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A Comprehensive Analysis on the Genetic Diversity of *Plasmodium Falciparum* MSP-1 Field Isolated from Different Regions in Sudan. A Cross-Sectional Study

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Abstract

Malaria caused by *P. falciparum* still posing a great risk in sub Saharan African countries. Persistence of malaria is considered as a major threat to health and blocks the path to economic development for individuals, communities and nations. In this study we investigated the genetic diversity of *P. falciparum* MSP-1 alleles based on frequency distribution across the different study sites. A prospective cross-sectional molecular and parasitological study carried out in different geographical regions in Sudan, including; Khartoum, New Halfa, Red Sea, White Nile, Al Qadarif, Gezira, River Nile and Ad Damazin. Blood samples were collected from these states during the malaria transmission season 2017 - 2018. A total of 419 blood samples were collected from all the study sites. Thick and thin blood film were prepared for each collected blood sample for microscopic diagnosis of malaria, Genomic DNA were extracted and examined using PCR for genotyping of the different MSP-1 alleles; MAD20, K1 and RO33. The amplified DNA products were visualized and analyzed using the agarose gel electrophoresis. Analysis of MSP-1 alleles showed great polymorphism of MAD20 and K1; 10 genotypes ranging from 200 - 650 bp and 8 genotypes ranging from 300 - 700 bp, respectively. Whereas, RO33 showed a monomorphic diversity among the different age groups and location; 1 genotype with size band of 200bp. For K1 frequency, Red Sea was the highest frequency (67.0%), followed by Ad Damazin (60.6%), Al Qadarif (61.4%), Khartoum (51.6%), New halfa (54.3%) and River Nile was (51.6%). MAD20 allelic family single infections were distributed across the study sites with different frequencies, Al Qadarif was having the highest frequency (72.7%) followed by Khartoum (67.7%), while White Nile had the lowest frequency (37.5%). RO33 showed highest frequency in samples collected from Gezira with frequency of (62.3%) and the lowest frequency was observed in White Nile with frequency of (37.5%). MOI values showed an insignificant difference among the locations although having the lowest value in samples collected from Gezira i.e. 2.3. MSP-1 alleles frequency showed statistically insignificant difference among the different locations, P value = 0.609. The prevalence of each allele separately also showed insignificant difference; RO33 (P value = 0.419), MAD20 (P value = 0.800) and K1 allele (P value = 0.076). The genetic diversity of *P. falciparum* showed great variation from location to another, with relation to age or/and gender, this study illustrated high polymorphism with different alleles at MSP-1 loci.

Keywords: *P. falciparum; MSP-1 Alleles; Frequency Distribution; Sudan*

Introduction

Malaria is a mosquito-borne disease caused by eukaryotic protozoa of the genus *Plasmodium*. it is transmitted to humans through the bite of infected female mosquitoes of the genus *Anopheles* [1]. It is known to be caused by one of four Plasmodia species, namely *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae* [2] with *P. falciparum* being the most lethal [3]. A fifth species been reported to transmit disease in human, it is known as *P. knowlesi*, which been reported in Southeast Asian countries [4-6]. Persistence of malaria poses a major

threat to health and blocks the path to economic development for individuals, communities and nations and it remains a serious human health and is particularly prevalent in tropical and subtropical regions, including parts of the Americas, Asia, and Africa [7,8]. In 2010, the World Health Organization (WHO) reported 216 million malaria cases with an estimated 655000 deaths, principally among children [9].

Malaria due to *P. falciparum* is the most common infectious disease in tropical countries [10]. It accounts for the high morbidity and mortality rate, over 80% of cases and deaths due to malaria found to be occurring in Africa, especially, among the high-risk groups i.e. children below five years of age and pregnant women [11-14]. In 2015, malaria killed an estimated 303 000 under-fives globally, including 292 000 in the African Region [15]. Between 2010 and 2015, the malaria mortality rate among children under 5 fell by an estimated 35%. Nevertheless, malaria remains a major killer of under-fives, claiming the life of 1 child every 2 minutes [16]. Malaria is prevalent in many regions of the world. It affects young and old; but children are particularly at high risk and it considered as one of their major health risk in tropical Africa [15].

Merozoite surface protein 1 (MSP-1) gene is the most commonly used genetic marker for the determination of the genetic diversity of the malaria parasite [17-19]. Twenty-four major versions of the MSP-1 gene have been identified [20,21] and its coding sequence divided into 17 blocks among which 7 are variable blocks interspersed with conserved and semi-conserved regions. In some variable blocks, the variation is dimorphic and sequences grouped into one of the two allelic families (K1 and MAD20). Block 2 represents an exception to dimorphism; it is the most polymorphic part of the gene having a third allelic family or variant RO33 [22,23]. Genetic diversity at the MSP-1 locus generated by exchanging blocks of sequences during sexual (meiotic) recombination and by putative strand-slippage events during asexual (mitotic) replication of the parasites, which lead to rearrangements of block 2 tripeptide repeats [24]. High meiotic recombination rates within MSP-1 estimated for parasites in areas of intense malaria transmission in Africa, where most human infections consist of mixture of genetically distinct allelic variants [25]. However, the meiotic recombination is rare between different allelic types, although it occurs in block 2 between MAD20 and RO33 and creates a fourth allele family known as MR [26]. The effect of altitude and estimated rainfall on indices of malaria infection/transmission described in a study carried out in Tanzania in which *P. falciparum* prevalence was negatively associated with altitude. However, the relationship varied according to ecological setting, climate, vector species, topography, and host and parasite genetics [27]. In this study we aimed to investigate the genetic diversity of *P. falciparum* MSP-1 gene across age and gender variation among different locations in Sudan.

