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RESEARCH ARTICLE OPEN ACCESS

Determining the Association Between MSP1/2 Variant and Multiplicity of Infection on Incidence of Severe Malaria in Sudanese Children in Gezira State, Sudan

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Abstract

The Almanagil province located in Gezira scheme, Gezira state, Sudan, represents a suitable environment for the breeding of malaria-carrying mosquitoes. An estimated 5.9% of Sudanese people suffer from malaria, with 87.6% of cases caused by *Plasmodium falciparum* and 12.4% by *Plasmodium vivax*. Clinical manifestation of malaria cases range from mild uncomplicated to severe and fatal complications and the genetic variants and multiplicity of falciparum infection can worsen the manifestations of malaria. The objective of this work is to determine the degree of genetic variation in *P. falciparum* infection in a high-transmission region of central Sudan by analyzing merozoite surface protein-1 (msp1) and merozoite surface protein-2 (msp2) variations. During the rainy season of 2022, Eighty-nine children with confirmed severe falciparum malaria whom admitted to Almanagil Pediatric Hospital were included in this study. Dry blood spots were used to extract the DNA and amplification of three msp1 and two of msp2 allelic subfamilies, namely K1, RO33 and MAD20 and FC27 and IC/3D7 respectively. The data was analyzed by using SPSS computer program (v 23.0). The three genetic subfamilies of msp1 (K1, RO33 and MAD20) and the two alleles of msp2 (FC27 and IC/3D7) were identified. Msp1 variants represent K1 (64/89, 71.9%), RO33 (56/89, 62.9%) and MAD20 (72/89, 80.9%), while msp2 diversity represents ICI/3D7 (52/89, 58.4%), FC27 (62/89, 69.6%) and ICI/3D7/FC27(33/89, 37.1%). The MAD20 and FC27 showed high genetic diversity among both genes, respectively. RO33 allele shows a strong association with severity of *falciparum malaria* (OR 2.572, P 0.045), while the K1 was the lowest risk factor for malaria severity. The allele subfamily K1 and MAD20 of msp1 were associated with hypoglycemia (OR 4.21 and 2.91), respectively. Our study revealed high genetic polymorphisms of msp1 and msp2. Among Central Sudanese children with high MOI of *P. falciparum* isolates, there was a significant frequency of msp1, a strong association between the K1 allele and hypoglycemia, and a substantial association between the RO33 and MAD20 alleles with the severity of the infection. These findings could help develop malaria control strategies.

Keywords: *Plasmodium falciparum*, Malaria, MSP-1, MSP2, Children, Sudan

INTRODUCTION

The human malaria parasite *Plasmodium falciparum* is a single-celled eukaryotic organism. Clinical signs of *Falciparum malaria* range from asymptomatic parasitemia to fever, headache, exhaustion, abdominal discomfort, vomiting, and diarrhea in the case of uncomplicated disease to coma and death in the case of complicated disease. Malaria burden is high, in some countries, worldwide, which causes high morbidity and mortality, approximately two hundred forty nine million cases and about 93.6% of cases and 95.4% of deaths globally; 78.1% of all deaths in African region were among children aged under 5 years in 2022 according to the WHO.[1]

The overall malaria prevalence in Sudan is 5.9%, with 87.6% attributable to *P. falciparum* and 5% attributable to a mixed infection with *P. vivax.*[2] A knowledge of the genetic structure of the malaria parasite population is necessary for determining its dynamics, diversity, distribution, and epidemiology[3,4] the identification of genetic diversity can help in controlling and elimination of malaria. Factors in the host, the parasite, and the environment all contribute to the severity of a malaria infection, the outcome of malaria treatment and recovery may affected by the variations in parasites like merozoite surface protein polymorphism (MSP). In many geographical contexts, *P. falciparum* displays highly variable genetic diversity.[5] The virulence and complex diversity of *P. falciparum* may interpret its ability to exhibit with a variety of clinical symptoms and signs. Malaria is made worse by the production of several chemokines and lymphokines, which may be affected by the multiplicity of infections.[6] At the surface of the Plasmodium merozoite, the MSP1 is the most important protein.[5,7] For Plasmodium blood stages, MSP-1 is necessary[8] and has an important role in erythrocyte invasion,[9] particularly at the merozoite's first contact with RBCs,[10,11] In addition to red blood cell rupture and parasite egress.[12] *In vitro* investigations have shown that antibodies to MSP-1 may reduce parasite development,[9,13] and other epidemiological

