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Molecular Mutations in *pfmdr1* Gene Codons (86, 184 and 1034) of *Plasmodium falciparum* related to Resistance to different Anti-malarial Drugs in Children attending to Pediatrics Teaching Hospital, Gezira State, Sudan

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Abstract: *Malaria is still one of most problem that threating life in Sudan. Depending on the annual report for 2018, there were 1,617,499 true positive cases and 5.003 death. P.falciparum is the most prevalent species in Sudan with percentage of 91.2%.P.fMDR1which is located in chromosome 5 is still not clearly understudy regarding its role in drug resistance. Tis study aimed to detect and analyze three codons in this gene (codon 86,184, and 1034) and correlate the mutation to some drugs used in Gezira state, Sudan. Sample wre140 dried blood spot (DBS) which taken for molecular analysis (Nested PCR and RFLP).The study found that the percentage of mutation in the gene was 20.7% (it was 6.4 %in codon 86,8.6%in codon 184 and 5.7% in codon 1034). There was no significant correlation between last treatment and mutation in the codons ,yet participant who Quartum therapy as last treatment ,had the highest percentage of mutation. The study revealed that PfMDR1shoud be concerned in a much attention that by doing massive scanning for it in endemic area with high observed drug failure. Also it revealed the drug policies needed to be revised again in this endemic area.*

Keywords: Malaria, *P.falciparum, P.fMDR1*, dried blood spot, Nested PCR, RFLP, mutation, codon 86, codon 184, codon 1034.