Materials and Methods

Study Design and Study Sites

A prospective cross-sectional molecular and parasitological study carried out in different geographical regions in Sudan, including; Khartoum (15°55'N and 32°53'E), New Halfa (15°35'N 35°39'E), Port Sudan (19°35'N 35°37'E), White Nile (13°10′N 32°40′E), Al Qadarif (14°02'N 35°23'E), Gezira (14°30'N 33°30'E), River Nile (18°27'N and 33°23'E) and Ad Damazin (11°46'N and 34°21'E) (Figure 1). The studied areas located in a poor savannah region, characterized by a short rainy season (July to September), winter season (October to March) and summer season (April to July). The transmission of malaria in these areas is seasonal and unstable following rainfall from May to October with peak transmission June to November and depends on the rains [28]. Blood samples were collected from these states during the malaria transmission season 2017 - 2018. This season was started from July to December in Khartoum, Gezira, Nile River state, but in Red Sea state the season of malaria transmission started from November to January.

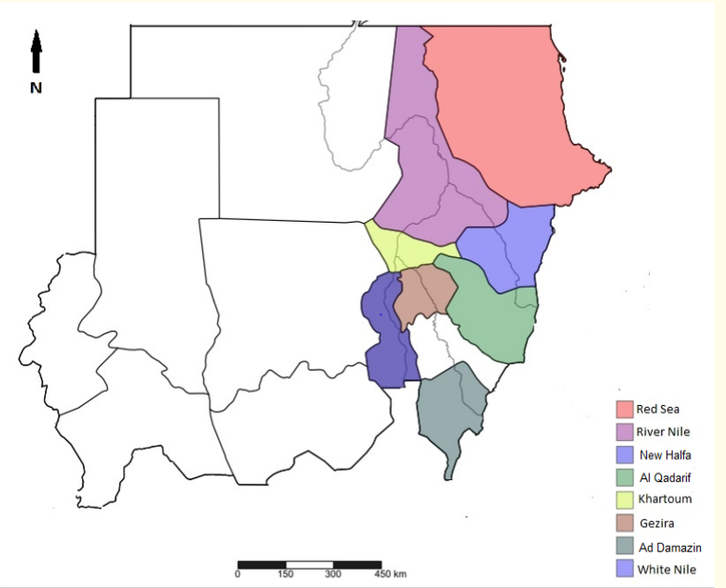

Figure 1: *Map showing the different locations selected to study the genetic diversity of P. falciparum MSP-1.*

Study Population, Sample Size and Sample Collection:

Febrile patients in representative health facilities were recruited. Those are clinically diagnosed by a physician as malaria cases (positive microscopy, temperature ≥ 37°C). Clinical phenotypes were assessed according to the WHO guidelines [29]. A total of 419 blood samples were collected from all the study sites. Two ml venous blood samples in sodium citrate anti-coagulated blood containers and/or finger prick spotted blood on Whatman 3 filter paper (Sigma-Aldrich, USA) been collected from each patient after obtaining writing informed consent from adult or from their behalf guardians in case of children. Demographical data, clinical data and other baseline information's has been collected using semi-structured questionnaire.

Diagnosis of malaria

Thick and thin blood film were prepared; thick film was prepared using the corner of a clean slide to spread the drop of blood in a circle (diameter 1 - 2 cm), thin film was prepared using a clean sterile spreader slide, held at a 45° angle, toward the drop of blood on the specimen slide the blood spreads along the entire width of the spreader slide for each collected blood sample noting all the instructions and guidelines of WHO [30] for preparing Giemsa stained blood films (one drop Giemsa + 1 ml PBS, pH 7.2 Sigma-Aldrich, USA). Using the thin blood film, the number of parasites was counted per 200 leucocytes using hand-tally-counters. The parasite count was then calculated according to the following formula:

No. of asexual stages X Total white blood cell count per microliter/No. of leucocytes counted.

Data were grouped into high parasitemia (> 10 parasite/1 oil field), moderate parasitemia (1 - 10 parasite/1 oil field) and low parasitemia (1 - 100 parasite/100 oil field) groups.

Isolation of *Plasmodium* parasite genomic DNA:

Genomic DNA was extracted and purified from blood samples using QIAamp DNA blood Mini Kit (Qiagen Inc., Germany), all steps were according to manufacturer instructions, Chelex DNA extraction method was used to extract DNA from finger prick spotted blood on Whatman 3 filter paper according to Bereczky., *et al.* 2005 [31]. DNA were re-suspended in 200 µl of 1X TE-buffer and stored at -20°C. The quality and concentration of extracted DNA have been determined by Nano-drop spectrophotometer apparatus (ND1000, USA).

