Original Research Article

Genetic polymorphism of Merozoite Surface Protein-2 (MSP-2) in *Plasmodium falciparum* isolates from endemic Sub-Counties in Kisii County, Kenya.

ABSTRACT

Background

Despite a lot of efforts being put in place globally to reduce malaria burden, malaria is still one of the major public health problems. Msp2 gene *P. falciparum* analysis gives paramount evidence in the evaluation of malaria epidemiology to assist in the ongoing malaria control and elimination programs. Therefore this study aimed at examining genetic polymorphism of msp-2 gene of *P. falciparum* and its multiplicity of infection (MOI) from endemic Sub-Counties in Kisii County, Kenya.

Methodology

Dried blood spots were collected from 249 study participants who were positive for *P. falciparum* parasite from January to April 2021. Parasites density/µl of blood was calculated by multiplying the number of parasites counted and 8000 white cells/µl then dividing by number of white cells counted. Parasite level was considered low if the parasite/µl of blood was < 500, moderate 501 ≤ 5000 and high if it was > 5000 Parasites DNA was extracted using chelex-100® method and nested PCR for amplification followed by fragment analysis using gel electrophoresis.

Results

246 samples were identified with msp2 alleles where 94% belonged to 3D7 and 83% belonged to FC27. 40 different polymorphic alleles were identified where 13 belonged to FC27 and 27 belonging to 3D7. 86% isolates had multiple msp2 alleles where the Multiplicity of infection (MOI) overall mean was 2.75.

Conclusion

The study isolates showed high genetic polymorphism. Therefore, this suggests that that there is high local malaria transmission, meaning there is failure to the scale up of malaria control interventions in the endemic areas of the County.

Key words: Malaria, alleles, 3D7, FC27, Multiplicity of infection.

INTRODUCTION

According to World malaria report [1] there was 241 million cases of malaria worldwide in 2020 compared to 227 million cases in 2019. The estimated number of malaria deaths stood at 627 000 in 2020 which is an increment of 69 000 deaths over the previous year. Globally, the malaria mortality rate rose from 13 deaths per 100 000 in 2019 to 15 deaths per 100 000 in 2020 [1].

Malaria deaths in the WHO African Region rose from 534 000 in 2019 to 602 000 in 2020 while the mortality rate increase from 56 per 100 000 population at risk in 2019, to 62 per 100 000 population at risk in 2020 [1].

In 2019, the most affected region in Africa by malaria was Sub-Saharan Africa, having 19 countries accounting for 85% worldwide malaria burden [2]. Kenya is among the Sub-Saharan countries with high cases of malaria prevalence accounting for 30% of all hospital attendance [3]. Countries surrounding coastal and Lake Victoria regions specifically the Western part of Kenya records the highest cases of malaria transmissions countrywide [4]. The level of transmissions in these parts is enormous with yearly entomological inoculation rates (EIR) of 30 to 100 infectious bites per person [5].

A lot of efforts have been put forward to eliminate malaria completely all over the world. However, the major problems which limit national effort to combat malaria are challenges to the implementation of major intervention strategies for malaria control [6]. In endemic region, malaria infection is characterized by having higher genetic diversity, which is implicated in its evolutionary fitness, and consequently having more survival advantage by overcoming the control efforts [7]. Genetic diversity is also an indicator of the intensity of transmission [8], and potential deficiencies in malaria control programs [9].

The common technique molecularly used for characterization of polymorphism in *P.falciparum* infection is the nested amplification of merozoite surface proteins (msp) like block 3 regions of msp-2 gene located on chromosome 2 [10]. It is made up of five blocks with the central block (block 3) being highly polymorphic [11]. Msp-2 is a highly polymorphic antigenic marker that has been used widely in description of the

P.falciparum populations, hence used as an informative marker for strain differentiation [12]. Msp2 gene exists in two main allelic families, FC27 and 3D7 [13]. Studies dealing with molecular investigation of *P.falciparum* genetic diversity its multiplicity of infection (MOI) could be used to describe the level of malaria transmission and the function of the targeted interventions [14].

Therefore to achieve successful malaria elimination, information on the genetic profile of the parasite population in different geographical area, and factors that determine gene flow between locations is of importance. Hence this study aims at examining genetic polymorphism of msp-2 gene of *P. falciparum* and its multiplicity of infection (MOI) from endemic Sub-Counties in Kisii County.

METHODOLOGY

Study Site

The study was conducted in rural areas of Kisii County where study subjects were drawn from Bonchari, Kitutu Chache North and South Mugirango sub-counties of Kisii County.

