Genetic diversity and allelic variability of pfmsp1 y pfmsp2 of P. falciparum and its association with syntomatology and antimalarial drugs resistance markers in Yambio county, South Sudan

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Research Article

Keywords:

Posted Date: February 21st, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3959166/v1

Additional Declarations: No competing interests reported.

Abstract

Background

Malaria genetic diversity is an important indicator of malaria transmission. *Pfmsp1* and *pfmsp2*are a frequent molecular epidemiology tool to assess the genetic diversity. This study aims to assess the genetic diversity and the description of multiplicity of infection (MOI) of *P. falciparum* in Yambio County, South Sudan. Additionally, it assesses the association of specific alleles or multiplicity of infection with antimalarial drugs resistance haplotypes and severity of infection, major challenges in malaria control strategies.

Methods

There were collected 446 malaria samples from patients in Yambio county. After *P. falciparum*confirmation, *pfmsp1* and *pfmsp2* allelic families were genotyped. Frequencies of each alleles were described and multiplicity of infection was calculated. The association between MOI and complicated malaria was assessed using U-Mann Whitney test. The Kruskal-Wallis test was used to compare MOI between collection sites, age groups and antimalarial resistance haplotypes.

Results

For *pfmsp1*, monomorphic K1 allele infection was predominant (37.0%) in every location and for *pfmsp2* locus, monomorphic 3D7 was predominant (44.8%). 71.9% of samples were polyclonal infections (overall MOI = 1.96). The high diversity and polyclonal infections are associated with molecular markers of resistance, and high MOI has been related with a lower risk of severity of infections. There was not find evidence of association between a specific allele and an infection trait.

Conclusion

High genetic diversity and high level of polyclonal infections have been found in this study, confirming the general high transmission, and highlighting the need for control measures to be intensified in Yambio county, South Sudan.

Introduction

Malaria remains one of the main global health concerns. It has been estimated that during 2022 malaria caused 608,000 deaths globally[1]. South Sudan is a malaria endemic country, which accounted for 1.3% of the global burden of malaria cases [2]. An increasing national malaria incidence trend has been observed in recent years, which may likely be linked to the long-standing humanitarian crisis, and other factors such as population movements. [3,4].

High levels of genetic diversity of *P. falciparum* are a challenge for malaria control interventions because the efficacy of programmatic strategies is reduced. Genotyping of *P. falciparum* parasites examines the

genetic diversity of circulating parasites, and provides a measure of the multiplicity of infection (MOI), i.e., the number of distinct strains per infection [5,6]. Genetic diversity and MOI are influenced by several factors, including transmission intensity, seasonality and host immunity. Areas with low levels of malaria transmission usually present less genetic diversity and more frequency of monoclonal infections. In contrast, high rates of malaria transmission increase the genetic diversity and the risk of polyclonal infections [7].

In addition, high malaria transmission intensity is associated with the appearance of new parasite strains, as a consequence of recombination and selection of more competent strains [8]. This also applies to the emergence and selection of antimalarial drug resistance mutations and the deletion of the genes encoding proteins detected by rapid diagnostic tests (RDT). MOI also has an important impact on molecular surveillance, because surveillance methods do not always distinguish between strains, giving an incomplete view of the circulating parasite population.

The study of polymorphism in the genes *pfmsp1 and pfmsp2,* encoding *P. falciparum* merozoite surface protein 1 (pfMSP1) and 2 (pfMSP2), is the most frequent molecular epidemiology tool to assess the genetic diversity of the parasite populations [9–11]. MSP1, a surface protein, and MSP2, a glycoprotein, are both blood – stage proteins involved in erythrocyte invasion [12]. These two proteins are also major malaria vaccine targets as they are targeted by the immune system [13].

Pfmsp1 gene, located in chromosome 9, has a highly polymorphic region in its block 2 conformed by three allelic families, K1, MAD20 and RO33. *Pfmsp2* gene, located in chromosome 2, block 3 is the most polymorphic region with two allelic families, FC27 and 3D7 [14].

Genotyping of current genetic structure of *P. falciparum* population could provide important insights to inform the design of malaria control strategies, identifying hot-spots of malaria and the strains circulating in an area [6]. Parasite sub-populations could be related with severity[15,16] or other characteristics such as antimalarial resistance and diagnosis evasion, that could play a key role to estimate the scope of the malaria epidemiological situation.

This study aims to assess the genetic diversity of *P. falciparum* in Yambio County, South Sudan, through the genotyping of *pfmsp1* and *pfmsp2* allelic families and the description of multiplicity of infection (MOI). Additionally, it assesses the association of specific alleles or multiplicity of infection with antimalarial drugs resistance haplotypes and severity of infection, major challenges in malaria control strategies.

Methods

Sample collection and study site

The study was conducted in 8 rural villages and the state hospital in Yambio County, Western Equatoria State, South Sudan, hyperendemic for malaria, with a transmission peak during the rainy season (Figure

1). Sample collection was carried out at the end of the malaria peak during the transmission season in 2019 (January to February). Blood samples were taken on Whatman 903TM paper (GE Healthcare Bio-Sciences Corp.) from patients with symptomatic malaria infections, diagnosed by pLDH-based-RDT (CareStart^{TM}), by finger prick.

Samples were part of a study designed to assess antimalarial resistance to the combination treatment, sulfadoxine-pyrimethamine (SP) + amodiaquine (AQ) (SPAQ), which was being given as Seasonal Malaria Chemoprophylaxis (SMC) [17]. The samples collected for this study were collected from patients with uncomplicated malaria according to the criteria established by WHO, except samples from Yambio hospital that were from patients with severe malaria.

DNA extraction and molecular diagnosis of *Plasmodium sp.*

DNA was extracted from dried dot blood spots using Chelex – Saponin method [18]. To ensure enough DNA 5 mm diameter punch was used. Malaria molecular diagnosis was performed on all samples by nested multiplex PCR to confirm and identify *Plasmodium sp.* infections [19].