studies have linked these antibodies to protection against malaria.[14,15,16] The diversity of protein on the merozoite surface *P. falciparum* may affect the transmission and immune evasion.[17] Many merozoite surface proteins of *P. falciparum* linked to virulence and parasite infection severity have been identified.[18,19] Vaccines have been developed targeting the proteins that are produced by the *P. parasite* throughout its pre-erthrocytic blood stages. The MSP-1 gene spans 17 sequence blocks on chromosome 9; of these, block 2 is the most polymorphic, splitting into the MAD20, K1, and RO33[20] allelic groups. The gene for the msp2 glycoprotein is located on chromosome 2. The protein has five distinct regions, the most pleomorphic of which is the middle region (block 3), and it has a molecular weight of about 30 kDa. There are two groups of msp2 alleles: 3D7/IC1 and FC27. The dynamics of malaria transmission and its virulence were predicted using the msp2 subfamily.[17]

The multiplicity of *P. falciparum* infection (MOI) is a challenge in treating the malaria infection extremely in area with high transmission season, which can aggravate resistant strain to antimalarial chemotherapy.[21,22] Microscopic analysis of thick and thin blood films is the "gold standard" method for parasite diagnosis and therapy recommendations.[23] There is a lack of information on the msp1 and msp2 genetic variants in *P. falciparum* that circulate in areas like Gezira state in central Sudan, even though malaria is very prevalent in that country. This study details the frequency and variety of the msp1 and msp2 genes in *P. falciparum* parasites found in *P. falciparum* positive patients in Almanagil, Sudan. It also examines the relationship between these alleles and the severity of sickness and the number of infections. These findings could help in treating, elimination and controling malaria in our study area.

MATERIALS AND METHODS

Study area and population

This was a hospital based cross-sectional study conducted in Almanagil, Gezira State, Central Sudan, between September and December 2022. The city located south of Khartoum about 156 kilometers in Gezira Agricultural Scheme's irrigated region, where a highly variable mesoendemic to hyperendemic malaria transmission pattern exists. Malaria is endemic in this region all year long, with *P. falciparum* being the most common kind and *P. vivax* being the second. Children who were treated at Almanagil Pediatric Teaching Hospital for malaria and meeting the inclusion and exclusion criteria were recruited for this study. Blood films (thin and thick) were stained with 10% Giemsa's at pH 7.2 and then examined microscopically by expert technician for the presence of malaria parasites; 200 fields under 100 x magnification was examined from the thick film before the slide was considered negative. The parasitemia was determined by counting only the a sexual stages against 200 white blood cells (WBC) and the number of parasites multiplied by 8000 then divided by 200, assuming the average of total WBC count of individuals equal to 8000 cells.[12] When microscopic examination revealed *P. falciparum* mono-infection in any child younger than 18 years old, regardless of gender, were included in this

Table 1. primers sequences using to amplify Pfmsp1

PCR cycle	The Primer	The sequence
First PCR	M1-OF	F:5- -CTAGAAGCTTTAGAAGATGCAGTATTG-3-
msp1	M1-OR	R:5- -CTTAAATAGTATTCTAATTCAAGTGGATCA-3-
Second PCR	M1-KF	5- -AAATGAAGAAGAAATTACTACAAAAGGTGC-3-
	M1-KR	5- -GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3-
	M1-MF	5- -AAATGAAGGAACAAGTGGAACAGCTGTTAC-3-
	M1-MR	5- -ATCTGAAGGATTTGTACGTCTTGAATTACC-3-
	M1-RF	5- -TAAAGGATGGAGCAAATACTCAAGTTGTTG-3-
	M1-RR	5- -CATCTGAAGGATTTGCAGCACCTGGAGATC-3-

The PCR cycle condition performed as described by Ananias et al. The product size was 1200-1400 bp[27]

study. After explaining the objectives of the study, how to collect the sample, and the minor harm that could occur, the verbal consent was taken from one of the parents, and then the patients were included in the study. The demographic, clinical and laboratory results were collected by using a well-structured questionnaire.