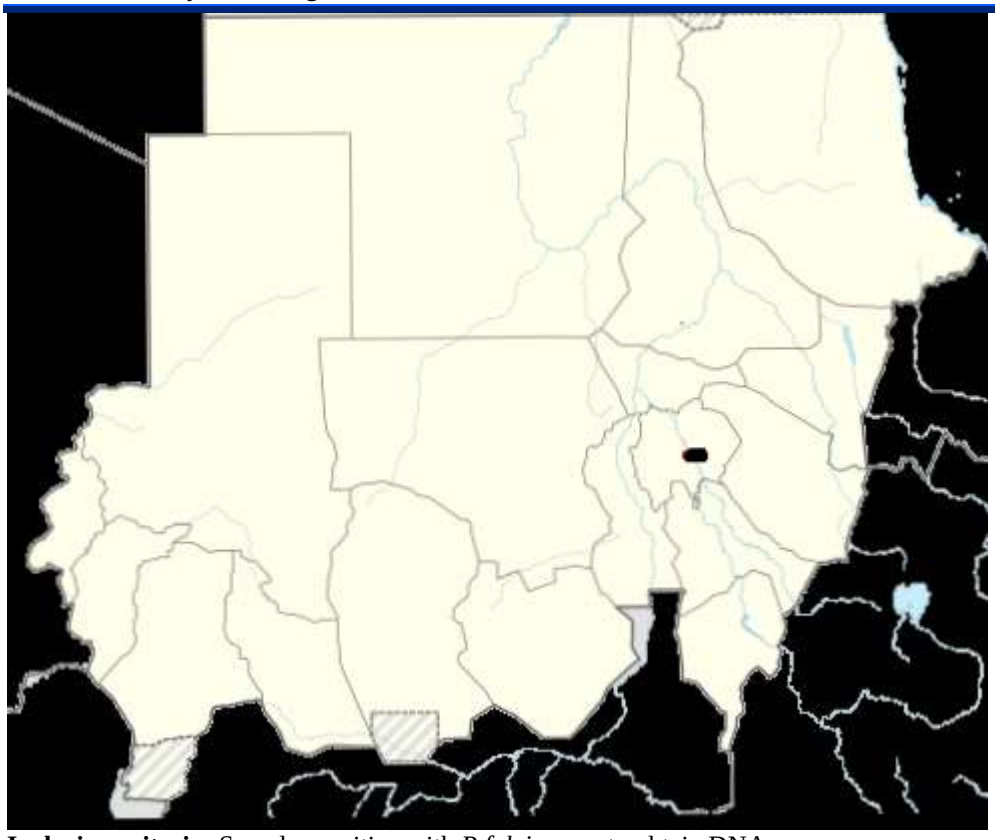
Introduction

In Sudan Malaria is still major divesting health problem .Depending on 2018 annual report, there were 1,617,499 true cases of malaria and 5.003 deaths. *Plasmodium falciparum* is the most prevalent species, 91.2% of malaria cases in Sudan are due to it (1,2) 97.9 of these cases are in Gezira state(1,3). Between 2015–2018 All progress in malaria control programs had failed lead to 228 million cases and 405, 000 deaths (4,5) .Antimalarial drug resistance regarding Plasmodium falciparum is a difficult obstacle in the plan of elimination of malaria(4,6,7). A lot of The factors are included in the elevating the rate of spread of antimalarial drug resistance include de novo mutation, human population movement and infection among migrants, drug use, and malaria transmission intensity (8,9). Nevertheless, poverty, beside to drug prescription, and misuse of drugs continue to be cornerstone in the development of resistance.(8,10). policy of malaria treatment in Sudan has been changed through times from chloroquine for the uncomplicated cases and quinine for complicated cases to sulfadoxine/ pyrimethamine (SP) antifolate drugs to other mono therapies which all failed through time until the introduction of artemisinin-based combination therapy (ACT) in 2005(11,12,13). *Pfmdr1* (*P.falciparum* Multidrug resistance 1), is located on chromosome five. It encodes a 162 kDa protein named *P. falciparum* homologue of the P-glycoprotein (Pgh1) (14,15). *Pfmdr1* Polymorphism in antimalarial drug resistance is still underestimated, out of spot light and debated (16). In this gene Five point mutations have been detected: N86Y, Y184F, S1034C, N1042D and D1246Y. The mutation in *Pfmdr1* (N86Y) linked with chloroquine failure was reported in different parts of Sudan (17,18,19,20). Many antimalarial drugs resistance such as quinine, mefloquine, halofantrine, artemisinin, lumefantrine, CQ, and amodiaquine have been linked to mutations in *pfmdr1* gene codon (N86Y, Y184F, S1034C) (8,21,22)

Methods and Materials

Study Design: The study was a cross-sectional hospital based study started in May 2021 up to April 2022 to detect *PfMDR1*mutations via the collected samples.

Study area: This study was carried out in Wad Medani City which is located in Gezira state, the center of Sudan. It lies in Blue Nile´s west bank between 33˚31' E and 14˚24' N The targeted area in Wad Medani city was Wad Medani Pediatrics teaching hospital. The area is characterized by its malaria endimicity due to its agricultural back ground .Gezira scheme for agriculture is around the area of the study and the main prevalent species is *Plasmodium falciparum*.



Inclusion criteria: Samples positive with *P.falciparum* to obtain DNA.
Exclusion Criteria: Samples negative with P.falciparum or positive for other species
Sample size: Samples were collected randomly based on two-sided hypothesis tests using Epi Info with 80% power and a confidence interval of 95%. The complete number of samples was 140 Dried blood spots on filter papers.
Ethical Considerations: Ethical approval was obtained from Faculty of Medical laboratory sciences, Gezira University and Ministry of Health Gezira State. Also an informed consent by mother, father or the responsible from the child was obtained.
Sample Collection: Finger puncture is used to obtain Capillary blood using new sterile lancet. 70% ethyl alcohol was used to clean each participant's finger. After wiping away the first drop, a triplets drops of blood added to the filter paper, let to dry and then each filter paper was sealed in special sterile plastic bag and stored at room temperature to time of lab work on it.
Laboratory Analysis
DNA Extraction was done by using TE Buffer . The circles of DBS were cut off by using sterile surgical plate, then put in a sterile eppendrof tube with 1.5 ml size. Then 100 µl of TE buffer added and the DBS were punched down with tips for many times. The mix was incubated at 97℃ for 15 minutes. Then the tube was centrifuged at high speed for 30 seconds. The supernatant which contained the DNA was used for the rest of the molecular test.
Nested-PCR was used to detect mutations in pfmdr1 and then restriction fragment length polymorphism (RFLP) was used to determine the polymorphisms. PCR reaction volume was 25-µl mixture which contain 200 µM each dNTP, 0.25 µM each specific primer, 16 mM (NH4)2SO4, 67 mM Tris-HCl (pH 8.8), 2 mM MgCl2, 1 U Taq DNA polymerase, and 2 µl of template DNA.(Table 1,Table 2 and Table 3).
Polymorphisms restriction sites at codon 86 were obtained by (ApoI and AflIII for 86N and 86Y, respectively), at codon 184 (DraI for 184F), at codon 1034 (DdeI for 1034S), (New England Biolabs, Ipswich, MA, USA) . For the ingestion , 2 µL of the restriction enzyme were added directly to 10 µL of the nested PCR product ,mixed well and incubated at 37°C for overnight. Then amplicons were analyzed by gel-electrophoresis.