Polymerase Chain Reaction (PCR) for *Plasmodium* species genotyping

PCR was used to confirm the microscopy results and to determine the multiplicity of infection using genus and species specific primers according to Snounou., *et al.* 1993 [32] (See table 1). Genus-specific primers (rPLU5 and rPLU6) were used to amplify the target genes then another reaction was carried out using the nested primers with the genus-specific PCR template (species-specific rFAL1, rFAL2 and rVIV1, rVIV2). The reaction mix contained: 17.3 H_2O, 1 µL of each primer (1 mM), 1 µL dNTPs (0.2 mM each), 1 µL $MgCl_2$ (50 mM), 2.5 µL PCR ViBuffer (10X), 0.2 µL Taq polymerase (5 u/µL), and 1 µL from DNA were added to each single reaction. With each run a positive and negative controls were included. PCR process were as follow; samples were subjected to an initial denaturation at 95ºC for 5 minutes, a step at 55ºC for 2 minutes, a step at 72ºC for 2 minutes that was followed by 37 cycles at 94ºC for 30 seconds, and a final step at 72ºC for 10 minutes in an MJ Research thermocycler machine (USA). The second reaction of the species-specific PCR was performed with the same program.

P. falciparum MSP-1 gene genotyping

As described previously by Ntoumi., *et al.* 2000 [23]; two pairs of primers were used for MSP-1 gene, an "outer'' pair and nested pairs (See table 1). The outer pair was used for the initial amplification, and the product obtained is used as template for the next amplification using the nested pair of primers. In the outer reaction, 1 µL of DNA template suspension was used to amplify the region spanning block 2 for MSP-1. Three separate additional amplification reactions were carried out using nested primers pair's specific for the three allelic families of block 2 polymorphic regions (MAD20, K1 and RO33). The PCR was performed in a final volume of 25 µL containing 1µL of extracted DNA or the first PCR product, 0.7 µL of each primer, 1 µL of dNTPs (0.2 mM each), 0.2 µL of Taq polymerase (0.2 Units), 1 µL $MgCl_2$ (1 mM), 2.5 µL PCR buffer (10X), and 17.9 µL H_2O. The profile for outer PCR for MSP-1 gene was 30 cycles as follows: 95°C, 5 minutes; 58°C, 2 minutes; 72°C, 2 minutes; 94°C, 1 minute followed by 1 cycle of 58°C for 2 minutes and 72°C for 5 minutes. And for the nested reaction was 35 cycles of 95°C for 5 minutes; 61°C, 2 minutes, 72°C for 2 minutes and 94°C for 1 minute then followed by one cycle of 61°C for 2 minutes and 72°C for 5 minutes. Amplification was performed using a MJ Thermo-cycler (USA).

Gene (PCR reaction)	Primers	Reference
P. falciparum genus (N1) rPLU5	CCTGTTGTTGCCTTAAACTTC	[32]
P. falciparum genus rPLU6	TTAAAATTGTTGCAGTTAAAACG	[32]
P. falciparum genus specific (N2) 1	TTAAACTGGTTTGGGAAAACCAAATATATT	[32]
P. falciparum genus specific 2	ACACAATGAACTCAATCATGACTACCCGTC	[32]
P. vivax genus specific rVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC	[32]
P. vivax genus specific rVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	[32]
P. falciparum MSP-1 (N1)	CTAGAAGCTTTAGAAGATGCAGTATTG	[23]
P. falciparum MSP-1	CTTAAATAATATTCTAATTCAAGTGGATCA	[23]
P. falciparum MSP-1 MAD20 (N2)	AAATGAAGAAGAAATTACTACAAAAGGTGC	[23]
P. falciparum MSP-1 MAD20	GCTTGCATCAGCTGGAGGGCTTGCACCAGA	[23]
P. falciparum MSP-1 RO33 (N2)	TAAAGGATGGAGCAAATACTCAAGTTGTTG	[23]
P. falciparum MSP-1 RO33	CATCTGAAGGATTTGCAGCACCTGGAGATC	[23]
P. falciparum MSP-1 K1 (N2)	AAATGAAGGAACAAGTGGAACAGCTGTTAC	[23]
P. falciparum MSP-1 K1	ATCTGAAGGATTTGTACGTCTTGAATTACC	[23]

Table 1: *Primers used for the amplification of the 16sRNA of P. falciparum and genotyping of MSP-1 gene.*

Visualization and analysis of *P. falciparum* MSP-1 gene PCR Products

The amplified DNA products were visualized and analyzed using the agarose gel electrophoresis (BioMetra, Germany). 2% agarose gel in 1X TBE buffer stained with 3 µl Ethidium bromide. 100 base pair DNA marker was run with the sample in parallel wells as a molecular weight marker for the distinguishing of different band sizes. The gel was run for 60 minutes in 1X TBE running buffer at 90 V and 30 A. Finally, the gel was photographed using UV trans-illuminator (BioDoc-it, BioMetra-Germany) (Figure 2).