Study Design

Experimental study design was used.

Study population

Patients of both gender and all age groups who attended health centers with signs of malaria were the target population, excluding malaria referral positive cases from other hospitals within and outside the sub-counties.

Sample Size Determination

Sample size was determined using the formula of Daniel *et al.,* [15];

$$n = \frac{z^2 P(I-P)}{d^2}$$

n represents desired sample size in which the population > 10,000

z represents the standard normal deviate at the required confidence level.

P represents the proportion in the target population estimated to have adequate knowledge and practices on malaria control. From the fisher recommendation 50% will be used where there is no estimates available of the proportion in the target population assume to have characteristic of interest.

q=1-p =0.5

d= the level of statistics significance =0.05

$n = \frac{(1.96)^2 (0.50) (0.5)}{(0.05)^2}$ =384 malaria cases

Sampling Technique

Snowball sampling technique was employed whereby the researcher was referred to malaria endemic Sub-County hospitals from the Kisii Teaching and referral hospital. From Sub-County hospitals, District Health Information System (DHIS) report was used to obtain the specific hospitals recording high cases of malaria where the researcher was referred to the specific hospitals. In the hospitals under study random sampling method was applied to collect 422 study participants.

Specimen collection

The sampled participants were issued with a voluntary informed consent forms to fill. After which, their blood samples were collected following the established venipuncture protocol and procedures as stated in Laboratory Procedure manual, [16]. These procedures included anchoring the patients' vein by holding their arm and placing a thumb below the venipuncture site where after, the vein is entered swiftly by a sterile 21 gauge needle at an angle of 30^o then 10 ml blood was collected before withdrawing the needle. After needle withdrawal study participants were advised to raise their arm above their head and put more pressure using the other hand with a small size cotton wool on the punctured part. Collected blood was put in ethylene diamine-tetracetic acid (EDTA) blood tubes where each EDTA bottle was coded with a specific number.

Malaria parasitemia testing using light microscopy

All study participants' blood was prepared in duplicates for light microscopy thick and thin blood smears. For thin smears, a drop of blood about a dime was put on the pre-cleaned, labeled slide. Spreader slide was then put at the end of blood drop at an angle of 45^0 for blood to spread on it and push forward

smoothly and rapidly. For thick smear small drop of blood about 1.5cm size of a dime was also put on the pre-cleaned, labeled slide. Spreader slide on one side corner was used to spread the blood in circular. Thin smears were then fixed with absolute methanol for 30 seconds. Smears were then stained with 4% Giemsa's stain and observed according to WHO, [17] standard guidelines. Thick blood smears were used to calculate parasites per microliter of blood (parasitemia level) using the procedure described by [18]. Each film was then examined using oil immersion X100. For thick film to be considered negative at least 100 high power fields were examined.

Parasitemia level and parasite density (Parasites/µl) calculations per positive selected case

Tally counter was used to count the number of parasites in a field. Using the assumed 8000 white blood cells /µL [19], parasite density/µl calculation was done using the formula below;

$$\text{Parasites/µl of blood} = \frac{\text{Number of parasites counted} \times 8000 \text{ white cells/µl}}{\text{Number of white cells counted}}$$

Parasite level was considered low if the parasite/µl of blood was < 500, moderate 501 ≤ 5000 and high if it was > 5000 [20]. Thin film was used to identify the parasite species.

Extraction of DNA from the dried blood samples (DBS)

DNA extraction from DBS was done using chelex-100® (Bio-Rad Laboratories CA) method following the procedure stated by Somé *et al.,* [21] with some modification. Each sample final volume was 200 µl then stored at -20^0.

Plasmodium falciparum allelic typing using msp2 gene

Amplification was done in two steps, first step being primary amplification for the entire polymorphic gene segments while the second one being Nested PCR targeting the allelic type -specific region. For the first amplification reaction (Nested 1/outer PCR), 1 µL of the template DNA extract from the blood samples spotted onto the filter papers was amplified with sterile ultrapure (PCR) water of 24.6 µl, PCR buffer of 3.0 µl, dNTPs of 0.6 µl, two primers S3 and S2 (*3D7*) each 0.3 µl and Taq PCR MasterMix (Aidlab Biotechnologies Co., Ltd., Beijing, China) of 0.2 µl coming up to a total volume of 30 µl per PCR tube. Nested PCR 2 also had a total volume of 30 µL, consisting of 24.6 µL of PCR water, 3.0 µL of PCR