Table 1: The primers sequences of codon 86 and 184

*Pf*mdr1_A1_F outer	TGTTGAAAGATGGGTAAAGAGCAGAAAGAG	1-21
*Pf*mdr1_A3_R outer	TACTTTCTTATTACATATGACACCACAAAC	648-619
*Pf*mdr1_A2_F nested	GTCAAACGTGCATTTTTTATTAATGACCATTTA	25-54
*Pf*mdr1_A4_F nested	AAAGATGGTAACCTCAGTATCAAAGAAGAG	584-556
*Pf*mdr1_NewF_outer	GTGTATTTGCTGTAAGAGCT	2834- 2853
*Pf*mdr1_NewRev_outer	GACATATTAAATAACATGGGTTC	3791-3769

Pfmdr1_N1_nested	CAGATGATGAAATGTTTAAAGATC	2921-2944
Pfmdr1_N2_nest	TAAATAACATGGGTTCTTGACT	3784-3763

Table 2: Protocol for PCR for codon 86 and184

	Codon 84	Codon 184
Initial denaturation	94°C, 3 min	94°C, 3 min
Denaturation	94°C, 30 sec	94°C, 30 sec
Annealing temperature	55°C, 30sec	60°C, 30 sec
Elongation	65°C, 1 min	65°C, 1 min
No of cycles	30	30
Final elongation	65 °C, 5 min	65 °C, 5 min

Table 3: Primers Sequences and PCR protocol for codon 1034

P*fmdr1* fragment 2, long (for SNPs at codons 1034)	Outer forward MDRFR2F1	5_-GTGTATTTGCTGTAAGAGCT	958	34 cycles of 94°C for 30 s; 55°Cfor 1 min; and 65°C for 1.5min; then 65°C for 5 min
	Outer reverse MDRFR2R1	GACATATTAAATAACATGGGTTC		
	Nested forward MDRFR2F2	CAGATGATGAAATGTTTAAAGATC	864	29 cycles of 94°C for 30 s; 60°C for 30 s; and 65°C for 1 min; then 65°C for 5 min
	Nested reverse MDRFR2R2 5	TAAATAACATGGGTTCTTGACT		

Data Analysis: Data were coded, entered, cleaned, and analyzed using excel and Statistical Package for Social Science Software version 20. Associations were considered as significant only if *P* value was equal or less than 0.05.

Results:

In this study the targeted gene was *PfMDR1* and three codons had been included and analyzed which are : N86Y, Y184F , and S1034c. Out of 140 samples ,after molecular analysis including Nested PCR and RFLP, 29 samples had been found with mutation. from those 29 samples ,9 were with mutation in codon 86, 12 in codon 184 and 8 in codon 1034.

Table 4: shows the mutation in codon 86, two samples were with hetero mutation

Mutation in codon 86

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	N/N	131	93.6	93.6	93.6
	N/Y	2	1.4	1.4	95.0
	Y/Y	7	5.0	5.0	100.0
	Total	140	100.0	100.0	

Table 5: shows the mutation in codon 184, 1 samples were with hetero mutation

Mutation in codon 184

	Frequency	Percent	Valid Percent	Cumulative Percent
Y/Y	128	91.4	91.4	91.4
Y/F	1	.7	.7	92.1
F/F	11	7.9	7.9	100.0
Total	140	100.0	100.0	

Table 6: shows the mutation in codon 1034, 1 samples were with hetero mutation

Mutation in codon 1034

	Frequency	Percent	Valid Percent	Cumulative Percent
S/S	132	94.3	94.3	94.3
S/C	1	.7	.7	95.0
C/C	7	5.0	5.0	100.0
Total	140	100.0	100.0	

