(25.7)          | -                  | -             |
| <i>Msp2</i>       | FC27          | 30(85.7)         | 250-800            | 11            |
|                   | IC1/3D7       | 23(65.7)         | 100-600            | 11            |
|                   | FC27/ IC1/3D7 | 20(57.2)         | -                  | -             |

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

## Multiplicity of infection (MOI)

In total, zero and 65.7% (23/35) of the isolates contained multiple infections in *msp1* and *msp2*, respectively. The MOIs for both *msp1* and *msp2* are summarized in Table 5 below. MOI for *msp2* was 2.2 (1.87-2.23) with a range of 1–6 strains, and was higher than MOI for *msp1*, i.e. 1.0 with only 1 strains.

Table 5: Multiplicity of infections for *msh-1* and *msh-2* genes in study isolates

| Gene | MOI             | Monoclonal infection % (n/N) | Polyclonal Infection % (n/N) |
|------|-----------------|------------------------------|------------------------------|
| Msh1 | 1.0 (1.0-1.0)   | 97.1 (34/35)                 | 0 ( 0/35)                    |
| Msh2 | 2.2 (1.87-2.23) | 34.3 (12/35)                 | 65.7 (23/35)                 |

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

PCR amplification and DNA sequencing of the *PfK13* gene to observe the common SNPs associated with artemisinin resistance revealed that all *P. falciparum* isolates carried the wildtype allele.

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* marker.

### Climate patterns

Figure 6 shows total annual rainfall for the 60 year period from 1961-2020. Annual precipitation ranged from 488mm – 1494mm. When data was averaged over ten-year periods from 1961 to 2020 in order to understand long-term patterns, one-way ANOVA revealed that differences in precipitation were not statistically significant ( $F(5, 54) = 1.29$ ,  $p = 0.28$ ). Mean minimum and maximum temperatures for the 60 year period from 1961-2020 are shown in Table 6. Mean annual minimum temperatures ranged from 9.9°C - 12.5°C while mean annual maximum temperatures ranged from 21.1°C - 24.1°C over the 60 year period. There were significant differences in the mean minimum and maximum temperatures averaged over ten-year periods from 1961 -2020 (One-way ANOVA  $F(5,54) = 27.02$ ,  $p = 0.00$  and  $F(5,54) = 16.60$ ,  $p = 0.00$ ; respectively).

Table 6 Mean Maximum and minimum temperature in the study area.

**Annual Mean Minimum Temperature**

| Period/Year | 1961-1970   | 1971-1980   | 1981-1990   | 1991-2000   | 2001-2010   | 2011-2020   |
|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Year 1      | 11.2        | 9.9         | 10.7        | 10.5        | 11.5        | 11.8        |
| Year 2      | 10.6        | 10.7        | 10.8        | 10.9        | 12.5        | 11.6        |
| Year 3      | 10.5        | 10.7        | 11.1        | 10.5        | 11.8        | 11.4        |
| Year 4      | 10.2        | 10.0        | 10.4        | 10.7        | 11.8        | 12.0        |
| Year 5      | 10.3        | 10.3        | 10.6        | 11.1        | 11.5        | 11.9        |
| Year 6      | 10.8        | 10.5        | 10.8        | 11.0        | 12.0        | 11.8        |
| Year 7      | 10.6        | 10.7        | 11.2        | 11.3        | 11.5        | 12.5        |
| Year 8      | 9.9         | 10.4        | 11.1        | 11.4        | 11.2        | 10.9        |
| Year 9      | 10.4        | 10.9        | 10.6        | 11.3        | 12.0        | 12.4        |
| Year 10     | 10.7        | 10.8        | 10.9        | 11.2        | 12.0        | 11.5        |
| <b>MEAN</b> | <b>10.5</b> | <b>10.5</b> | <b>10.8</b> | <b>11.0</b> | <b>11.8</b> | <b>11.8</b> |
| <b>SD</b>   | <b>0.1</b>  | <b>0.1</b>  | <b>0.1</b>  | <b>0.1</b>  | <b>0.1</b>  | <b>0.1</b>  |