buffer, 0.6 µL of dNTPs, 0.3 µL of S1 primer 0.3 µL of S4 primer, Taq of 0.2 µL and 1 µL of the product of the first amplification reaction which was used as a template DNA. Negative and positive controls to the specific alleles were included in each reaction. Amplification was involved in three stages; first stage of denaturation, second stage being annealing and the third stage being extension. The cycling conditions were done as it appears in Table 1 below. Nested PCR products separated using 1.5 % agarose gel and stained using 2 µL of ethidium bromide were visualized under ultraviolet (UV) trans-illumination and photographed. Estimation of the fragment size was related to a 100bp DNA ladder. *Plasmodium falciparum* infection was categorized into polyclonal infection or monoclonal infection depending on the presence of alleles. Alleles which are within the fragment size of 20 bp in each family were considered the same [22]. Multiplicity of infection (MOI) was calculated by the average number of PCR fragments per infected individual.

Ethical consideration

Ethical approval was granted by the University of Eastern Africa, Baraton while research permit was obtained from the National Commission for Science, Technology and Innovation (NACOSTI). Further, approval to carry out the research was granted by the Kisii County government. Voluntary informed consent was sought from the respondents before participation to the study. Balloon inflation and ball squeezing were used to reduce pain and fear for children below 12 years, teenagers and adults respectively.

Data analysis

All the data was entered into excel where parasitological and demographical data was calculated as proportions and mean $\pm$ standard error (SE).The frequency of *msp2* alleles was calculated as the proportion of the allelic family out of the alleles detected in the isolates. Chi-square (χ^2) tests (SPSS), was used to compare statistically the Multiplicity of infection (MOI) with parasitemia levels/density and age of the study participants, where P-values of $P \leq 0.05$ were considered significant.

RESULTS

Demographical data

Gender of the study participants

Out of the total study participants who were positive for *P. falciparum* 58.23% were female and 41.77% were male (Fig 1).

Age of the study participants

Study participants in the age of 0-5 years and were positive for *P. falciparum* parasite were 18.47% (2.74 ± 0.21) while those with the ages of 6-15 years were 54.22% (9.75 ± 0.26) (Fig 2).

Among 18.47% (2.74 ± 0.21) who were in the age of 0-5 years, 3.21% (2.59 ± 0.63), 1.61% (3.63 ± 0.48), 2.81% (3.45 ± 0.51), 6.83% (2.44 ± 0.47), 0.80% (3.4 ± 0.50) and 3.21% (2.74 ± 0.55) came from Eramba, Sieka, Kiaruta, Nyamagiri, Moticho and Suguta respectively. (Fig 3).

Microscopy clinical characteristics

Out of the 384 study participants, 65% turned out to be positive while 35% turned out negative (Fig 4)

Parasite density (p/µl) and parasitemia levels

P. falciparum parasite density in the samples collected and confirmed positive was ranging between 280-26400 parasites/µl with a geometric mean ± standard error of 10972.14 ± 408.64. Highest parasitaemia was 15298.69 ± 534.52 while the lowest parasitaemia was 435.68 ± 11.40 (Fig 5)

Highest parasitemia was seen among study participants who were between 6 and15 years old. (Table 2).

Out of the total study participants with high parasitemia level 10.84% (18302.63 ± 1461.35), 11.24% (13585.57 ± 1235.79), 11.64% (14397.86 ± 1193.48), 15.66% (15635 ± 1086.40), 5.62% (14003.29 ± 1973.52) and 11.64% (15228.97 ± 1202.98) were from Eramba, Sieka, Kiaruta, Nyamagiri, Moticho and Suguta respectively. (Fig 6).

Alleles frequencies

Plasmodium falciparum msp2 gene allelic diversity

249 samples which were positive for *Plasmodium falciparum* were all genotyped for nested PCR Merozoite Surface Protein-2. Alleles were determined by family type and size. 40 different polymorphic aleles were identified in the study participants where 13 belonged to FC27 ((100-600 bp) (Fig 7) and 27 belonging to 3D7 (100-650 bp) (Fig 8). 98.80% (246) samples were identified with msp2 alleles where

93.50% (230) belonged to 3D7 (Plate 1) and 83.33% (205) belonged to FC27 (Plate 1). Isolates with only FC27 alleles were 5.5% (16) while with only 3D7 alleles were 16.67% (41). Isolates with both 3D7 and FC27 alleles were 76.83% (189). 1.2% (3) samples did not show to have any of the two families' alleles despite several amplifications. 85.94% (214/249) isolates had multiple msp2 alleles where the Multiplicity of infection (MOI) overall mean was 2.75 (2.59 for FC27 and 2.91for 3D7) (Table 3).