Allelic genotyping of *pfmsp1* and *pfmsp2* genes

Amplification of *pfmsp1* and *pfmsp2* genes was carried out by nested PCR as previously described by Huang et al [20]. The allelic families studied for each gene were R033, K1 and MAD20 for *pfmsp1* (block 2) and 3D7 and FC27 for *pfmsp2* (block 3). After the nested PCR, electrophoresis was carried out using 2% agarose gel stained with Pronasafe (Pronadisa SA, Spain). The PCR products were separated by electrophoresis and visualized under ultraviolet (UV) transilluminator (ChemiDoc^{TM} XRS, BioRad). The number and size of each DNA fragment was estimated for further analysis through the software ImageLab (Biorad) [21].

Monoclonal infections were defined by detection of a single PCR fragment for each locus and polyclonal infections were defined as those having more than one fragment for, at least, one locus.

Haplotypes associated with resistance to antimalarial drugs

The single nucleotides polymorphisms (SNPs) located in different genes related with antimalarial drug resistance were studied by Nested PCR as described by Plowe et al., with minor modifications [18]. After Nested PCR, a restriction fragment length polymorphism (RFLP) analysis was performed to determine if there were mutations in the plots of interest. The amplification fragments were digested with different restriction enzymes (New England BiolabsR Inc.) to analyse restriction fragment length polymorphisms (RFLPs). PCR products were separated by electrophoresis on a 2% agarose gel, stained with Pronasafe (Pronadisa, Spain) and visualised under an ultraviolet transilluminator (ChemiDocTM XRS. BioRad).

To study resistance to sulfadoxine and pyrimethamine, SNPs in *pfdhfr* and *pfdhps* genes were studied. The haplotypes were classified according to the number of SNPs as **partially resistant** with four

mutations (*pfdhfr* 51I/59R/108N + *pfdhps* 437G), **fully resistant** with five mutations (*pfdhfr* 51I/59R/108N + *pfdhps* 437G/540E) and **super resistant** with six mutations (*pfdhfr* 51I/59R/108N + *pfdhps* 437G/540E/581G)[22]. To study resistance to chloroquine and amodiaquine, 86 and 1246 SNPs of *pfmdr1* and 76 SNP of *pfcrt* were analysed. The haplotypes of these genes included one with double mutation in *pfmdr1* (86 Y/1246 Y) and a combination of two single mutations in different genes (*pfmdr1* 86Y + *pfcrt* 76T).

Statistical analysis

All data were managed in Excel and then, statistical analyses were conducted using R 4.0.0 software.

The *pfmsp1* and *pfmsp2* allele frequencies were calculated as the number of fragments assigned to the same allelic family (MAD20, K1 and RO33 for *pfmsp1* and 3D7 and FC27 for *pfmsp2*) divided by the total number of alleles detected in each gene (*pfmsp1* or *pfmsp2*) [24].

Multiplicity of infection (MOI) was defined as the number of parasite genotypes per infection. The mean MOI was calculated as the sum of total number of parasites genotypes divided by number of *P. falciparum* positive samples. The expected heterozygosity (He), which describes the possibility of being infected simultaneously by two parasites with different alleles at one locus, was calculated using the formula: $H_E = [n / (n - 1)] * [(1 - \Sigma pi^2)]$, where 'n' is the total number of samples and 'pi' the frequency of the allele (%) at the given locus [25].

The proportion of alleles observed at each locus within each group was compared using Chi-square test statistic or Fisher's exact test as convenient. The association between MOI and complicated malaria was assessed using U-Mann Whitney test, as MOI did not follow a normal distribution. The Kruskal-Wallis test was used to compare MOI between collection sites, age groups and antimalarial resistance haplotypes.

All analyses used a 95% confidence level and a p-value of ≤ 0.05 for statistical significance.

Ethics

This study was approved by the MSF and the South Sudan Ministry of Health Ethics Review Boards.

Results

Among the 535 samples confirmed by PCR for *P. falciparum*, 446 samples were included for the analysis from eight different locations in Yambio County, in order to ensure enough DNA quality and quantity. The mean age of patients was 4.41, as the majority of the samples were collected from children (Table 3).

Allelic genotyping of *pfmsp1* and *pfmsp2* genes

The frequency of *pfmsp1* and *pfmsp2* allelic families was 91.9% (410/446) and 83.6% (373/446), respectively. 61.2% (251/410) of the samples positive for *pfmsp1* were single - allelic infections (Table 1).

From the total sample, monomorphic K1 allele infection was predominant (37.0%, 152/410) in every location (Figure 2a). Among the polyclonal infections (47.6%, 195/410) the most frequent was RO33+K1 identified in 20.9% (86/410) of the samples, followed by trimorphic infections (RO33+K1+MAD20). However, these polymorphic genotypes were detected in different frequencies by location.

Among isolates positive for *pfmsp2* locus, 60.9% (227/373) were monomorphic, being single allele 3D7 predominant (44.8%, 167/373). 39.1% of isolates presented double-allelic genotype 3D7+FC27, with a wide range of number of fragments per locus from 2 to 11 (Table 1). The frequency of different allele combinations varies among different collection sites (Figure 2b).

Table 1. Frequency of genotypes of allelic families of *pfmsp1* and *pfmsp2* genes.

Family alleles	Genotype frequency % among *pfmsp* family (N_{pfmsp1} = 410; N_{pfmsp2} = 373)	Number of alleles per genotype
Pfmsp1		
RO33	13.7% (56)	1
K1	37.1% (152)	1 – 3
MAD20	5.1% (21)	1 - 3
RO33+K1	21.0% (86)	2 – 6
RO33+MAD20	4.6% (19)	2 – 6
K1+MAD20	7.3% (30)	2 – 8
RO33+K1+MAD20	10.2% (42)	3 – 9
Pfmsp2		
3D7	44.8% (167)	1 – 5
FC27	16.1% (60)	1 – 6
3D7/FC27	39.1% (146)	2 – 11

pfmsp1 and *pfmsp2* genes allelic polymorphism, diversity and multiplicity

Combining results of *pfmsp1* and *pfmsp2,* 71.9% of samples (321/446) presented polyclonal infections, while 28.1% (126/446) had monoclonal infections. The overall MOI was 1.96; the MOI of *pfmsp1* and *pfmsp2* was 1.53 and 1.85 respectively (Table 2).