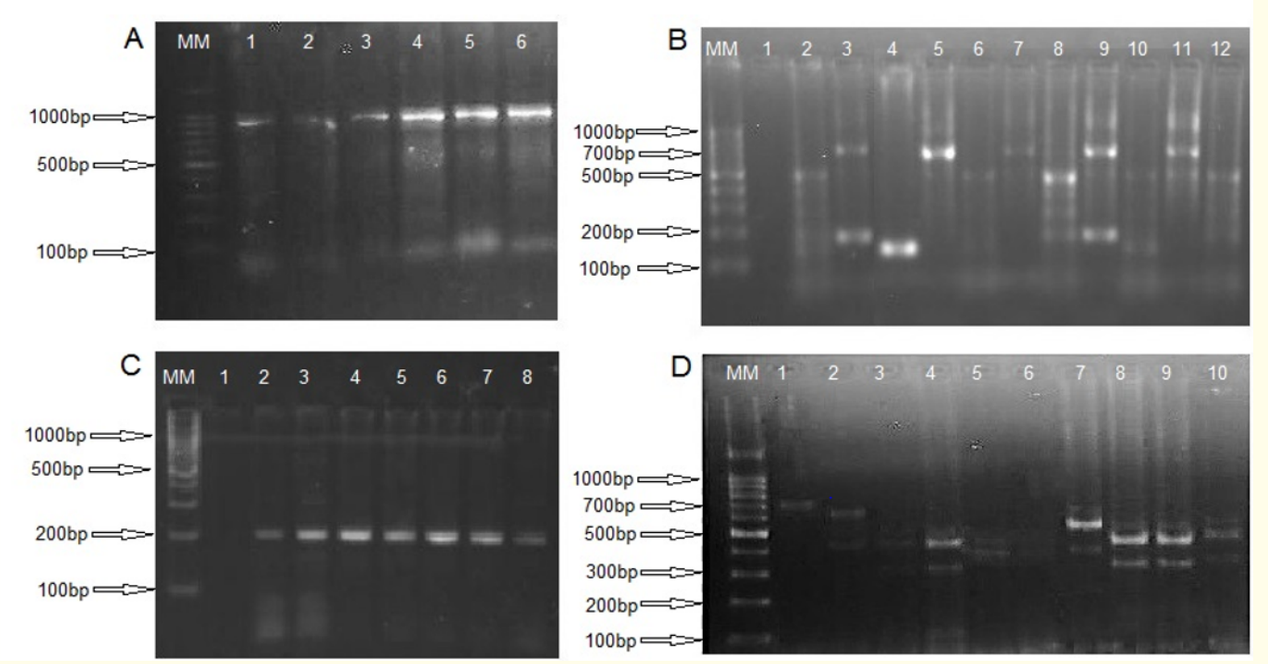

Figure 2: *Picture showing the different band sizes of the different genotypes detected. A: shows the PCR product of the outer MSP-1 with a band size of 1091 bp. B: shows the different genotypes detected for MAD20, band size range from 200 - 650 bp. C: shows the genotype detected for RO33 with a band size of 200 bp. D: shows the varing genotypes of K1 allele with a band sizes range from 300 - 700 bp.*

Statistical analysis

Descriptive and inferential data were analyzed using the Statistical Package for the Social Sciences (SPSS v20). Multiplicity of infection (MOI) of MSP-1 gene was determined by calculating the number of different alleles at each locus; single infections were those with only one allele per locus at all of the genotyped loci. Multiclonal infections were defined as those having more than one allele in at least one locus out of the loci genotyped [33].

Results and Discussion

Characteristics of study population

The genetic diversity of *P. falciparum* in Sudan is still not completely understood although a number of studies investigated this diversity in several regions [33,34]. In this study, a total of the 450 patients (250 males and 169 females) were included, the mean age of participants was 32.44 ± 15.19 (Figure 3). Most of the participants were from Red Sea with a total of 115 patients followed by Gezira of 114 patients, male and female were inconsistent among the study sites. Their mean age differences among the locations were statistically significant, P value = 0.000 (Figure 3).