Association between MOI and parasite density, age and gender

There was no statistical difference between MOI and parasite density ($X^2 = 3.713$; d.f = 4, P < 0.477) and MOI and age ($X^2 = 7.024$; d.f = 8, P < 0.566) (Table 4).

DISCUSSION

This study found out a higher genetic diversity of *P. falciparum* from the isolated samples. This study was in agreement with the study of Mohammed *et al.,* [23], Mayengue *et al.,* [22] and Soulama *et al.,* [24] which took place in Ethiopia, Congo and Burkina Faso respectively. However, this study was not in consistent with the studies of Mohammed *et al.,* [25] and File *et al.,* [14] which took place in North East Ethiopia, Oromia, Ethiopia respectively. This difference might be brought up by the different study areas, the frequency of MOI and levels of malaria transmissions. In this study *P. falciparum* msp2 genotyping specific allele has proved that there is high diversity of malaria parasite in the County. This means that populations residing in areas with high malaria transmissions automatically harbor multiple malaria parasite strains. The above findings might be a good indicator to elaborate more on the effects of the interventions control in the County.

This study also showed the predominance of 3D7 allelic family when compared to FC27 allelic family. This study has been in concordance with the study of Mohammed *et al.,* [25], File *et al.,* [14] and Mayengue *et al.,* [26] which took place in North East Ethiopia, Oromia, Ethiopia and Congo respectively. Although this study did not agree with the studies of Ogouyèmi-Hounto *et al.,* [27] and Hamid *et al.,* [28] which took place in South of Benin and Central Sudan respectively. This variation may be due to the sample population, transmission intensity and geographical location.

Additionally, this study reported high MOI of 2.75 overall mean like the study of Mohammed *et al.,* [23] in Ethiopia which reported an overall MOI of 2.8 and the study of Mara *et al.,* [29] in western Côte d'Ivoire

which reported an overall mean ranging from 2.32 to 3.42. This study was in agreement with the study of Babiker *et al.,* [30] in Tanzania which reported an increase level of MOI with increase endemicity. Unlike this study, the studies of Mohammed *et al.,* [25] in North East Ethiopia and File *et al.,* [14] in Oromia, Ethiopia reported low MOI of 1.2 and 1.4 respectively. This difference is likely associated with transmission intensity this is because MOI is usually used as a proxy for malaria transmission where areas with high MOI are associated to transmission intensity [31]. This study high MOI suggests high local malaria transmission and failure due to the scale up of malaria control interventions to reduce MOI levels just like the study of Bakhiet *et al.,* [32] which contrasts the efforts of malaria control.

This study showed no association between MOI and age. This means that age has no effect on the episode of malaria infection in the study participants. This study was in consistent with the study of Mohammed *et al.,* [23], Mohammed *et al.,* [25], and Sondo *et al.,* [33] which took place in North West Ethiopia, North East Ethiopia and Burkina Faso. It was in contrast with the study of File *et al.,* [14], Karl *et al.,* [34] and Pinkevych *et al.,* [35] which took place in Oromia, Ethiopia, Madang Papua New Guinea and Coastal Tanzania respectively. This is because age effect on MOI does not go hand in hand with the sporadic immunity acquisition. Although the study of Smith *et al.,* [36] showed that malaria transmission intensity highly affects the influence of age on MOI. Hence this explains that File *et al.,* [14], Karl *et al.,* [34] and Pinkevych *et al.,* [35] are perennial and meso-endemic areas.

Moreover, this study did not show an association between MOI and parasite density. This means increase in age has no correlation with MOI. This study showed that study participants on younger age (0 -15 years) were having high parasite density class compared to study participants from 16 years of age and above (Table 2). This study was in agreement with the study of Ogouyèmi-Hounto *et al.,* [27] and Mayengue *et al.,* [22] unlike the study of Ojurongbe *et al.,* [37]. This variation may be due to immune status being a determining factor.

CONCLUSION

P. falciparum diversity assessment plays a role in the malaria control evaluation. This study isolates showed high genetic polymorphism meaning that three quarter of the isolates had multiple clones. Therefore, this suggests that that there is high local malaria transmission in the endemic areas of the County and this means that there is failure to the scale up of malaria control interventions in the County.