In the *pfmsp1* locus, K1 allelic family was the most frequent (75.6%) and the most diverse, with 189 different allele fragments ranging from 70 bp to 629 bp (Figure 3a). RO33 family showed less diversity, ranging from 100 to 284, the 150 bp fragment was the most predominant. The expected heterozygosity (H_E) of *pfmsp1* was 0.63.

In the *pfmsp2* locus, 3D7 was the predominant allelic family (83.9%, 313/373), the predominant fragment size was between 100 and 119 (Figure 3b). FC27 allelic family, identified in less frequency (55.2%, 206/373), presented more diversity with 242 different fragments. The expected heterozygosity was 0.48.

Table 2. Genotyping of allelic variants of *pfmsp1* and *pfmsp2*. H_E : expected heterozygosity; MOI : Multiplicity of infection

Allelic family	Allelic family frequency	Fragment size	Number of fragments per locus	Number of alleles per family	H_E	MOI
pfmsp1 **gene** (N = 410)						
RO33	49.5% (203)	100 - 284	1 - 3	56		
K1	75.6% (310)	70 – 629	1 - 5	189		
MAD20	27.3% (112)	100 - 464	1 - 6	122		
Total *pfmsp1*					0.63	1.53
pfmsp2 **gene** (N = 373)						
3D7	83.9 % (313)	100 - 848	1 – 6	178		
FC27	55.2 % (206)	101 - 948	1 - 7	242		
Total *pfmsp2*					0.48	1.85
Total *pfmsp1 & pfmsp2*						1.96

Variability of multiplicity of infection by age and village

No statistical significant difference was observed in multiplicity of infection (MOI) by location, although *pfmsp2* showed a wide range of MOI (1.48 – 2.25) (Table 3).

Contrastingly, the MOI significantly varied according to age, increasing from the under-five age group to reach the highest MOI in the age group between 5 and 14 years (Figure 4).

Table 3. Multiplicity of infection by location and age group

	pfmsp1 MOI	*pfmsp2* MOI	*pfmsp1 & pfmsp2* MOI
Location			
Nambia	1.57	1.95	2.14
Gitikiri	1.37	1.84	2.01
Bakiwiri	1.55	2.25	1.95
Mamboi	1.55	1.99	2.07
Masumbu	1.67	1.78	1.96
Birisi	1.69	1.79	2.01
Yambio State Hospital	1.27	1.85	1.42
Kasia	1.52	1.48	1.91
p-value	0.111	0.217	0.163
Age group			
< 5 (N=119)	1.49	1.86	1.91
5 – 14 (N=170)	1.67	1.93	2.30
>14 (N=138)	1.41	1.73	1.81
p-value	**0.007**	0.107	**0.022**

Distribution of the allelic variants and multiplicity of infection by malaria infection severity

The overall mean MOI of uncomplicated malaria infections (2.01) was significantly higher than the overall mean MOI of severe malaria infections (1.40) (Figure 5). However, neither *pfmsp1* MOI nor *pfmsp2* MOI was independently associated with severe malaria (Table 4).

The frequency of *pfmsp1* and *pfmsp2* alleles according to severity of infection is presented in Table 5. No significant differences in allelic distribution between both groups were detected.

Table 4. Relation between multiplicity of infection (MOI) and severity of malaria infection.

	pfmsp1 MOI		*pfmsp2* MOI		*pfmsp1 & pfmsp2* MOI	
	N	MOI	N	MOI	N	MOI
Severity of infection						
Severe malaria	17	1.40	8	1.93	18	1.40
Uncomplicated malaria	429	1.54	360	1.85	400	2.01
P - value	0.251		0.289		**0.011**	

Table 5. Distribution of *pfmsp1* and *pfmsp2* allelic families by infection severity.

	Severe malaria		**Uncomplicated malaria**		*p - value*
	Fragment size (bp)	F% (n)	Fragment size (bp)	F% (n)	
***pfmsp1* allele family**					
	N=17		N = 378		
RO33	102 - 151	41.2% (7)	100 - 284	51.9% (196)	0.620
K1	133 - 383	70.6% (12)	70 - 842	78.8% (298)	0.779
MAD20	234 - 320	23.5% (4)	100 - 461	28.6% (108)	0.916
***pfmsp2* allele family**					
		N = 7		N = 359	
3D7	106 - 513	85.7% (6)	100 - 848	84.1% (302)	0.883
FC27	286 - 560	57.1% (4)	101 - 948	54.9% (197)	0.912

Allelic frequencies and multiplicity of infections relation with antimalarial drug resistance haplotypes

All 446 samples included in the study were analysed for molecular haplotypes associated with resistance to sulfadoxine – pyrimethamine, which included SNPs of *pfdhfr* and *pfdhps* genes, and to amodiaquine and chloroquine, which included SNPs of *pfmdr1* and *pfcrt* genes.

The overall mean MOI showed a gradual increase following the level of resistance, from 2.36 for the partially resistant haplotype 51I/59R/108N + *pfdhps* 437G), to 3.03 for the fully resistant haplotype (3.03) (*pfdhfr* 51I/59R/108N + *pfdhps* 437G/540E), and 3.88 for the super resistant haplotype (*pfdhfr* 51I/59R/108N + *pfdhps* 437G/540E/581G), (Figure 6). Nevertheless, the difference between the mean MOI of the haplotypes was not significant (Table 6).

Concerning the frequency of alleles of the *pfmsp1* family, no significant differences were observed between the resistance haplotypes (Table 7). For *pfmsp2* family alleles, although the differences between the four groups were not statistically significant, the three haplotypes associated with resistance showed high frequency of the allelic family 3D7 gene (> 80%) than isolates without resistance haplotypes (10.6%). Parasite infections that did not have any of the 3 resistance haplotypes presented lower *pfmsp1* MOI (1.71 and 1.79, respectively) but higher *pfmsp2* MOI (2.94 and 3.15, respectively) (Table 6).