**Annual Mean Maximum Temperature**

| Period/Year | 1961-1970   | 1971-1980   | 1981-1990   | 1991-2000   | 2001-2010   | 2011-2020   |
|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Year 1      | 22.3        | 21.8        | 22.4        | 22.8        | 22.8        | 23.3        |
| Year 2      | 22.1        | 22.1        | 22.4        | 22.3        | 23.1        | 23.2        |
| Year 3      | 21.8        | 22.7        | 22.8        | 22.4        | 23.7        | 23.1        |
| Year 4      | 21.9        | 22.1        | 22.1        | 22.2        | 23.0        | 23.2        |
| Year 5      | 22.1        | 22.2        | 21.9        | 22.7        | 23.5        | 23.2        |
| Year 6      | 22.2        | 22.6        | 22.3        | 22.8        | 23.0        | 23.5        |
| Year 7      | 21.8        | 22.2        | 23.1        | 22.6        | 23.1        | 24.1        |
| Year 8      | 21.1        | 22.0        | 22.8        | 22.5        | 22.9        | 21.7        |
| Year 9      | 22.3        | 22.0        | 21.8        | 22.8        | 23.7        | 24.0        |
| Year 10     | 22.0        | 22.4        | 22.1        | 23.1        | 23.4        | 23.0        |
| <b>MEAN</b> | <b>22.0</b> | <b>22.2</b> | <b>22.4</b> | <b>22.6</b> | <b>23.2</b> | <b>23.2</b> |
| <b>SD</b>   | <b>0.1</b>  | <b>0.1</b>  | <b>0.1</b>  | <b>0.1</b>  | <b>0.1</b>  | <b>0.2</b>  |

## 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. We therefore 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 of vectors of malaria in close proximity to human dwelling may be potent for the establishment of an active malaria transmission cycle in the area in the face of climate change and in view of the presence of clinical disease. Although the long-term pattern of annual rainfall did not differ significantly over the 60 year period analyzed, we found statistically significant increases in both average minimum and maximum temperature over the period. Increased temperatures affect malaria transmission in various ways including increasing the reproduction rates and shortening parasite incubation periods within vectors as well as effecting mosquito biting rates, resting and mating behaviors and dispersal, which increase transmission (Martens *et al.* 1995). In Kenya, studies found that the increase of malaria outbreaks from 3 to 13 districts in western Kenya for example occurred at a time when the mean monthly maximum temperatures in that region increased by 2°C (Githeko *et al.* 2000). More recent studies have also revealed temperature-related abundance of mosquitoes and shown the impact of climate extremes on mosquito-borne disease transmission (Lim *et al.*, 2021; Nosrat *et al.* 2021). In our analysis, we found an increase of about 1.3°C in both the mean minimum and maximum temperatures over the 60 year period analyzed consistent with forecasts from sophisticated climate modeling which indicate that the observed trends in global warming will continue in the foreseeable future and that average global temperatures will have risen by 1.0–3.5°C by 2100 (IPCC, 2001; Watson *et al.*, 1998).

For the duration of the study, 41 plasmodium infection cases, representing a 5.6% infection rate, 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% among the suspected cases cannot be ignored. The question that follows is whether the identified parasite population is locally established or imported and spontaneous. Of the 41 participants with plasmodium infections, only 5 (representing 12.2%) reported a history of travel to a malaria endemic destination with the previous 30 days before testing. This suggested an emerging malaria transmission scenario requiring further detailed investigation. The circulating parasite strains showed full sensitivity to the available treatment option with no observed delayed parasite clearance, parasitemia at Day 3, indicating no risk of antimalarial drug resistance was observed.

Overall, the three allelic families (K1, MAD20 and RO33) of *Msp1* gene and two (3D7 and FC27) of *Msp2* gene were observed in this study. A total of forty nine alleles types were detected for the two genes in all localities: twenty seven for *Msp1* (Fig. 1) and twenty two for *msp2* (Fig. 3).