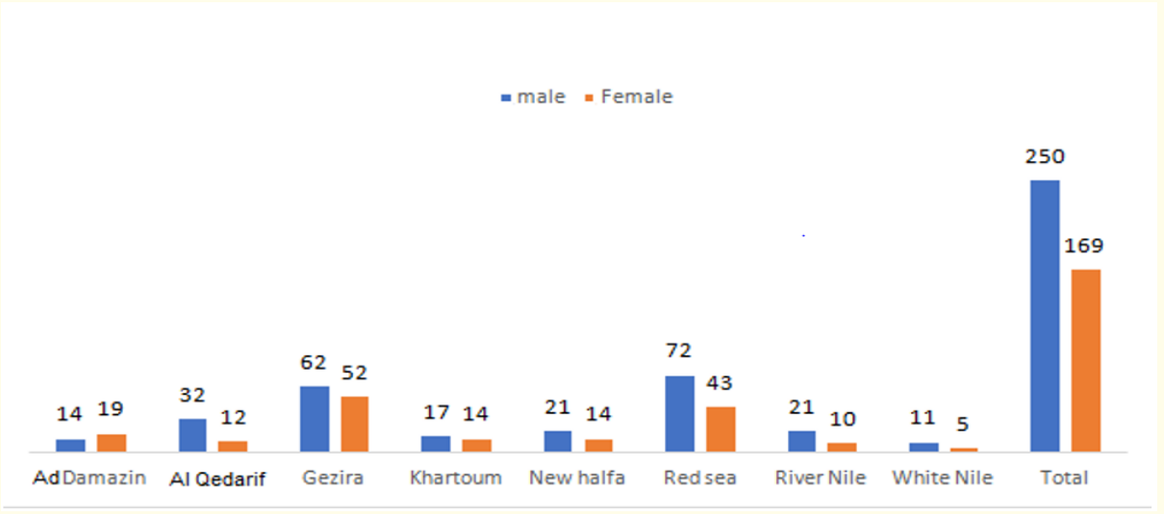

Figure 3: *Shows the gender distribution across the different study sites.*

Red Sea state were counted as having the highest parasitemia level among males when compared to males from the other locations, while Khartoum females were having the highest parasitemia level when compared to females of the other sites. Hemoglobin levels were not of high variation when compared based on the location. However, No Statistical significance difference between the study sites was obtained for parasitemia or hemoglobin levels differences. P values = 0.986 and 0.977 respectively (Table 2). These results were in agreement with previous study conducted by Kalu., *et al.* 2012 in Nigeria [35], found no significant difference in parasitemia among males and females ($p > 0.05$). The distribution of parasite densities across the different study sites showed that the parasite densities did not had any variation among the different location, no significant difference, P value = 0.977. Based on the frequency distribution of parasite densities among the locations, the density of more than 5000 parasite per 200 WBCs was the most density detected across the locations (Table 3). Although the overall estimate of infected males versus females were in agreement with Kalu., *et al.* 2012 [35], This could as a result of the high rate of males exposing themselves more than the females cause of the hot weather, thereby exposing themselves more to malaria vector bites than the females [35-38]. Notwithstandingly, the distribution of the different parasite densities among the different age groups was statistically insignificant, P value = 0.301, although, the most frequent parasite density was found among (21 - 30 years) followed by (1 - 10 years) and (11 - 20 years) (Table 4). These results were in agreement with several studies on which the high infection rates were observed in age groups of 10 - 20 and 21 - 30 years; this could be due to insufficient fortification towards malaria vector bites or scarce knowledge about malaria transmission. Moreover, these age groups composed of young individuals whose manners and activities influence them to persist contact with malaria vectors [35,39].

Locations	No. of Patients (%)	Mean haemoglobin	Mean Age	Mean Parasitemia
Khartoum				
Male	17 (4.1%)	11.2 ± 3.3	20 ± 6.93	6598.18 ± 4379.4
Female	14 (3.3%)	10.2 ± 2.1	23 ± 9.08	6869.92 ± 7593.1
River Nile				
Male	21 (5.0%)	10.7 ± 2.3	27 ± 17.46	5526.19 ± 4861.4
Female	10 (2.4%)	11.6 ± 2.9	37 ± 17.83	4347.00 ± 4103.6
Gezira				
Male	62 (14.8%)	12.2 ± 2.7	21 ± 16.21	4345.64 ± 4403.9
Female	52 (12.4%)	13.6 ± 2.2	23 ± 16.03	4972.30 ± 4047.4
Al Qadarif				
Male	31 (7.4%)	11.5 ± 2.6	12 ± 12.25	5064.37 ± 4562.4
Female	13 (3.1%)	10.2 ± 3.2	15 ± 12.34	4586.67 ± 4413.4
New Halfa				
Male	21 (5.0%)	12.3 ± 2.4	39 ± 18.64	4839.52 ± 8867.1
Female	14 (3.3%)	10.7 ± 2-7	42 ± 16.46	4178.62 ± 3962.3
Red Sea				
Male	72 (17.2%)	11.6 ± 2.5	39 ± 17.38	7379.61 ± 9219.1
Female	43 (10.3%)	10.1 ± 3.1	36 ± 13.57	6067.86 ± 5663.9
Whit Nile				
Male	11 (2.6%)	12.6 ± 2.8	28 ± 17.60	5947.27 ± 4769.3
Female	5 (1.2%)	12.5 ± 1.6	35 ± 20.32	2204.00 ± 2245.7
Ad Damazin				
Male	14 (3.3%)	12.7 ± 2.1	18 ± 10.23	5010.00 ± 4120.9
Female	19 (4.6%)	11.1 ± 2.6	19 ± 9.68	3085.26 ± 3334.3
P. value		0.000	0.977	0.986