Table 6. Relation between multiplicity of infection (MOI) and antimalarial drugs resistance haplotypes.

	Pfmsp1 **MOI**	***Pfmsp2*** **MOI**	***pfmsp1 & pfmsp2*** **MOI**
pfdhfr & pfdhps **mutated haplotypes**			
Partially resistant	1.65	2.44	2.36
Fully resistant	2.20	2.82	3.03
Super resistant	2.50	3.13	3.88
Non - associated with resistance haplotype	2.06	2.87	2.90
p-value	0.383	0.8	0.061
pfmdr1 **haplotype**			
Pfmdr1 86Y+1246Y	1.71	2.94	2.95
Non - associated with resistance haplotype	2.16	2.83	2.99
p-value	0.185	0.785	0.665
Pfmdr1 + pfcrt **haplotype**			
Pfmdr1 86Y + pfcrt 76T	1.79	3.14	3.21
Non - associated with resistance haplotype	2.17	2.81	2.97
p-value	0.157	0.790	0.647

Table 7. Distribution of *pfmsp-1* and *pfmsp-2* allelic families according to sulfadoxine – pyrimethamine resistance haplotypes.

	Non - associated with resistance haplotype	Partially resistant	Fully resistant	Super resistant	P - value
	F% (n)	F% (n)	F% (n)	F% (n)	
pfmsp1 allele family					
	N = 123	N=20	N = 247	N = 16	
RO33	45.5% (56)	50% (10)	51% (126)	68.8% (11)	0.342
K1	77.2% (95)	65% (13)	77.3% (191)	68.8% (11)	0.547
MAD20	23.6% (29)	20% (4)	30.4% (75)	25% (4)	0.462
pfmsp2 allele family					
	N = 104	N = 16	N = 237	N = 16	
3D7	10.6% (11)	81.3% (13)	81% (192)	81.3% (13)	0.284
FC27	45.2% (47)	50% (8)	54.9% (130)	62.5% (10)	0.913

Discussion

This study describes the polymorphisms and diversity of *pfmsp1* and *pfmsp2* to give in-depth information about the genetic nature and structure of the *P. falciparum* population in Yambio county, South Sudan. The information produced will be translated into insightful information for malaria control decision-making not only in terms of epidemiological data, as malaria intensity of transmission, but also of parasite population characterisation, showing their diversity and composition.

This study showed a high proportion of polyclonal infections (72%), slightly lower than the reported in border country Sudan (80%) [26] but more than in Ethiopia (54%) [27]. Multiplicity of infection is considered a key indicator of malaria transmission intensity, in fact, the high intensity of malaria transmission results in a high MOI [28] [29]. In our study all locations showed a high MOI, and no significant differences were detected between locations. This high MOI indicated that the area had a high level of malaria prevalence with different parasite populations circulating at the same time. Besides the prevalence of malaria, other factors also influence the MOI, such as seasonality, patient age, patient immunity and parasite density. In fact, higher MOI is expected after the peak of transmission, as it is also the moment with more genetic diversity[30].

MOI tends to change by age, being higher in children under 5 years old or between 6 – 10 years old in highly endemic areas [31]. This is consistent with the results of our study, where MOI gradually increased during childhood, until the age of 14 years after which it started to decrease. These differences on MOI

could be due to the fact that individuals with continuous exposure to *Plasmodium* sp infection develop a protective immunity against malaria that decreases the risk of being infected by multiple clones [32].

Nevertheless, the persistent exposition to multiclonal infections might increase the tolerance to antigenically diverse infections[33] , which explains that higher MOI is associated with less severity in high transmission areas as South Sudan[34], but not in low transmission areas[35]. Another hypothesis that could support higher severity in lower MOI infection is that virulent strains are more competitive and avoid infections by other strains [36]. However, there is still no agreement about the relationship between MOI and severity [26]. For example, it has also been reported that higher MOI increased the probability of recombination and selection of more virulent strains [37]. Additionally, there are reports of high multiplicity of infection in asymptomatic children [9], that could be explained by the high malaria prevalence in that area. Interpretation of our results should be done considering that we have only used samples collected after malaria peak and from symptomatic children.

The recombination between different simultaneous strains could also be related with resistance to antimalarial drugs[8]. According to our results, MOI was gradually higher with the incorporation of more resistance molecular markers, so polyclonal infections are related with selection of parasites with resistance mutations [37]. However, although multiclonal infections are caused by different strains, through the molecular analysis of resistance haplotypes based on amplification and sanger sequencing we are picking up the information from the main strain, and probably missing some of the others clones if they are in too low parasitaemia[38].

MOI is also one of the determinants of genetic diversity given that recombination rates depend on the different simultaneous strains in the mosquito[39], where sexual reproduction and genetic recombination of the parasite take place. Consequently, genetic diversity in *pfmsp1* and *pfmsp2* have also been used to assess variations in malaria transmission and as an indicator of malaria control measures [11]. For example, antibodies to MSP-1 have been used as a significant biomarker of malaria exposure[40].

Also, circulating parasite subpopulation and its antigen diversity is essential information for the design of effective vaccines with MSP1 and MSP2 as target [5]. Therefore, characterization of this antigenic family is the first step, as vaccines should be based on their variability.

Heterozygosity found in our study (He = 0.49) was moderate compared with higher *He* level in other Sub-Saharan countries[34,41]. But it is consistent with the ones reported in Rwanda (He = 0.49) [42] and Ethiopia (He = 0.5) [27]. Additionally, the results agree with previous studies in that *pfmsp1* show higher heterozygosity than *pfmsp2* [44].

About frequency of alleles, the K1 *pfmsp1* and 3D7 *pfmsp2* were the predominant alleles, agreeing with previous reports from other Sub-Saharan countries[45], such as Uganda[46], but differing from Sudan were MAD20 and RO33 are the most frequent for *pfmsp1* and for FC27 *pfmsp2*.