CONSENT

All authors declared that written informed consent was obtained from each study participant. Consent was obtained during questionnaire interviews in the specific hospitals under study and they all declared the report of the study to be published. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Table 1: *Plasmodium falciparum Msp*2 cycling conditions and allelic families used to identify genetic diversity and allele frequencies in parasites isolated from endemic Sub-Counties in Kisii County

Gene	PCR round	Primer name	Sequence	Cycling conditions	Allelic family (annealing temperature
Msp2	Primary (Outer/Nested 1)	S3	5'-ATGAAGGTAATTAAAACATTGTCTATTATA-3')	initial denaturing period of 94°C for 3 min; 30 cycles of 94°C for 25 sec, 42°C for 1 min, 65°C for 2 min; extension period of 72°C for 3 min; 4°C for infinity.	42°C and 65°C
		S2	(3'-ATATGGCAAAAGATAAAACAAGTGTTGCTG-5')		
	Secondary (Inner/Nested 2)	S1 (3D7)	5'-GCAGAAAGTAAGCCTTCTACTGGTGCT-3'	initial denaturing period of 94°C for 3 min; 30 cycles of 94°C for 24 sec; 50°C	50°C and 68°C
		S1	3'-		

	(3D7)	GATTTGTTTCGGCATTATTATGA-5’	for 35 sec, 68°C for 2 min 30 sec; extension	
	S4 (FC27)	5’-GCAAATGAAGGTTCTAATACTAATAG-3’,	period of 72°C for 3 min; 4°C for infinity.	50°C and 68°C
	S4 (FC27)	3’-GCTTTGGGTCCTTCTTCAGTTGATTC-5’		

Table 2: *Plasmodium falciparum* density class stratified per age of the study participants

	Age in years (Percentage)				
Parasite density class	**0-5 (%)**	**6-15 (%)**	**16-30 (%)**	**31-50 (%)**	**51 and above (%)**
low	1(0.4)	7(2.81)	11(4.42)	9(3.61)	6(2.41)
high	43(17.27)	104(41.77)	15(6.02)	3(1.20)	1(0.4)
moderate	2(0.80)	24(9.64)	166.43	6(2.41)	1(0.4)

Table 3: Distribution and mean MOI of *msp2* allelic families of *P. falciparum* isolates from endemic Sub-Counties in Kisii County

Allelic families	**Number of isolated samples (%)**	**Fragment size (bp)**	**Number of alleles**	**Mean MOI ± SE**
Msp2	246 (100)			2.75 ± 0.03
FC27	16 (6.50)	100-600 bp	13	2.59 ± 0.05
3D7	41 (16.67)	100-650 bp	27	2.91 ± 0.03
FC27+3D7	189 (76.83)			
Without msp2	3 (1.2)			

Table 4: Multiplicity of infection and P values of *P.falciparum* stratified by age, gender and parasite density class

	Multiplicity of infection (Percentage)			
Age in years	**<2 (%)**	**12.1-2.9(%)**	**>3.0(%)**	**P value**
0-5	1(0.47)	13(6.07)	25(11.68)	0.566
6-15	5(2.34)	50(23.36)	63(29.44)	
16-30	2(0.93)	18(8.41)	16(7.48)	
31-50	0	5(2.34)	9(4.21)	
51 and above	0	1(0.47)	6(2.80)	
Parasite density class				
low	1(0.47)	16(7.48)	12(5.61)	0.477
high	6(2.80)	55(25.70)	80(37.38)	
moderate	1(0.47)	16(7.48)	27(12.62)	