Sample collection

Under aseptic conditions, containers containing EDTA were used to collect 3 mL of venous blood from each participant, for 89 samples. Prior to storage in appropriate sealed containers with desiccant, blood samples were spotted onto filter paper (Whatmann® No. 3, Sigma-Aldrich, Germany), allowed to air dry, and then preserved.[24] Blood films were stained by Giemsa and parasite density was quantified according to Monica-cheesbrough as mentioned above.

DNA extraction

Small pieces of filter paper with dried blood spots (DBSs) on them were sniped off using scissors and placed into 1.5 mL microtubes. After adding about 500 µL of dH_2O to the test tubes, they were allowed to sit at room temperature for a time of 5 minutes. We threw out the dH_2O and did it twice more. Five hundred µL of extraction buffer (PBS or 10 mM Tris-EDTA (TE)) was added to filter paper. The filter sheets were treated with 100 µL of red blood cell lysis solution, then 10 µL of the same buffer was mixed with 1 mg/mL of proteinase K, and the combination was left to incubate at 37°C overnight. A clean pipette tip was used to repeatedly push the filter papers against the tube bottom. Cell lysates were heated to 95 degrees Celsius for 15 minutes to maximize extraction. The filter sheets were discarded after a short centrifugation (only 2-3 seconds) and the DNA-rich supernatant was frozen at 80°C for further analysis.[25,26]

Electrophoresis procedure for msp1 gene subfamilies

The primers sequences as shown in Table 1, were used for the amplification of msp1 of *P. falciparum* and subfamilies (Table 2 and 3).

The amplified products were mixed thoroughly with loading buffer, which included

Table 2. The PCR conditions used to amplify msp1 of *P. falciparum*

msp1 outer	X1	Temperature	
H_2O	9.0	95°C/5 min	
Mix	4.0	94°C /30 sec	
msp1-O1	1.0	55°C/30 sec	35 Cycles
msp1-O2	1.0	72°C/1.0 min	
DNA	5.0	72°C/5 min	

Table 3. Amplification of Pfmsp1 gene subfamily

msp1 nested	X1	nested Pfmsp1 (K1, RO33, and MAD 20)	
H_20	12.0	95°C/5 min.	
Mix	4.0	94°C /30 sec.	
MSP1-O1	1.0	56°C/30 sec.	35 Cycles
MSP1-O2	1.0	72°C/1.5 min.	
DNA	2.0	72°C/5 min.	

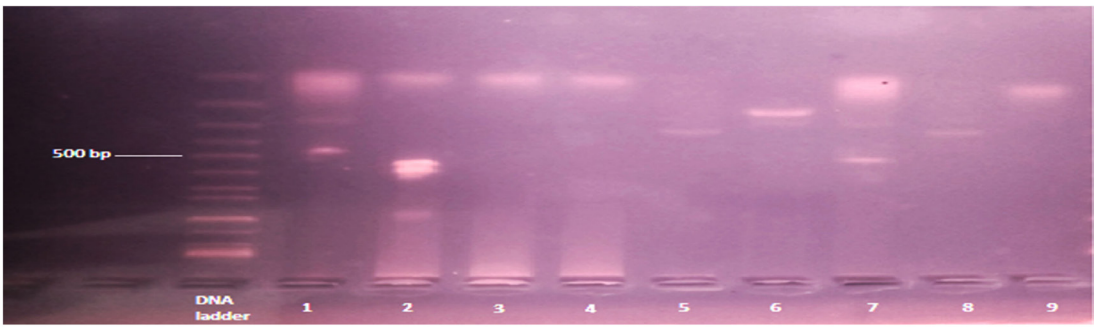

Figure 1. Shows RO33 allele of msp1: The band size ranged between 100-500 bp. numbers 1 (size 100/250 bp), 2 (size 100/500 bp) and 7 (size 100/500 bp) are positive with multiple clones, while the remaining was monoclonal RO33 allele