Different alleles in *pfmsp1* and *pfmsp2* have also been associated with different parasite properties and clinical outcomes. Association between alleles and severity is still unclear, and no association has been found in this study, moreover the number of severe malaria cases was low to achieve any robust conclusion. However, association between K1 allele in children [47] or mixed infections of RO33 and K1[48] and severity has been previously reported. Some influence of *pfmsp1* in severe malaria is expected because *pfmsp1* family participates in the invasion of erythrocyte cells by malaria parasite and in the modulation of parasite multiplication [49]. Consequently, different variants of *pfmsp1* could increase parasitaemia, which generally provokes complicated cases in children below the age of five years. For example, fragments of MAD20 around 200 bp have been associated with severe malaria[50]. Our results support this, as all the samples originating from severe malaria cases, except one, had MAD20 allelic fragment between 230 and 270 bp. Although the current study did not find any association between *pfmsp2* variability and severity of infection, previous studies suggest higher frequency of FC27 allele in uncomplicated malaria[51]. This disagreement could be related with the multifactorial cause of severe malaria or with the low number of severe cases included in this study.

No significant association was found between allelic families and molecular haplotypes associated with antimalarial drug resistance. However, some relation between complexity of infection, genetic polymorphisms and resistance is expected [8,43,52]. As antimalarial resistant parasites are probably under evolution, genetic polymorphisms could play two important roles, being an indicator of selection or providing some advantage by influencing the development of resistance.

Limitations

This study has some limitations, first of all the small number of isolates, and the collection of samples only at the end of the peak of transmission and in a single location may not be representative of the true diversity in the country. Therefore, a wider study would be necessary to reach stronger conclusions. In addition, the relation between specific alleles and infection and parasite traits were difficult to clarify due to limited data, especially for severe malaria samples. Moreover, clinical data including parasite clearance was not available. Nevertheless, the study presents the first description of genetic diversity of *P. falciparum* in an area of South Sudan and its potential association with major malaria control problems such as disease severity and molecular haplotypes associated with antimalarial drug resistance.

Conclusion

High genetic diversity and high level of polyclonal infections have been found in this study, confirming the general high transmission in Yambio County, South Sudan. The high diversity and polyclonal infections are associated with molecular markers of resistance, leading to increased risk of antimalarial drug resistance. On the other hand, despite the small number of severe malaria samples, high MOI has been related with a lower risk of severity of infections. The results of the study did not find evidence of association between a specific allele and an infection trait.

The high genetic diversity, understood as a relevant malaria indicator, highlights the need for control measures to be intensified in Yambio county, South Sudan.

Declarations

Authors Contributions: EL, MJS, PB conceived the study and participated in its design, literature research. IMF, PB conceived the idea and drafted the manuscript and coordination. IMF and PB curated and verified laboratory data; IMF, LG, VG and PB performed laboratory analysis. MJS, BT, EL and AA supported the study implementation, sample transportation, data collection and curation. All other authors provided rigorous review and approved the final draft prior to submission.

Declaration of interests

The authors declare they have no conflict of interest.

Data sharing

The study protocol and statistical analysis data that underlie the results reported in this article will be made available upon request. Proposals should be directed to the corresponding author. Requests will be reviewed and sharing of the data will follow the conditions required by all applicable laws and the possible prior signature of any necessary agreement, in accordance with the legal framework set forth by Médecins Sans Frontières (MSF) data sharing policy, which ensures that all security, legal, and ethical concerns are addressed.

Acknowledgments

The authors thank the population of Yambio County that participated, the South Sudan Ministry of Health and MSF Spain teams in Barcelona, Juba and Yambio for their support in organising this campaign. Also, the authors thank Kim West for her suggestions to improve this manuscript.

Funding

The intervention was funded by Médecins Sans Frontières and samples were analysed through a research agreement between Médecins Sans Frontières and National Centre of Tropical Medicine/ Institute of Health Carlos III. Agreement No: TRVP 121/20. IMF received a research fellowship (FPU-2019) from the University of Alcalá, Spain that enables her to develop this study.

Ethics

This intervention was approved by the Ethical Review Boards of the Ministry of Health of the Republic of South Sudan and Médecins Sans Frontières (ID: 1953).

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Figures

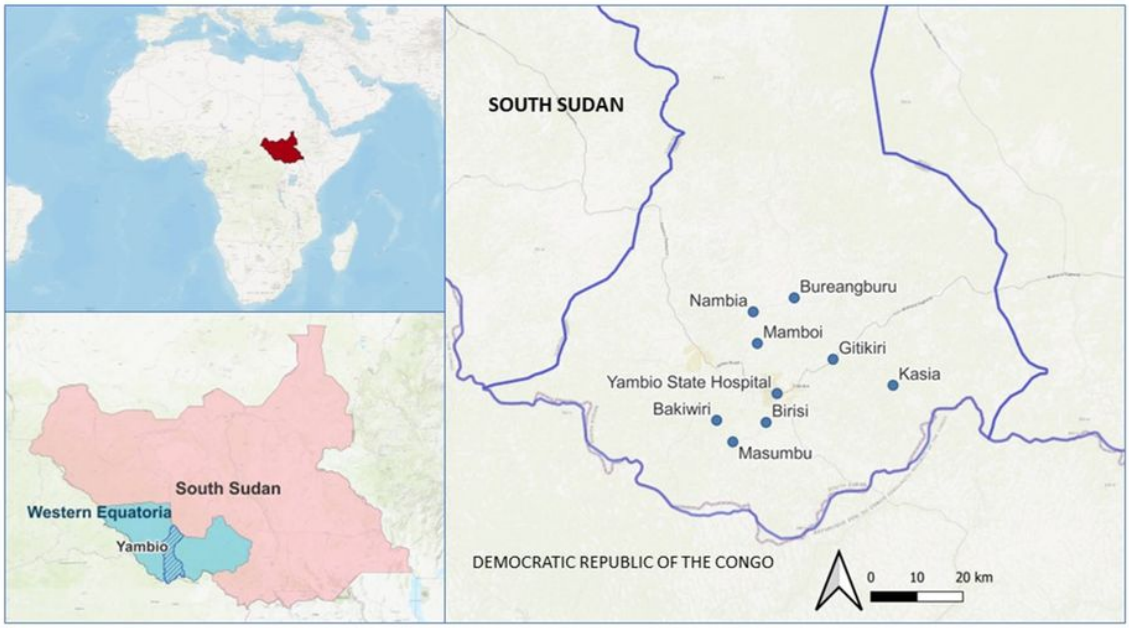

Figure 1

Map of areas of collection samples

Figure 2a. Distribution of genotypes of *pfmsp1* allelic family.