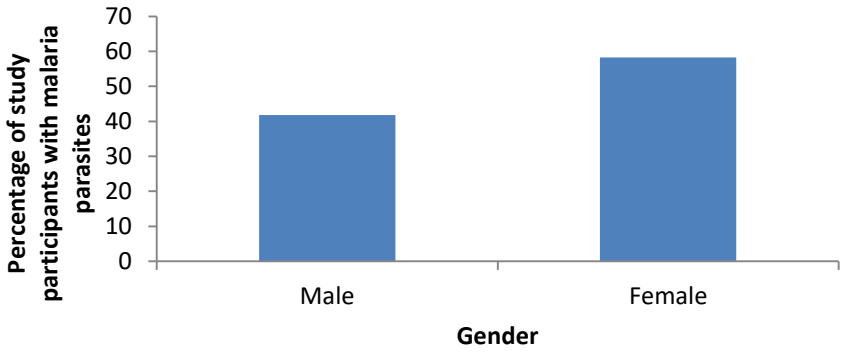


Fig 1: Prevalence of malaria parasites among participants

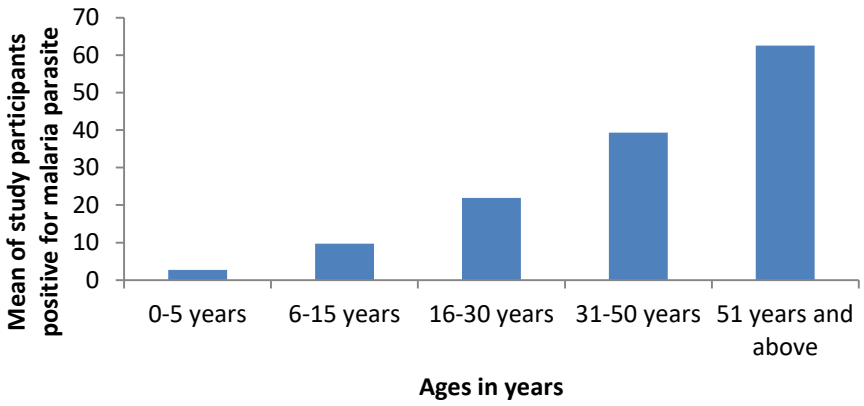


Fig 2: Mean of study participants who were positive for malaria parasite against ages in years

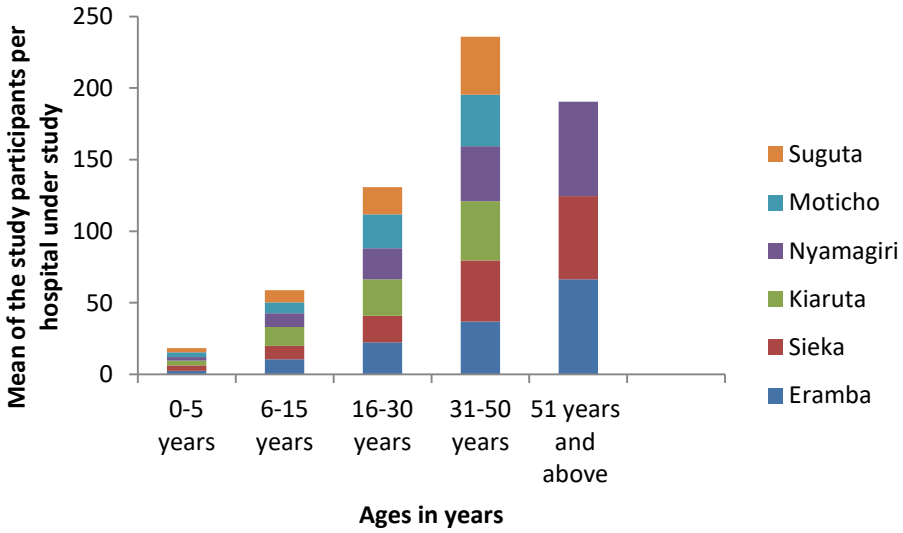


Fig 3: Mean study participants who were positive for malaria parasite per hospital under study against their ages in years

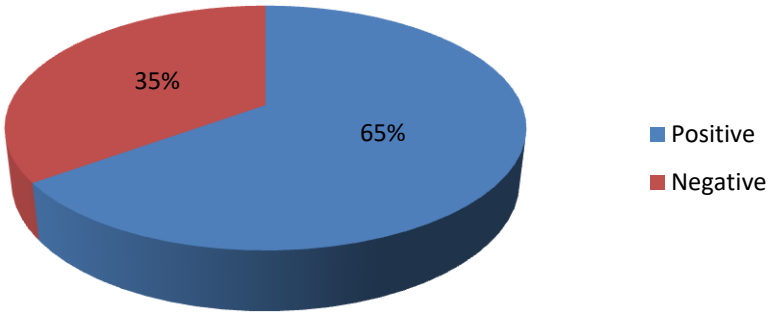


Fig 4: Study samples with and without malaria parasites after microscopy blood test

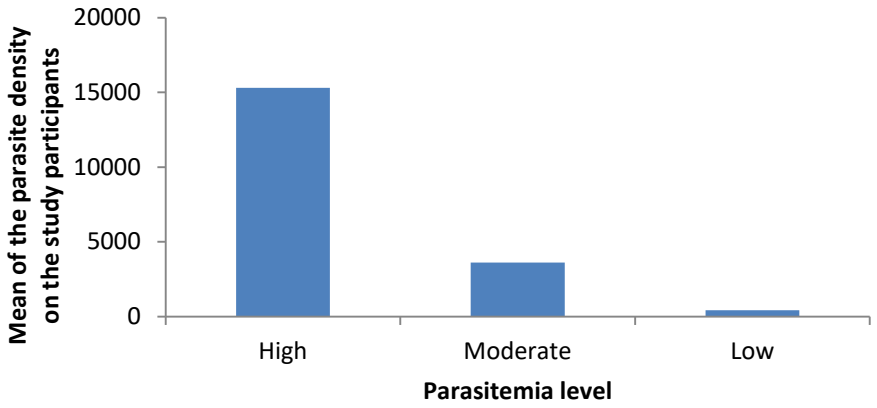


Figure 5: Mean of the parasite density on the study participants against parasitemia level