Table 7: reveal the correlation between last treatment and mutation in codon 86

Last Treatment (L T)			Codon 86		Total
			Presence of mutation	Absence of mutation	
	Artusnate	Count	3	18	21
		% within L T	14.3%	85.7%	100.0 %
	Artemither	Count	0	2	2
		% within L T	0.0%	100.0%	100.0 %
	Quartum lumefantrine	Count	2	41	43
		% within L T	4.7%	95.3%	100.0 %
	QUININE	Count	0	4	4
		% within L T	0.0%	100.0%	100.0 %
	With no history of treatment	Count	4	66	70
		% within L T	5.7%	94.3%	100.0 %
Total		Count	9	131	140
		% within L T	6.4%	93.6%	100.0 %

There was no significance correlation between presence or absence of mutation in codon 86 and last treatment used by patients. P.value was (0.298)

Table 8: shows the correlation between last treatment and mutation in codon 184

Last Treatment (L T)			codon 184		Total
			Presence of mutation	Absence of mutation	
	Artusnate	Count	2	19	21
		% within L T	9.5%	90.5%	100.0 %
	Artemither	Count	0	2	2
		% within L T	0.0%	100.0%	100.0 %
	Quartum lumefantrine	Count	4	39	43
		% within L T	9.3%	90.7%	100.0 %
	QUININE	Count	1	3	4
		% within L T	25.0%	75.0%	100.0 %
		Count	5	65	70

	With no history of treatment	% within L T	7.1%	92.9%	100.0 %
Total		Count	12	128	140
		% within L T	8.6%	91.4%	100.0 %

There was no significance correlation between presence or absence of mutation in codon 184 and last treatment used by patients. P.value was (0.746)

Table 9: shows the correlation between last treatment and mutation in codon 1034

Last Treatment (L T)			Codon 1034		Total
			Presence of mutation	Absence of mutation	
	Artusnate	Count	2	19	21
		% within L T	9.5%	90.5%	100.0%
	Artemither	Count	0	2	2
		% within L T	0.0%	100.0%	100.0%
	Quartum lumefantrine	Count	1	42	43
		% within L T	2.3%	97.7%	100.0%
	QUININE	Count	0	4	4
		% within L T	0.0%	100.0%	100.0%
	With no history of treatment	Count	5	65	70
		% within L T	7.1%	92.9%	100.0%
Total		Count	8	132	140
		% within L T	5.7%	94.3%	100.0%

There was no significance correlation between presence or absence of mutation in codon 184 and last treatment used by patients. P.value was (0.943)

Discussion

Drug resistance regarding *P.falciparum* is becoming a critical issue if not solved the results will be a disaster. It is a real problem leads to many deaths not due to the disease its self only but due to the drug resistance and failure. *PfMDR1* is a gene that has been out of light spot and debated for long period of time. In this study ,three codons out of the whole five codon of this gene had been isolated and analyzed,(codon 86,184,and 1034).The percentage of the mutation in *PfMDR1* codons is 20.7% (6.4% in codon 86 , 8.6% in codon 184, and 5.7% in codon 1034) which is conceded as high incidence comparing to sample size and in compare with studies done before this study agreed with slightly decrease in percentages due to difference in the sample size, *Mohamed et al* in 2018 in Blue Nile state , Sudan had a percentage of 21.7% of mutation. Although there were no significant correlation between last treatment had been taken by participants and mutation in the gene, but it observed that most mutations were found in samples who their participants had taken artusnate or lumefantrine. Most of studies observed that mutation in codon 184 increase sensitivity to lumefantrine which disagree with this study that observed mutation in codon 184 decrease sensitivity to this drug, this may due to other reasons beside the drug resistance , it might be due to misuse ,incomplete dose or even bad keeping of the drug . Most of studies pointed that the gene had a very doubtful and unclear role with quinine resistance ,in this study there no mutation found in participants used quinine as last treatment except one with mutation in codon 184, which supports the opinions of other studies. So depending on results of this study quinine and artmither are less risky with coming drug resistance.

Conclusion:

This study concluded that: Out of 140 (100%) samples, 29 (20.7%) had mutation in *PfMDR1* gene. The percentage of the mutation in PfMDR1 gene code 86 was 6.4%, in codon 184 was 8.6% and in codon 1034 was 5.7%. There were no significant correlation between last treatment and mutation in these codons.

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