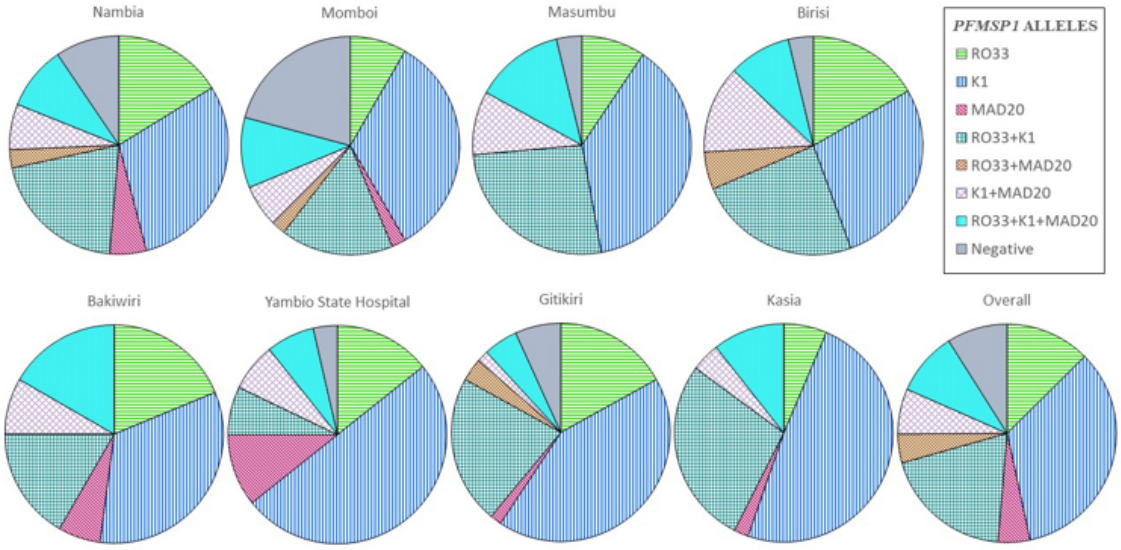

Figure 2b. Distribution of genotypes of *pfmsp2* allelic family.

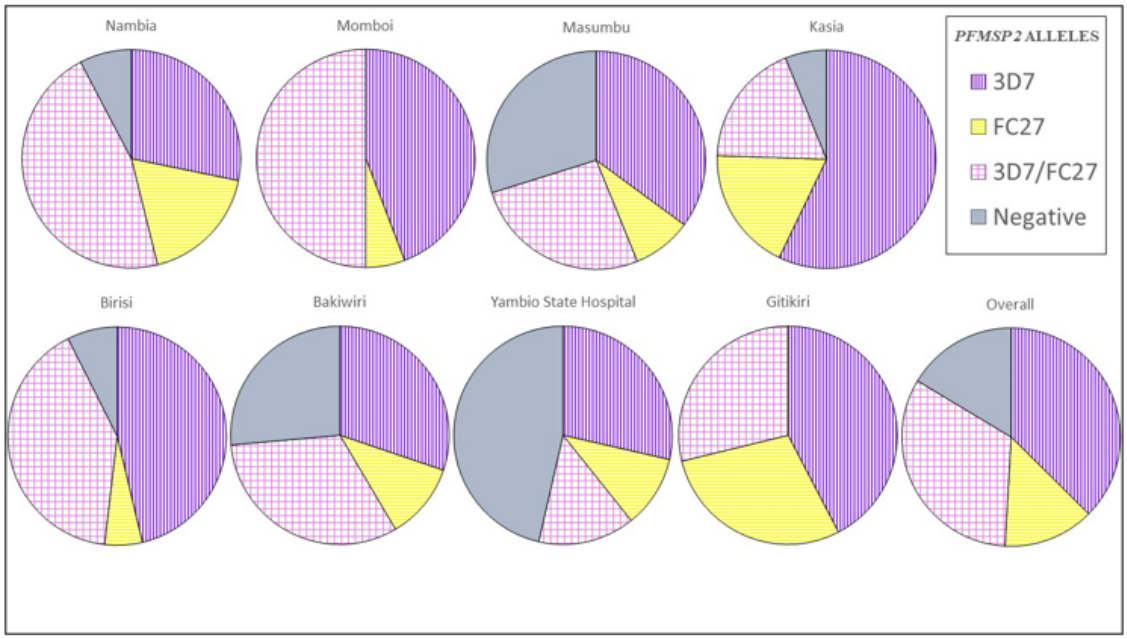

Figure 2

Distribution of allelic genotypes by location.

Figure 3a. Distribution of allelic fragments of *pfmsp1* (K1, RO33 and MAD20)