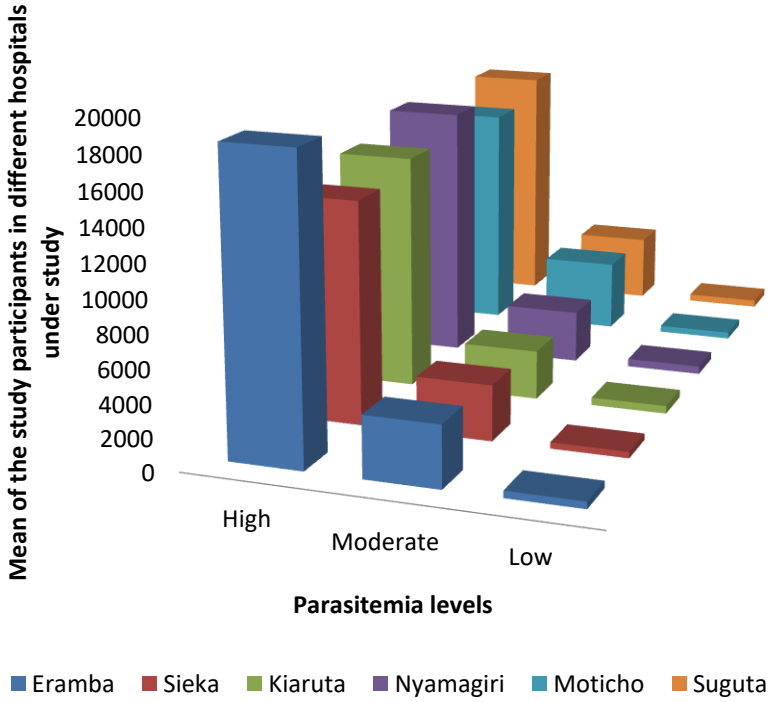


Figure 6: Mean of the study participants in different hospitals under study against parasitemia level

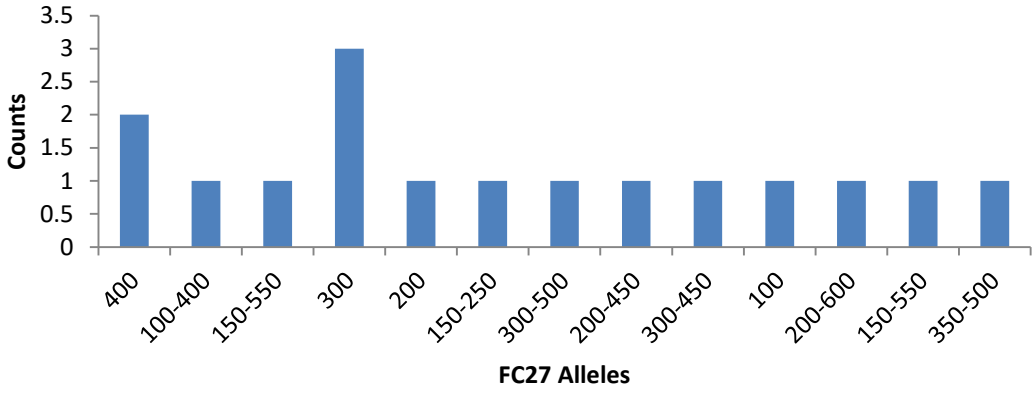


Fig 7: Counts of all isolates with only FC27 alleles only classified according to the size of their amplified fragment