Allele genotyping demonstrated the highly polymorphic nature of *P. falciparum* in Kikuyu isolates with respect to *msp-1* and *msp-2*. Among *msp-1* isolates, 77.1% (27/35) were polyclonal infections. Among polyclonal infections carrying two allelic types, the frequency 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. Among *msp-2* isolates, 57.1% (20/35) were polyclonal FC27-IC1/3D7 infections. 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.

MOI is an indicator of malaria transmission level because it has been shown to be higher in high malaria transmission areas and decreased when transmission is low. In total, zero and 65.7% (23/35) of the isolates contained multiple infections in *msp1* and *msp2*, respectively. MOI for *msp2* was 2.2 (1.87–2.23) and the MOI for *msp1* was 1.0, an indication of very low transmission in this area.

The main limitation of this study was the use of *msp* genotyping which, as others marker based on DNA fragment size, could reduce the genetic diversity evaluation of the parasites strains. Nevertheless, *msp1* and *msp2* genes are robust polymorphism markers and can be used successfully to characterize genetic *P. falciparum* strains populations.

## Conclusion

Although the positivity rate observed in the study site was very low, 87.8% of participants who tested positive did not report history of travel from the area 30 days prior to testing positive. This coupled with the finding of highly competent known vectors of malaria in the area 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 in the area will help 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 of parasites to available treatment options is good news from a malaria management perspective.

## Declarations

### Ethical Approval

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.

### **Authors' 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.

### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Authors affiliations**

All the authors are affiliated to Centre for Biotechnology Research and Development (CBRD) at the Kenya Medical Research Institute (KEMRI), Nairobi Kenya, as Research scientists or student Research assistants.

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### **Competing interests**

The authors declare that they have no competing interests.