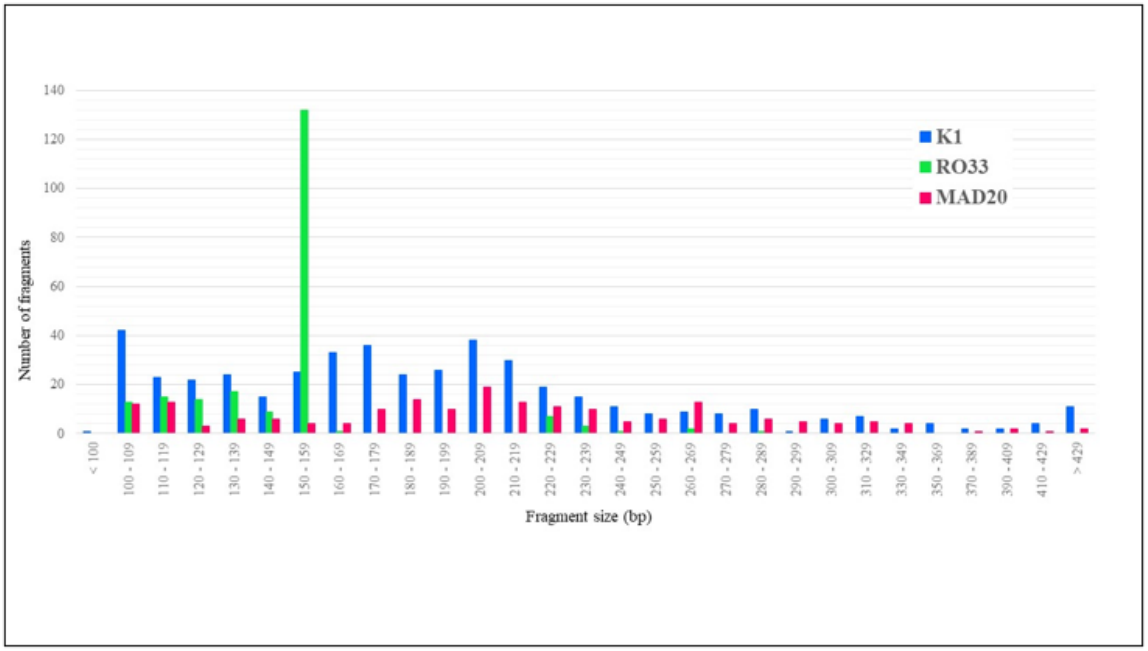

Figure 3b. Distribution of allelic fragments of *pfmsp2* (3D7 and FC27)

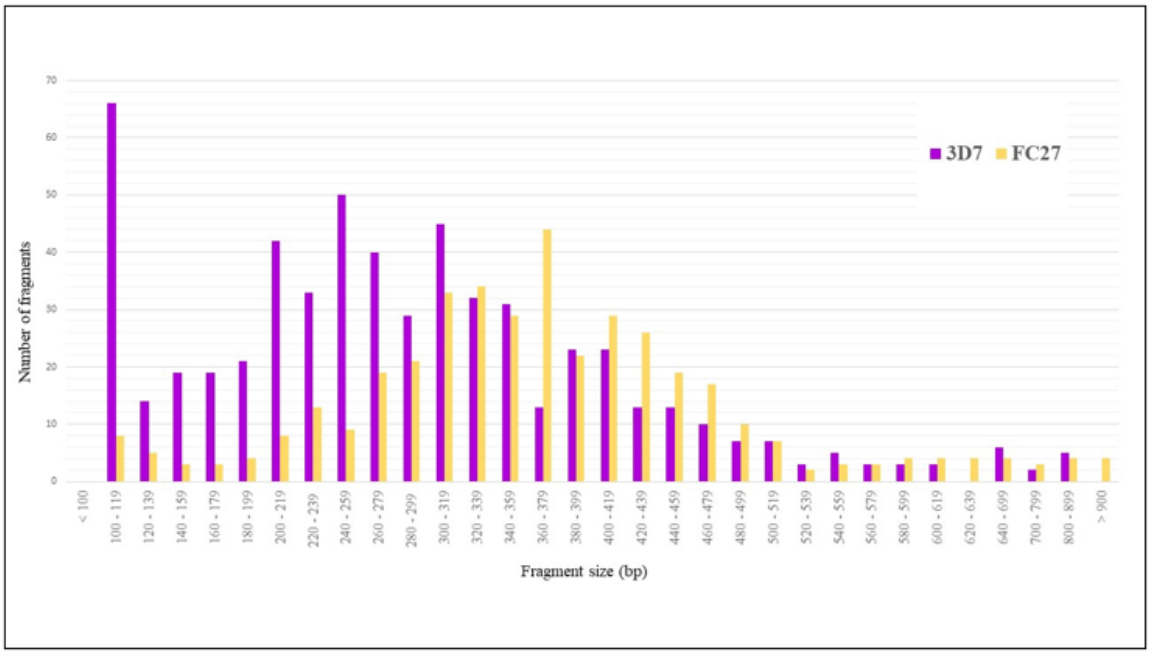

Figure 3

Distribution of allelic fragments of *pfmsp1* and *pfmsp2* by fragment length in base pairs (bp).

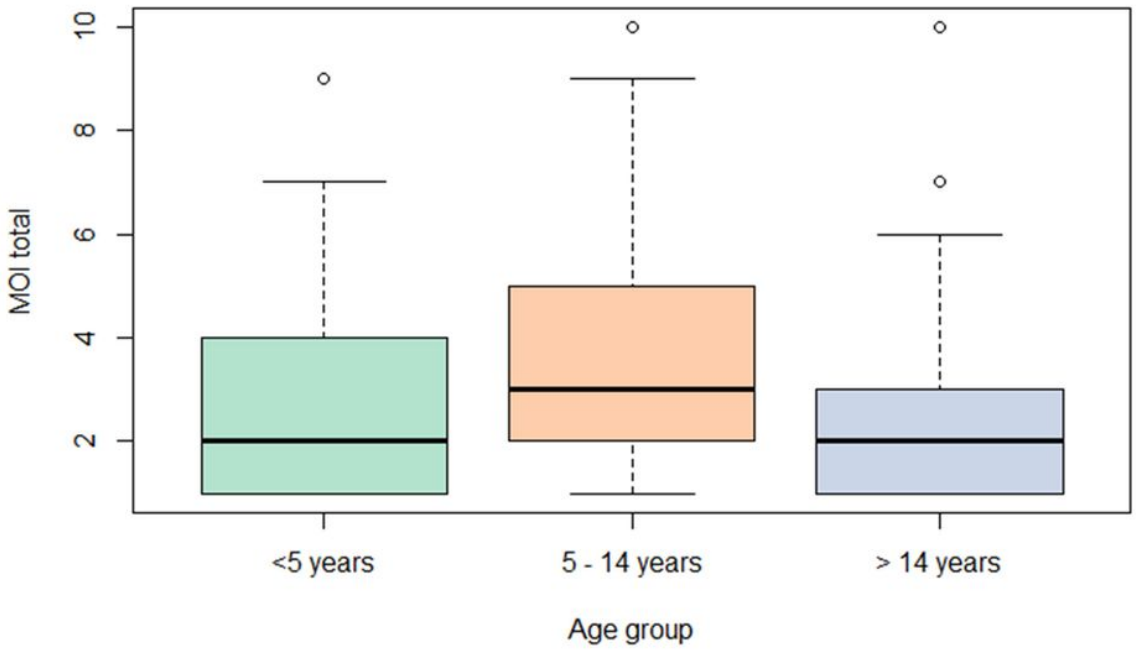

Figure 4

Box plot of total MOI by age group.