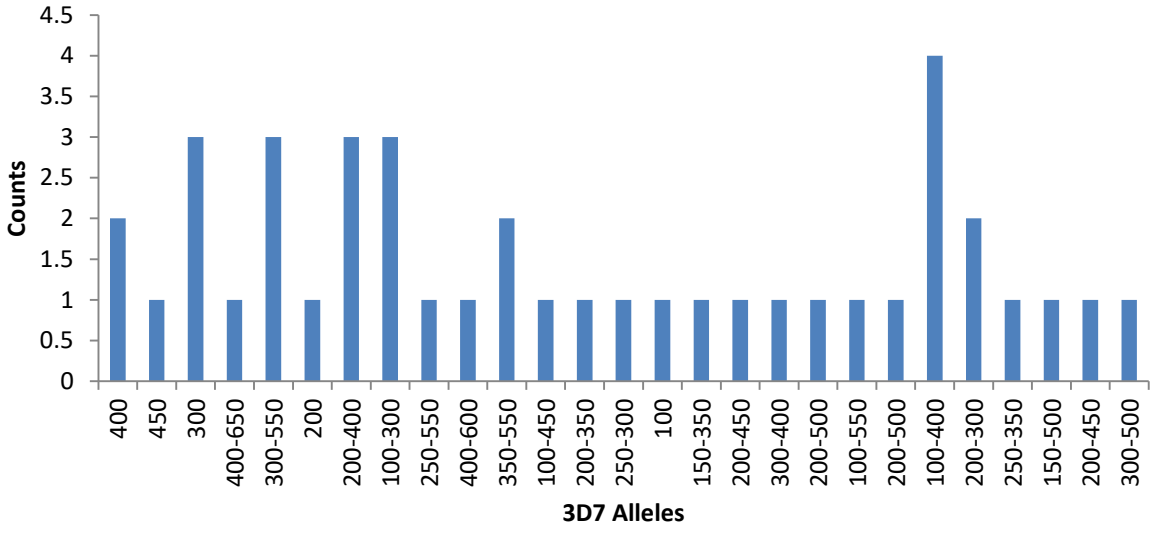
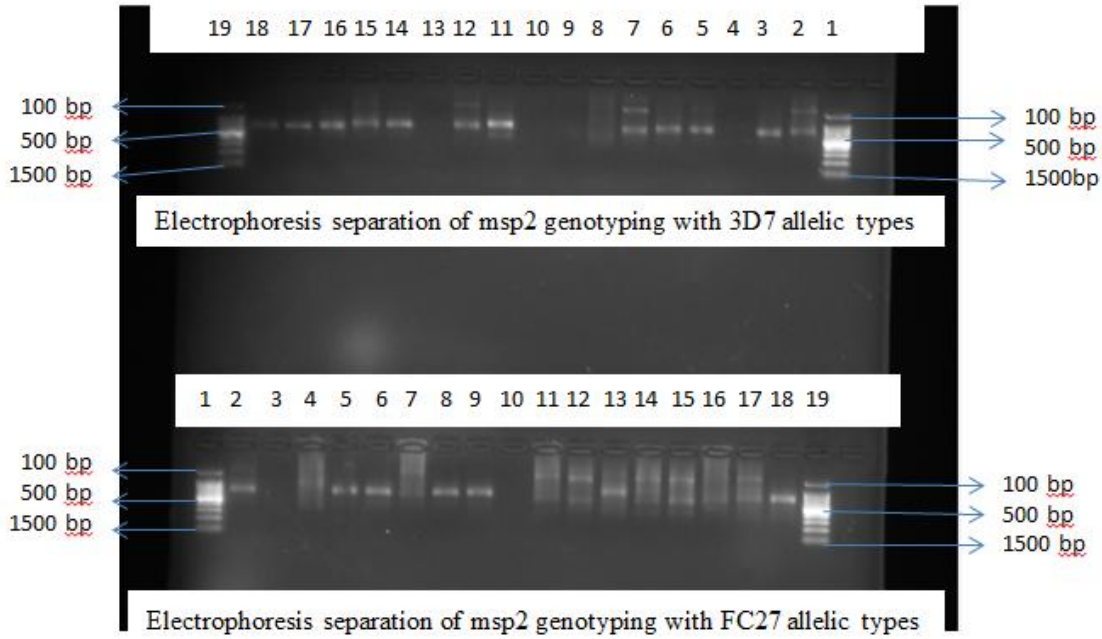


Fig 8: Counts of isolates with 3D7 alleles only classified according to the size of their amplified fragment



3D7 allelic types: Lanes 1 and 19 100-1500 base pairs ladder in 2% agarose gels, lanes 2 (100-400 bp), lane 3,5,6,11,12,14,15 (400 bp), lane 7 (100 -450 bp), lane 16,17 (450bp), lane 18- Positive control, lane 4,8,9,13 negative and lane 10 negative control.

FC27 allelic types: Lanes 1 and 19 100-1500 base pairs ladder in 2% agarose gels, lanes 2,13 (300 bp), lane 5,6,8,9 (400 bp), lane 11,12,14 (100-bp), lane 15,17 (100-500bp), lane 18- Positive control, lane 3,4,7,16 negative and lane 10 negative control.

Plate 1: Electrophoresis separation of msp2 genotyping of 16 isolates with 3D7 allelic types and FC27 allelic types