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## Figures





Figure 2

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

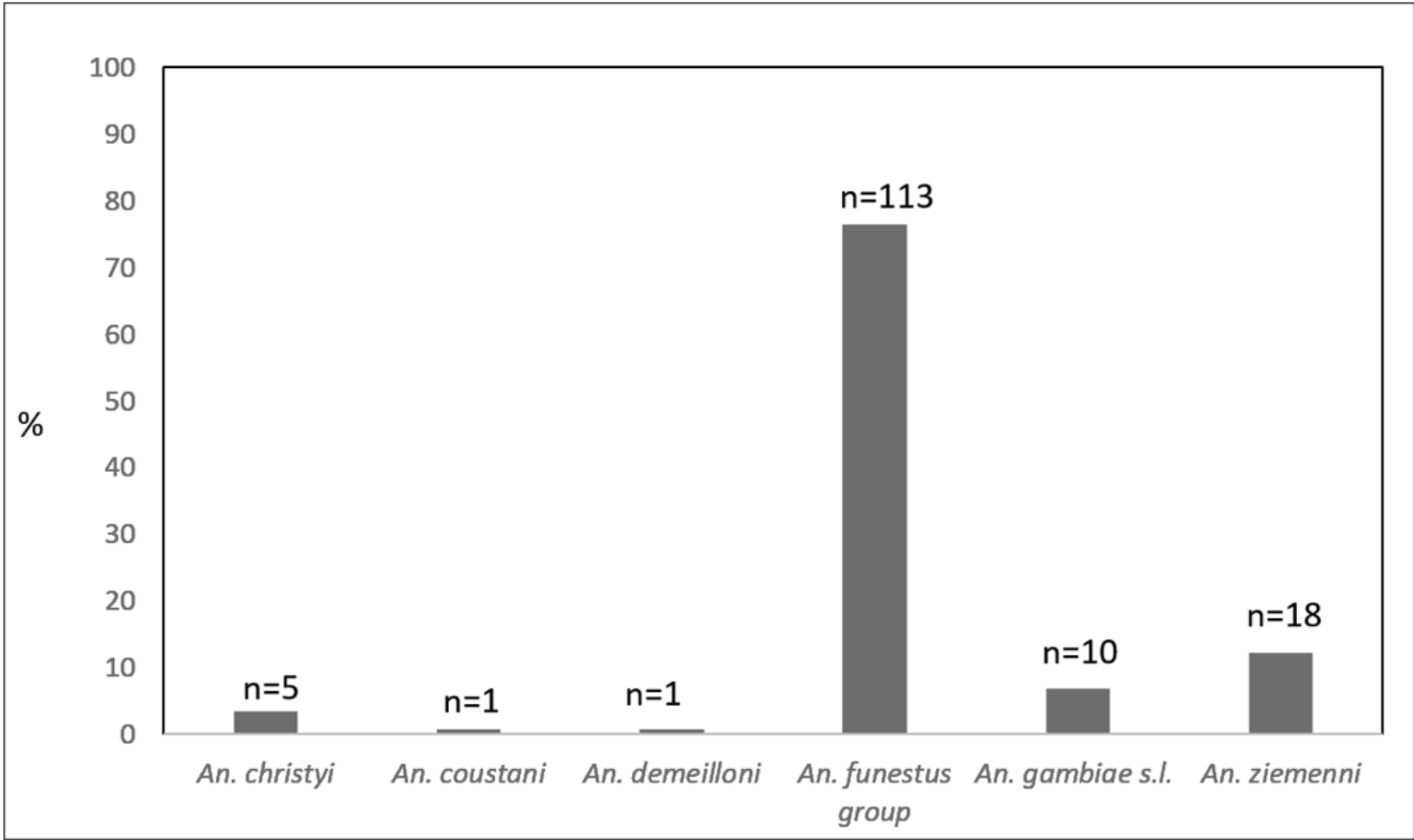
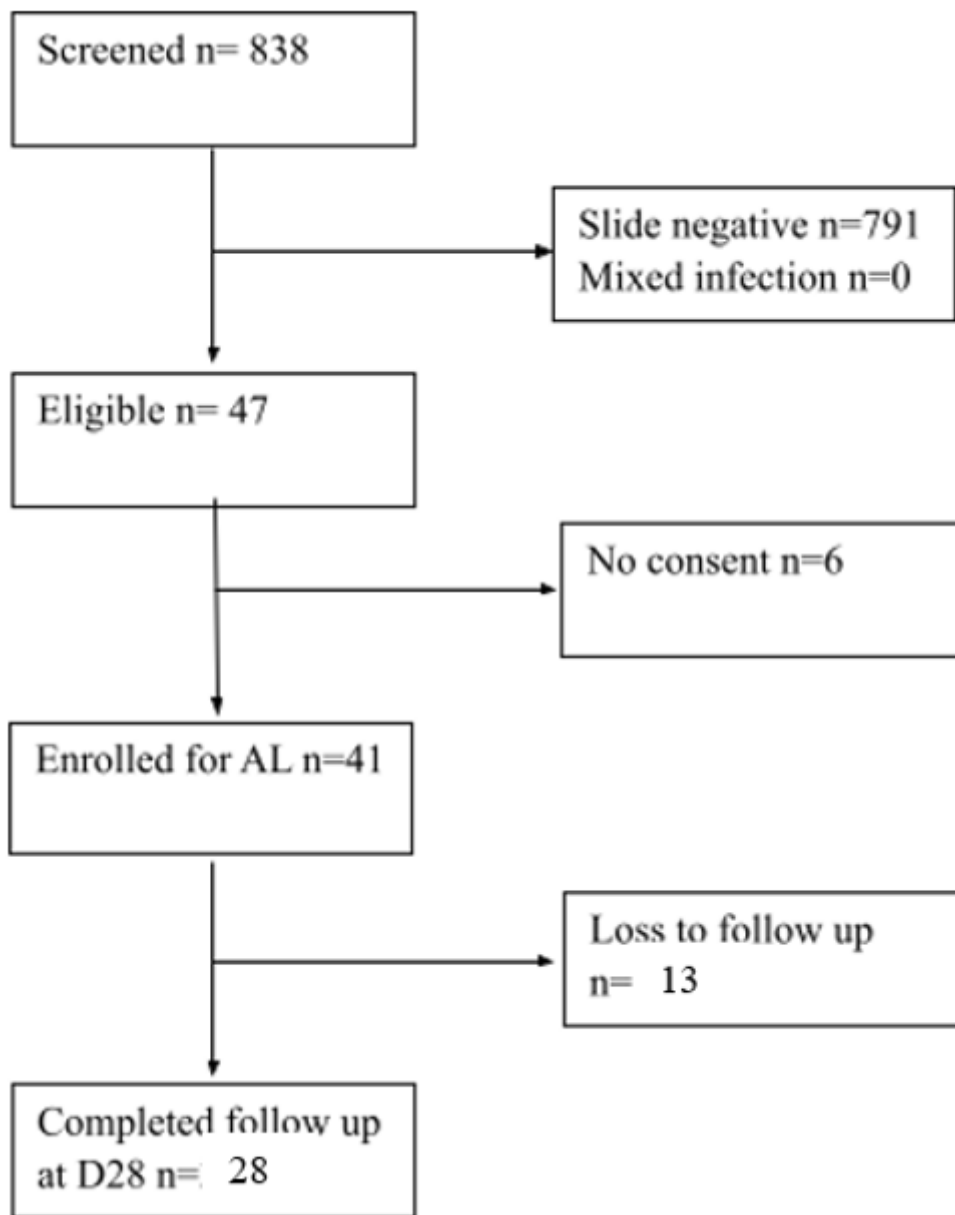