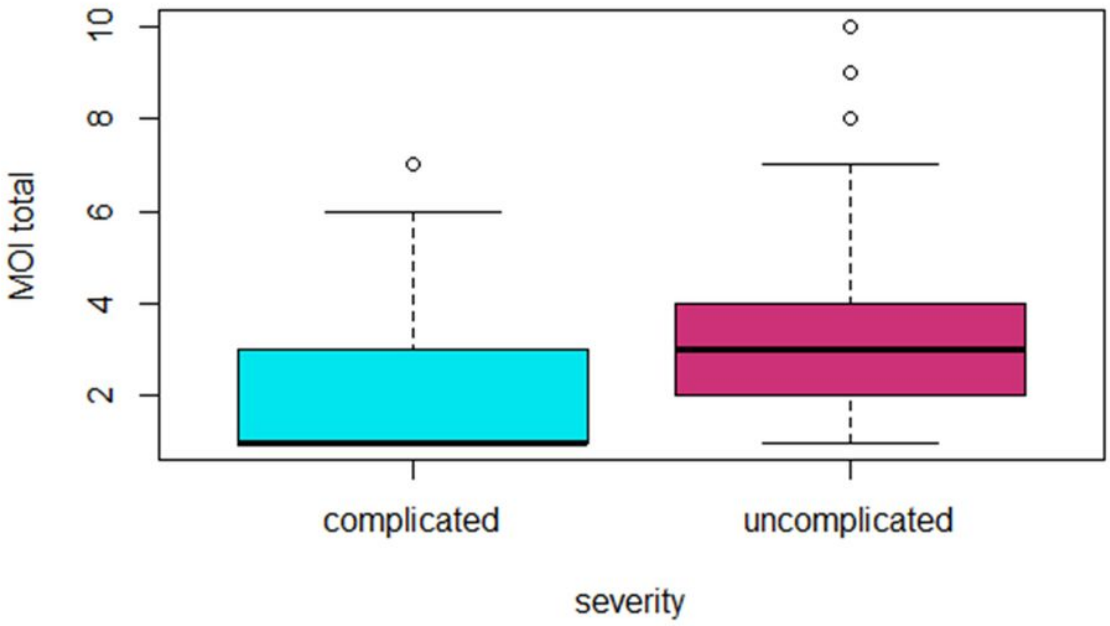

Figure 5

Box plot of total MOI by malaria severity.

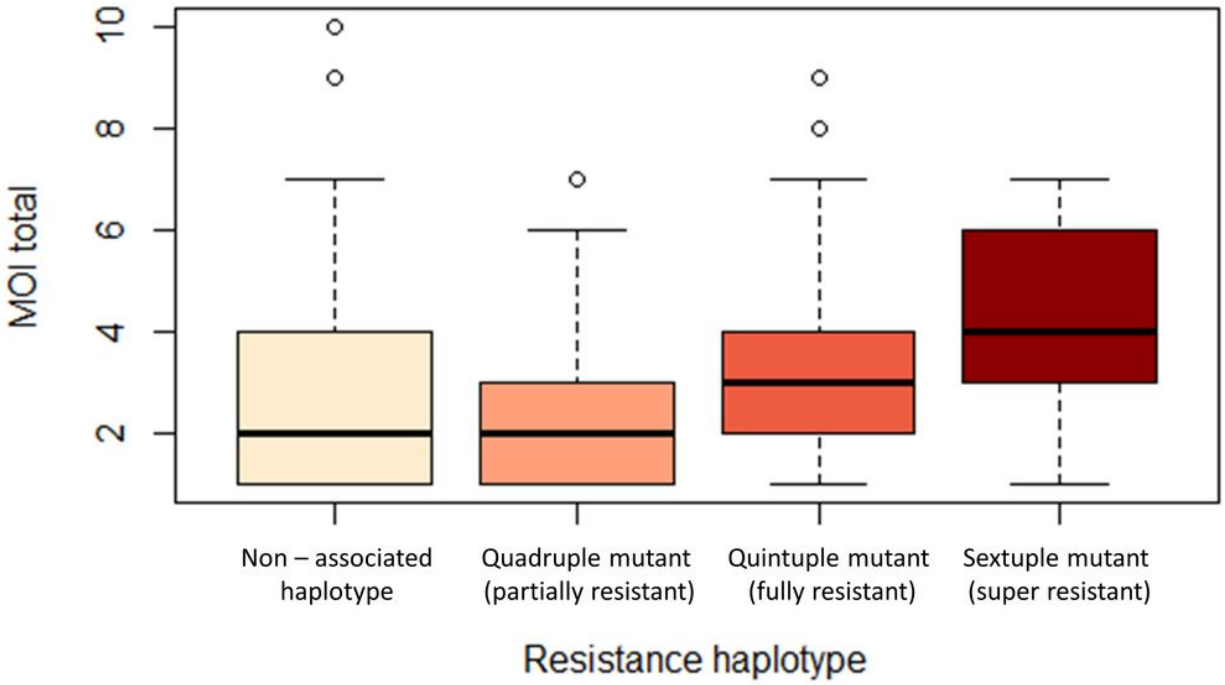

Figure 6

Box plot of total MOI by antimalarial resistant haplotypes.