Table 4. Characteristics of study participants

		Frequency	Percent (%)
Age ranges	<5 Years	47	52.8
	≥5 to ≤12 years	34	38.2
	>12 to ≤18 years	8	8.9
	Total	89	100
Gender	Male	48	53.9
	Female	41	46.1
	Total	89	100

Bromophenol blue 0.25%, ficoll 25%, 10 mM Tres, and 1 mM EDTA. Five microliters of each product were then added to a 2% agarose gel. The gel was electrophoresed using a buffer containing Tris-Borate-EDTA, which contained 0.09M boric acid, 0.09M Tris, and 0.002M EDTA (Figure 1). The DNA fragments were stained with ethidium bromide. The marker of DNA (100 pb ladder) was used to identify the fragments size (Boehringer Mannheim, U.K). The condition of electrophoresis gel as follows: run for 10-120 minutes in buffer and 120 volts. The products size was visualized by the photo documentation system. R fragment sizes were estimated according to migration (Amersham Pharmacia Biotech).

msp2 amplification and genotyping

The genus and species-specific primers were used to identify the species of Plasmodium by using 18S rRNA gene based nested PCR as described by Atroosh *et al*.[28] The nested PCR was used to amplify the polymorphic area of Pfmsp2 (block 3) and subfamily of alleles (FC27 and IC1/3D7). The PCR conditions and reactions were as follows: 1. Included in the 25 µl reaction volume are 2 µl of falciparum DNA, 0.2 mM of dNTPs, 2 mM of MgCl2, 1 µl of each primer, and 1 unit of Taq Polymerase (Vivantis, Selangor Darul Ehsan, Malaysia). Second, the outer PCR needs the following conditions: three minutes of denaturation at 94°C, thirty seconds of annealing at 55°C, and two minutes of extension at 72°C. In a thermal cycler (SensoQuest, Gottingen, Germany), the most recent cycle extension was at 72°C for 10 minutes (Figure 2 and 3).

Table 5. The frequency of the msp1 and msp2 gene alleles among study subjects

Alleles	Status	Frequency	Percent (%)
K1	+	64	71.9
	-	25	28.1
MAD20	+	72	80.9
	-	17	19.1
RO33	+	56	62.9
	-	33	37.1
FC27	+	62	69.7
	-	27	30.3
ICI/3D7	+	52	58.4
	-	37	41.6

Table 6. The Pfmsp1 and Pfmsp2 multiplicity of *P. falciparum* infection

89i	Frequency	Percent
K1+RO33	7	7.8
K1+MAD20	6	6.7
MAD20+RO33	5	5.6
MOI of msp1	1.35	
IC1/3D7/FC27	33	37.1
Multiclonal isolates of IC1/3D7/FC27	14	15.7
Mix infections IC1/3D7/FC27	35	39.3
MOI of msp2	1.48	

Detection of FC27and IC1/3D7alleles

As mentioned before, in the electrophoresis technique for the msp1 gene subfamily, the amplified PCR products were subjected to electrical analysis. The method for determining the multiplicity of infection (MOI) was outlined by Abukari *et al*.[29]

Data analysis

The data was analyzed by using SPSS program (version 20.0). Allelic distribution of both genes was counted as described by Abukari *et al*.[29] There was an application of the 95% confidence interval and degree of certainty.