Figure 3

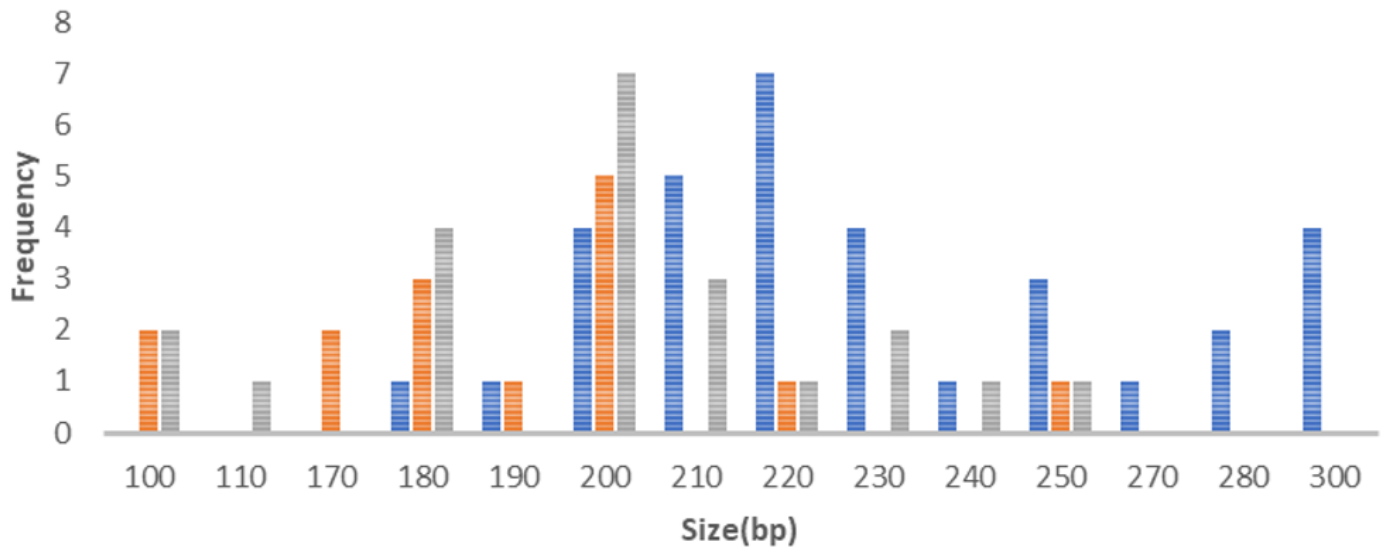


**Figure 4**

*Study participant recruitment and follow up flowchart*

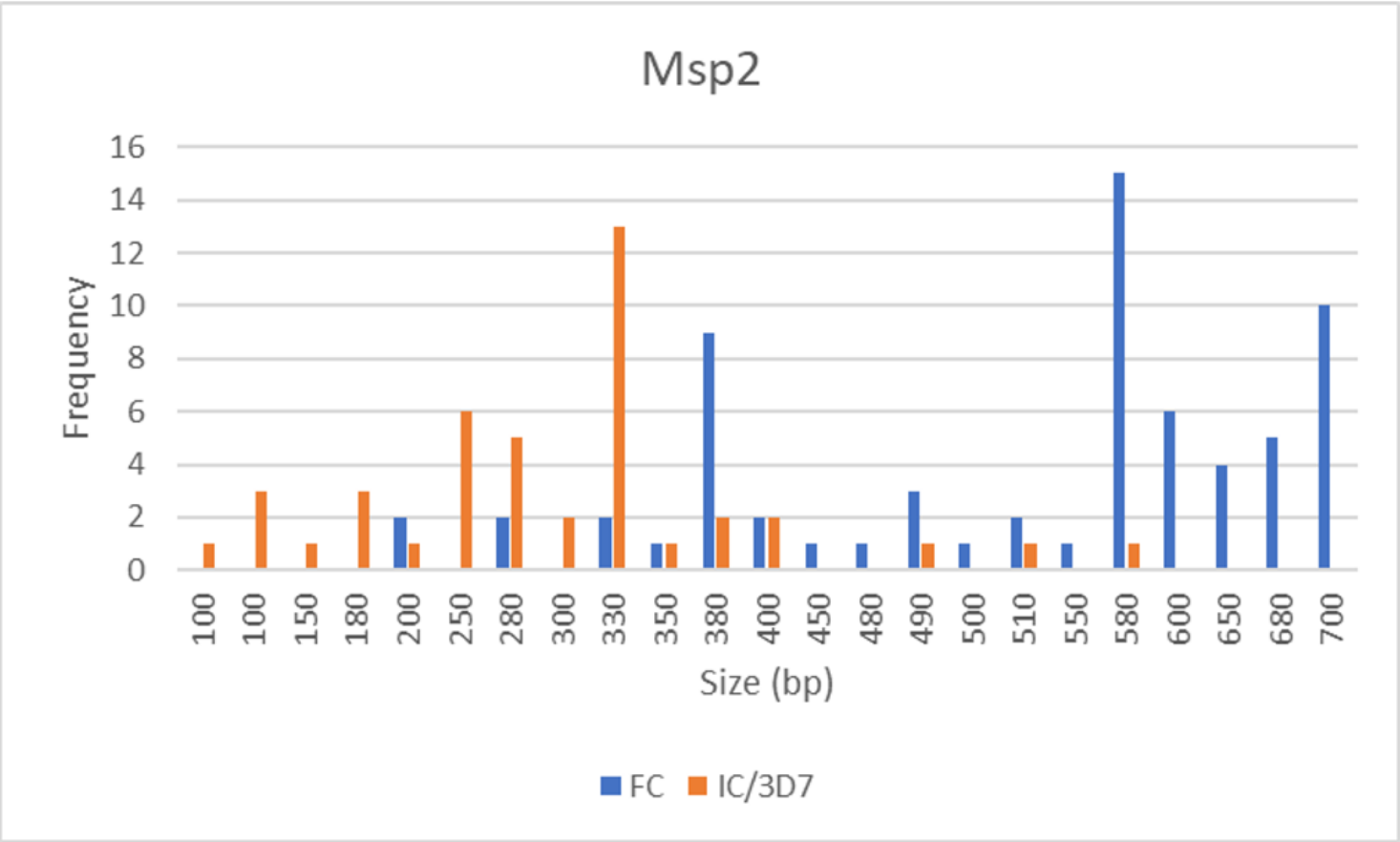
# MSP 1

■ K1 ■ R033 ■ MAD20

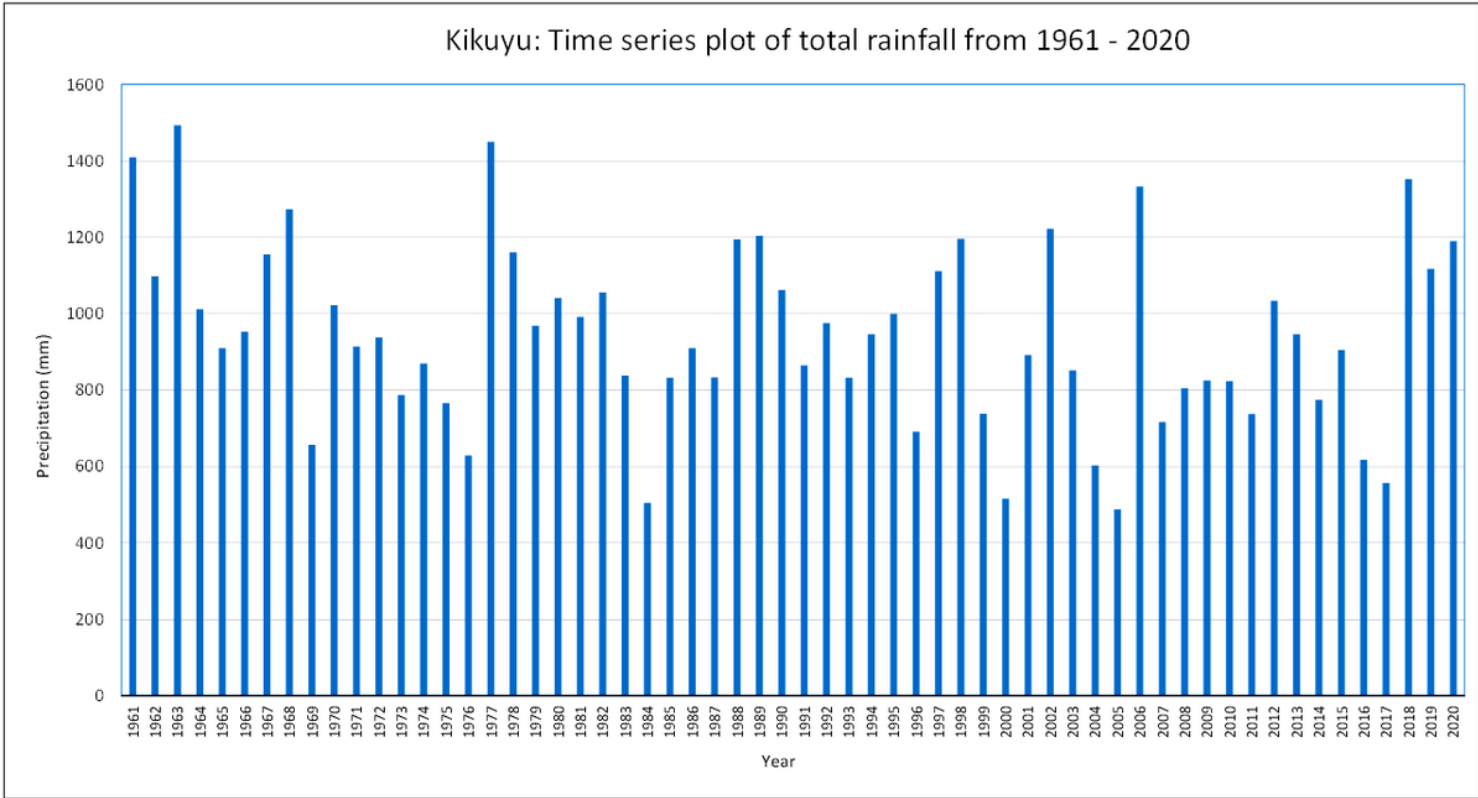


**Figure 5**

*Frequency distribution of msp-1 allelic families*



**Figure 6**  
*Frequency distribution of msp-2 allelic families*



## Figure 7

*total annual rainfall for the 60 year period from 1961-2020.*