Table 2: *Demographic data among the different study sites.*

Location	Parasite Density (%)*			Total
	< 500	501 - 5001	> 5001	
Ad Damazin	10 (30.3%)	10 (30.3%)	13 (39.4%)	33 (7.9%)
Al Qadarif	10 (22.7%)	14 (31.8%)	20 (45.5%)	44 (10.5%)
Gezira	28 (24.6%)	35 (30.7%)	51 (44.7%)	114 (27.2%)
Khartoum	6 (19.4%)	6 (19.4%)	19 (61.3%)	31 (7.4%)
New Halfa	9 (25.7%)	10 (28.6%)	16 (45.7%)	35 (8.4%)
Red Sea	22 (19.1%)	34 (29.6%)	59 (51.3%)	115 (27.4%)
River Nile	7 (22.6%)	9 (29.0%)	15 (48.4%)	31 (7.4%)
White Nile	4 (25.0%)	5 (31.2%)	7 (43.8%)	16 (3.8%)
Total	96 (22.9%)	123 (29.4%)	200 (47.7%)	419 (100%)

Table 3: *Parasite density detected by microscopy from the different study sites.*

**P. value = 0.977*

Age groups	Parasite Density*			Total
	< 500	501 - 5001	> 5001	
1 - 10 years	15 (19.2%)	26 (33.3%)	37 (47.4%)	78 (18.6%)
11 - 20 years	31 (33.0%)	26 (27.7%)	37 (39.4%)	94 (22.4%)
21 - 30 years	18 (18.8%)	30 (31.2%)	48 (50.0%)	96 (22.9%)
31 - 40 years	12 (19.7%)	13 (21.3%)	36 (59.0%)	61 (14.6%)
41 - 50 years	10 (20.8%)	17 (35.4%)	21 (43.8%)	48 (11.5%)
> 50 years	10 (23.8%)	11 (26.2%)	21 (50%)	42 (10.0%)
Total	96 (22.9%)	123 (29.4%)	200 (47.7%)	419 (100%)

Table 4: *Age group among Parasite density.*

**P. value = 0.301*

Analysis of *P. falciparum* MSP-1 gene alleles' frequency

In this study, we investigated the genetic diversity of *P. falciparum* based on the frequency distribution of the MSP-1 gene alleles across the different locations of Sudan and also across different age groups. Alleles frequency was defined as the number of alleles that been encountered from a single sample. Our results were in agreement with other studies investigated the diversity of *P. falciparum* in relation to age as a susceptibility factor for harboring the infection [40,41], also agree with previous study investigated the disease severity and founded no significant of the parasite density with age [42,43].

Interestingly, the results of MSP-1 allelic diversity were similar to previous results of several studies investigated the allelic diversity of MSP-1 among different locations. Alleles of MSP-1 loci were classified according to the size of their PCR-amplified fragments. The genetic marker and the corresponding allelic families were very diverse. A total of 19 different genotypes for MSP-1 gene were detected across the study sites, in which 10 were belonging to the MAD20 family with band size ranging from 200 - 650 bp, 8 were belonging to K1 allelic family with band size ranging from 300-700 bp and the last allelic family RO33 were only present with single allelic genotype with a band size of 200 bp. Allele polymorphism was detected by counting the different band sizes obtained by PCR for each of the different allelic families, MAD20 was having one allele per infection in a total of 241 patient, while in 113 it was having two different alleles per infection, and a total of 65 patient were having allelic infections of the other allelic families i.e. K1 and RO33. These results correspond to previous reports in which MAD20 and K1 were reported to be the most frequent allelic families in India [44-46], Also, in a previous study conducted in Sudan, MAD20 was the most prevalent allelic family [33]. also were similar to other studies reported from different regions showing that MAD20 is the most Prevalent [47-49] and disagree with other study [41]. For K1 allelic family, single infections were detected among 256 while multiple infections were detected among 45 patients. On the other hand, RO33 allelic family was only having single band, detected in a total of 221 patients. And a total of 198 patients were negative for the presence of RO33 allele presence. RO33 allelic family was most prevalent in Gezira when compared to its prevalence in the different locations. Similar results were also observed in previous reports from Ghana [50] and India [51]. This variation in the number of genotypes isolated were different comparing to other studies, in which K1 genotypes were most frequent than MAD20 and RO33, single infections were detected among 256 while multiple infections were detected among 45 patients which is in accordance with Kanungnit., *et al.* 2014 reported from Thai-Myanmar borders [52], Honduras [53], Senegal [54], India [55], Peru [56], Haiti [48], Lao PDR [57], Western Cambodia [58], Pakistan [47], Gabon [59] and Kenya [60]. While in other studies in Tanzania [40], Thailand [61], India [51], Myanmar [62], Bangui [63] and Mauritania [64] MAD20 was the most frequent genotype. In this study, RO33 allelic family was only having single band, detected in a total of 221 patients and a total of 198 patients were negative for the presence of RO33 allele, this results were in accordance with other reports showed RO33 was the most infrequent MSP-1 allelic family [40] whereas in some other studies RO33 was not recorded [61,62] and another study showed that RO33 is the most prevalent allele [65].