Ethical statement

In Gezira State, Sudan, our research was authorized by the Scientific and Research Ethics Committee. The patient's parent or legal guardian

Table 7. Shows the association between msp1 and msp2 allelic subfamilies and severity of falciparum infection

Allelic subfamilies of msp1 gene		Severity of infection				Total	P. value	Odd ratio
		Severe		Mild				
		Frequency	%	Frequency	%			
RO33	+	22	76	33	55	55	0.045	2.572
	-	7	24	27	45	34		
	Total	29	100	60	100	89		
K1	+	21	72	44	73	65	0.559	0.955
	-	8	28	16	27	24		
	Total	29	100	60	100	89		
MAD20	+	24	83	48	80	72	0.037	2.900
	-	5	17	12	20	17		
	Total	29	100	60	100	89		
FC27	+	21	72	41	68	62	0.835	0.977
	-	8	28	19	32	27		
	Total	29	100	60	100	89		
ICI/3D7	+	17	59	35	58	52	0.642	0.819
	-	12	41	25	41	37		
	Total	29	100	60	100	89		

Table 8. Shows the association between msp1 allelic subfamilies and severe anemia and hypoglycemia

Complications		Present		Absent		P. Value	Odd ratio
		Count	%	Count	%		
				K1 allele			
Severe Anemia	Yes	17	26.2	5	20.8	0.414	1.40
	No	48	73.8	19	79.2		
	Total	65	100.0	24	100.0		
Hypoglycemia	Yes	10	15.4	1	4.2	0.142	4.21
	No	55	84.6	23	95.8		
	Total	65	100.0	24	100.0		
				MAD20 allele			
Severe Anemia	Yes	4	24	18	25	0.586	0.923
	No	13	76	54	75		
	Total	17	100	72	100		
Hypoglycemia	Yes	4	24	7	10	0.128	2.91
	No	13	76	65	90		
	Total	17	100	72	100		
				RO33 allele			
Severe Anemia	Yes	12	22	10	29	0.228	0.670
	No	43	78	24	71		
	Total	55	100	34	100		
Hypoglycemia	Yes	5	9	6	18	0.194	0.467
	No	50	91	28	82		
	Total	55	100	34	100		

Table 9. The association between msp2 allelic subfamilies and severe anemia and hypoglycemia

Complications		Present		Absent		P. Value	Odd ratio
		Count	%	Count	%		
				FC27 allele			
Severe Anemia	Yes	14	22.5	6	22.2	0.575	0.777
	No	48	77.5	21	77.8		
	Total	62	100.0	27	100.0		
Hypoglycemia	Yes	7	11.3	5	18.5	0.139	0.761
	No	55	88.7	22	81.5		
	Total	62	100.0	27	100.0		
				3D7 allele			
Severe Anemia	Yes	11	21.2	4	10.8	0.438	0.572
	No	41	78.8	33	89.2		
	Total	52	100	37	100		
Hypoglycemia	Yes	4	9	6	18	0.394	0.465
	No	48	91	28	82		
	Total	52	100	37	100		

gave their informed permission. Throughout, we made sure that patient records remained secret.

RESULTS

Out of 89 children with falciparum malaria participated in this study. (48/89, 53.9%) were male and (41/89, 46.1%) were female. The common age group was less than 5 years representative (47/89, 52.8%) (Table 4).

The results of genetic diversity among the isolates of falciparum malaria showed the MAD20 was (72/89, 80.9%), K1 (64/89, 71.9%) and RO33 (56/89, 62.9%) of msp1 and FC27 (62/89, 69.97%) and ICI/3D7 52/89, 58.4%) of msp2. The MAD20 and FC27 showed high genetic diversity

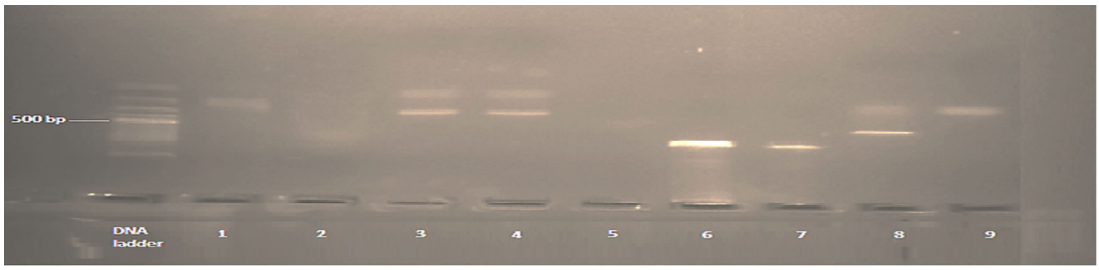

Figure 2. Shows K1 allele of msp1. The band size ranged between 100-650 bp. Numbers 3 (fragment size 300/500 bp), 4 (fragment size 300/500 bp) and 8 (fragment size 600/500 bp) are positive K1 allele with multiple clones, while the remaining were positive