Influence of age and gender on genetic diversity

The distribution of MSP-1 alleles over the different age groups showed that MAD20 allelic family was the most frequent among the age group of 1 - 10 years with frequency of 64.1%. K1 was more prevalent among the ages of 21 - 30 years, 31 - 40 years and 41 - 50 years with frequencies of 62.5%, 60.7% and 68.8%, respectively. For RO33, the alleles' distribution was most among ages between 11 - 20 years and ages above 50 years with frequency of 58.5% and 64.3% respectively. Although, with the varieties on the distribution of MSP-1 alleles among the different age groups, no significant difference was observed, P value = 0.622. These findings agree with previous studies done in Sudan studying the genetic diversity of *P. falciparum* and its interference with age and gender [33,66-68].

Multiplicity of infection among age groups were insignificantly different, P value = 0.742. although showing higher value in the age group of 21-30 years with value of 2.51 and lower value among 1 - 10 years and 31 - 40 years with value of 2.44 (Table 5). When stratifying the gender of the study participants, K1 and RO33 allelic families were found to be more frequent among males than in females with frequency of (60.8%) for K1 and (56.8%) for RO33 in males and (61.5%) for K1 and (46.7%) for RO33 in females. While MAD20 allelic family was most frequent in females with frequency of (65.7%) than in males with frequency of (52.0%). Multiple parasite infection (MOI) was found to be having higher value in males (2.51) than in females (2.41). The different distribution of MSP-1 allelic families among males and females showed a significant difference for both MAD20 and RO33 allelic families, P values = 0.021 and 0.027 respectively, while K1 showed insignificant difference, P value = 0.755 (Table 6). Various studies were in harmony in reporting similar results [33,67,68].

Age groups	RO33		MAD20			K1			MOI
	0	1	0	1	2	0	1	2	
1 - 10 years	38 (48.7%)	40 (51.3%)	11 (14.1%)	50 (64.1%)	17 (21.8%)	22 (28.2%)	46 (59.0%)	10 (12.8%)	2.44
11 - 20 years	39 (41.5%)	55 (58.5%)	22 (23.4%)	48 (51.1%)	24 (25.5%)	26 (27.7%)	54 (57.4%)	14 (14.9%)	2.48
21 - 30 years	52 (54.2%)	44 (45.8%)	8 (8.3%)	59 (61.5%)	29 (30.2%)	26 (27.1%)	60 (62.5%)	10 (10.4%)	2.51
31 - 40 years	35 (57.4%)	26 (42.6%)	6 (9.8%)	32 (52.5%)	23 (37.7%)	20 (32.8%)	37 (60.7%)	4 (6.6%)	2.44
41 - 50 years	19 (39.6%)	29 (60.4%)	9 (18.8%)	30 (62.5%)	9 (18.8%)	11 (22.9%)	33 (68.8%)	4 (8.3%)	2.46
> 50 years	15 (35.7%)	27 (64.3%)	9 (21.4%)	22 (52.4%)	11 (26.2%)	13 (31.0%)	26 (61.9%)	3 (7.1%)	2.45
Total	198 (47.3%)	221 (52.7%)	65 (15.5%)	241 (57.5%)	113 (27.0%)	118 (28.2%)	256 (61.1%)	45 (10.7%)	2.47
P. value	0.622								

Table 5: *Age group among MSP-1 alleles.*

Gender	RO33		MAD20			K1			MOI
	0	1	0	1	2	0	1	2	
Male	108 (43.2%)	142 (56.8%)	44 (17.6%)	130 (52.0%)	76 (30.4%)	73 (29.2%)	152 (60.8%)	25 (10.0%)	2.51
Female	90 (53.3%)	79 (46.7%)	21 (12.4%)	111 (65.7%)	37 (21.9%)	45 (26.6%)	104 (61.5%)	20 (11.8%)	2.41
Total	198 (47.3%)	221 (52.7%)	65 (15.5%)	241 (57.5%)	113 (27.0%)	118 (28.2%)	256 (61.1%)	45 (10.7%)	2.47
P. value	0.027		0.021			0.755			

Table 6: *MSP-1 allele's frequencies among participants.*

Influence of the parasitemia on the genetic diversity

K1 allele was the most predominant allele among the parasite density of less than 500 parasites per 200 WBCs, while MAD20 was the most predominant among the parasite density group of more than 500 and less than 5001 parasite per 200 WBCs. Also, noted that RO33 was the lowest frequent allelic family when categorizing allele frequency based on the parasite density.

Analysis of the distribution of MSP-1 alleles across the different parasite density groups showed no significant difference for the different MSP-1 alleles K1, MAD20 and RO33 with P value of 0.223, 0.652 and 0.707 respectively. Although, MOI were of no significant difference between the different parasites densities, parasitemia of more than 5001 parasites per 200 WBCs was having a higher multiple parasite allele infection (Table 7). Previous reports in Sudan were in agreement with these results but only disagree with the overall prevalence of MSP-1 across the studied sites [33,67].