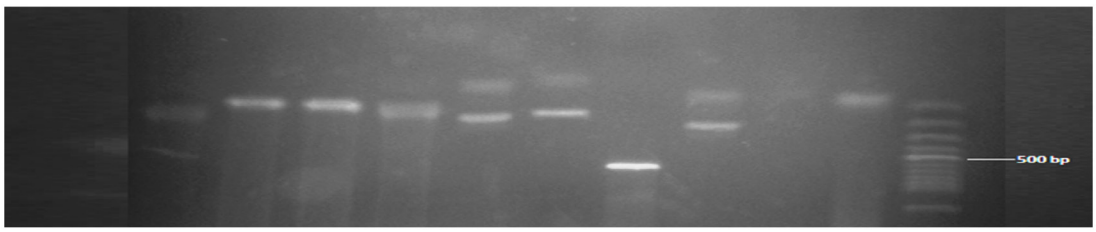

Figure 3. Showed MAD20 allele of msp1. The band size ranged between 90-600 bp. Numbers 5 (fragment size 90/100 bp), 6 (fragment size 90/100 bp), 8 (fragment size 100/300 bp) were positive with multiple clones, while the remaining were positive monoclonal MAD20 all

among both msp1 and msp2 alleles, respectively, as shown in Table 5.

Our results revealed that the multiplicity of K1+RO33, K1+MAD20 and MAD20 of msp1 were 7.8%, 6.7% and 5.6% respectively, while the multiplicity of ICI/3D7+FC27 was 37.1% of msp2 as shown in Table 6.

The result showed that the percentage of msp1 alleles among patients with severe malaria were (24/29, 83%), (22/29, 76%), and (21/29, 72%) for MAD20, RO33 and K1, respectively, while the correlation between the presence of the MAD20 followed by RO33 alleles and the severity of illness P = 0.037, Odd ratio 2.900 and P=0.045; Odds ratio = 2.572 respectively. Also, the percentage of msp2 alleles among severe malaria cases was 72% for FC27 and 59% for 3D7, when compared between both types of malaria infection Table 7.

Patients with severe malaria showed high risk for hypoglycemia with (Odd ratio 4.2 and 2.91) for K1 and MAD20 alleles of msp1, respectively, while severe anemia was slightly liked to msp1-K1 allele as shown in Table 8.

Among patients carrying FC27 and ICI/3D7 alleles of msp2, showed no association between genetic diversity of msp2 and severity of malaria infection (Odd ratio 0.777 and 0.572). Also, there was no association between FC27 and ICI/3D7 alleles of msp2 and hypoglycemia (Odd ratio 0.761 and 0.465), respectively, as shown in Table 9.

DISCUSSION

Human erythrocyte invasion by *P. falciparum* is likely facilitated by the merozoite surface protein 1 and 2 (MSP1 and MSP2). Examining the polymorphism and the fluctuating variety of this crucial gene in malaria-endemic locations could shed light on the disease's toxicity, epidemiology, and diagnosis.[30]

The MSP1 gene is located on chromosome 9 and encodes the major surface protein 1 (MSP1) of *P. falciparum*. This protein is composed of 17 blocks of sequences that are separated by conserved regions.[17] It is often targeted by the immune system and plays a crucial role in erythrocyte invasion.[17,31] The MSP2 gene is located on chromosome 2, and it encodes merozoite surface protein 2, the second most abundant Glycophosphatidylinositol (GPI) anchored merozoite surface protein. The gene is composed of five blocks, with the greatest polymorphism occurring in the middle block, block 3.[32] It is present in two allelic subfamilies namely (3D7-like and FC27-like).[33]

In our research region, we found the allelic subfamilies of msp1 (K1, RO33 and MAD20) and msp2 (FC27 and 3D7). Some *falciparum malaria* isolates have shown a tendency toward predominance for msp1 and msp2, respectively; they include MAD20 and K1 of msp1 and FC27 of msp2. This conclusion was consistent with research conducted in the Republic of the Congo (Mayengue *et al*), but not with research conducted in Tanzania,[17,34] Thailand[35] and Central Africa in Sudan.[31]

The study region yielded variants of Pfmsp2 alleles, including FC27 (62/89, 69.7%) and ICI/3D7 (52/89, 58.4%), with 33 out of 89 samples (37.1%) testing positive for both FC27 and IC1/3D7. Our study area had a higher prevalence of the FC27 allelic family compared to the IC1/3D7 allelic family. This finding aligns with previous research in White Nile State in Central Sudan, Gublak, Northwest Ethiopia, and Jazan, Saudi Arabia.[36-38] On the other hand, studies in Northwest Ethiopia, Boset and Badewacho Districts, Southern Ethiopia, and Southwestern Nigeria[39,40] have shown different results. These discrepancies could be attributed to differences in transmission dynamics, sample size, or study area.

The severity of malaria is considered as defined by WHO (WHO 2015), the result showed a significant correlation between the presence of the MAD20 followed by RO33 alleles and the severity of illness P = 0.037, Odd ratio 2.900 and P=0.045; Odds ratio = 2.572 respectively, when compared between severe and mild malaria cases. Researchers have shown that individuals with the RO33 allele had higher levels of tumor necrosis factor alpha, which is linked to the development of severe malaria (Robert *et al* 1996). The link between allelic backgrounds and illness severity was investigated. Samples positive for the RO33 allelic family of MSP1 among patients with mild malaria infection were represented (33/60, 55%) and severe malaria illness were (22/29, 76%). Positive samples for the K1 allelic family were found in (21/29, 72% and 44/60, 73%) with severe

and mild malaria, respectively. The positivity of MAD20 allelic family is detected (24/29, 83%) of patients with severe malaria and (48/60, 80%) of patients with mild malaria. This contradicts recent research in Senegal that linked the K1 allelic family to severe malaria.[38,41] The current study's modest size, especially in the most severe cases of malaria, may explain this discrepancy. Consistent with previous research in Bobo-Dioulasso.[42] Severe anemia and hypoglycemia are among the most common complications of malaria in our study area. The study revealed no link between MSP1 alleles and severe anemia in individuals carrying the K1 allele (p=0.414), MAD20 (P=0.586) and RO33 (p=0.228). Our report showed no association between hypoglycemia among patients carrying K1, MAD20 or RO33 alleles p. value 0.142, 0.128 and 0.194, respectively. K1 allele is both strongly associated with hypoglycemia and severe anemia, with odds ratios of 4.21, and 1.4, respectively. The result exhibited that the subjects carrying the K1 allele of msp1 had a greater risk of hypoglycemia than severe anemia. The RO33 allele was not associated with hypoglycemia (odd ratio 0.467) or severe anemia (odd ratio 0.670). This confirms what previous studies in Indonesia have shown.[20] Our study found that 15.7% of samples had multi-clonal isolates, 39.3% had mixed infections, and the MOI was 1.48. This could be because of the high rates of malaria transmission and the frequency of multiple infections; people living in areas that typically harbor multiple parasite strains are likely to be affected. Consistent with previous research it indicates that *P. falciparum* infection diversity may vary with respect to geographic location, transmission intensity, and sample population.[43]

CONCLUSION

Our study revealed high genetic polymorphisms of msp1 and msp2. Among children in Central Sudan with high MOI of P. falciparum isolates, there was a substantial connection between the RO33 and MAD20 alleles and the severity of the illness, and a high frequency of msp1, K1 allele was related with hypoglycemia. Our findings highlight the need for constant monitoring of *P. falciparum* polymorphism in the study area and the findings could help in treating, eliminating and controling malaria in our study area.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee of Planning and Development, Ministry of Health, Gezira State, Sudan (Reference No K1/T/44).

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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