Parasite Density	RO33		MAD20			K1			MOI
	0	1	0	1	2	0	1	2	
< 500	44 (45.8%)	52 (54.2%)	16 (16.7%)	57 (59.4%)	23 (24.0%)	23 (24.0%)	66 (68.8%)	7 (7.3%)	2.45
501 - 5001	62 (50.4%)	61 (49.6%)	20 (16.3%)	74 (60.2%)	29 (23.6%)	32 (26.0%)	73 (59.3%)	18 (14.6%)	2.46
> 5001	92 (46.0%)	108 (54.0%)	29 (14.5%)	110 (55.0%)	61 (30.5%)	63 (31.5%)	117 (58.5%)	20 (10.0%)	2.49
Total	198 (47.3%)	221 (52.7%)	65 (15.5%)	241 (57.5%)	113 (27.0%)	118 (28.2%)	256 (61.1%)	45 (10.7%)	2.47
P. value	0.652		0.223			0.707			

Table 7: *Parasite density among MSP-1 alleles.*

Genetic diversity of MSP-1 among the different study sites

The distributions of MSP-1 alleles among the different study sites were different from site to another. Across the 8 different sites K1 allele was the most predominant allelic family with different frequencies. In White Nile K1 single allele infection had the lowest frequency (50.0%), while in Red Sea it was the highest frequency (67.0%). On the other locations K1 frequency were vary, in Ad Damazin was (60.6%), Al Qadarif was (61.4%), Khartoum (51.6%), New halfa (54.3%) and River Nile was (51.6%). MAD20 allelic family single infections were distributed across the study sites with different frequencies, Al Qadarif was having the highest frequency (72.7%) followed by Khartoum (67.7%), while White Nile had the lowest frequency (37.5%). In Red Sea MAD20 allele was the most frequent allele with frequency of 97 followed by K1 with frequency of 84, while in White Nile MAD20 and K1 were having the less number of alleles per

infection. RO33 allelic family was most prevalent in Gezira when compared to its prevalence in the different locations. The frequency of MSP-1 alleles observed showed that MAD20 is the most common allele followed by K1 allele and lastly less prevalent is RO33 allelic family.

RO33 allelic family was only presence as a single allele infection i.e. no other allele with different band size on the PCR detected, RO33 showed the highest frequency in samples collected from Gezira with frequency of (62.3%) and the lowest frequency was observed in White Nile with frequency of (37.5%). MOI values showed an insignificant difference among the locations although having the lowest value in samples collected from Gezira i.e. MOI = 2.3.

MSP-1 alleles frequency showed statistically insignificant difference among the different locations, P value = 0.609. The prevalence of each allele separately also showed insignificant difference; RO33 (P value = 0.419), MAD20 (P value = 0.800) and K1 allele (P value = 0.076) (Table 8).

Location	RO33		MAD20			K1			MOI
	0	1	0	1	2	0	1	2	
Ad Damazin	17 (51.5%)	16 (48.5%)	6 (18.2%)	18 (54.5%)	9 (27.3%)	7 (21.2%)	20 (60.6%)	6 (18.2%)	2.54
Al Qadarif	21 (47.7%)	23 (52.3%)	3 (6.8%)	32 (72.7%)	9 (20.5%)	11 (25.0%)	27 (61.4%)	6 (13.6%)	2.54
Gezira	43 (37.7%)	71 (62.3%)	23 (20.2%)	69 (60.5%)	22 (19.3%)	35 (30.7%)	73 (64.0%)	6 (5.3%)	2.35
Khartoum	15 (48.4%)	16 (51.6%)	5 (16.1%)	21 (67.7%)	5 (16.1%)	7 (22.6%)	16 (51.6%)	8 (25.8%)	2.54
New halfa	17 (48.6%)	18 (51.4%)	4 (11.4%)	15 (42.9%)	16 (45.7%)	13 (37.1%)	19 (54.3%)	3 (8.6%)	2.57
Red sea	58 (50.4%)	57 (49.6%)	18 (15.7%)	62 (53.9%)	35 (30.4%)	31 (27.0%)	77 (67.0%)	7 (6.1%)	2.43
River Nile	17 (54.8%)	14 (45.2%)	3 (9.7%)	18 (58.1%)	10 (32.3%)	9 (29.0%)	16 (51.6%)	6 (19.4%)	2.58
White Nile	10 (62.5%)	6 (37.5%)	3 (18.8%)	6 (37.5%)	7 (43.8%)	5 (31.2%)	8 (50.0%)	3 (18.8%)	2.51
Total	198 (47.3%)	221 (52.7%)	65 (15.5%)	241 (57.5%)	113 (27.0%)	118 (28.2%)	256 (61.1%)	45 (10.7%)	2.46
P. value	0.419		0.800			0.076			

Table 8: *Describe the allelic diversity of MSP-1 allelic families among the study sites.*

Conclusions

Although the genetic diversity of *P. falciparum* showed great variation from location to another, with relation to age or/and gender, this study illustrated high polymorphism with different alleles at MSP-1 loci. Infections were highly complex, malaria attacks being characterized by a multiple genotypes per infection. Clonal variation increases infection complexity and makes polymorphism analysis much difficult. These results illustrate *P. falciparum* complexity in high transmission areas with variation on location leading to the difference of the *P. falciparum* strains circulating in that specific region.

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Conflict of Interest

The authors declare there is no any conflict of interest exists.

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Volume 14 Issue 9 September 2018