

Dedication

To

Dedicated to my parents father

/mother

To

My wife

To

My kids Yousria and Mohamed

Acknowledgments

I am greatly indebted to my supervisor Dr. Ibrahim Mohamed Elhasan, Head department of Parasitology and Medical Entomology Institute of Endemic disease, University of Khartoum for his guidance my postgraduate studies, and whose expertise, understanding, and patience, added to and the valuable insights into this work. Without his stimulating suggestion, encouragement and constant guidance this research would not have been possible.

Sincere and genuine thanks also go to my, co supervisor Dr Humodi Ahmed Saeed, Dean Faculty of Medical laboratory Science, Sudan University for Science Technology, who extended all kinds of support, assistance during study and I had pleasure to work with.

Thanks go to the staff members of the Institute of Endemic Disease, University of Khartoum a wonderful work place Profeser. Montasir Ibrahim, Dr. hiba Salah Eldeen and Badria Saeed, deserves special mention for providing skilful technical assistance.

My appreciation extended to the staff members Sennar Hospital Dr Mohamed Fadel Elseed Nasir and Nada Chief medical laboratory technician

Thanks go to Abyay medical health center, Abongo Chief Technician and Samia.

My thanks are also extended to the staff members of Sennar Malaria center, Dr Ali, Abed Elmonim Faza.

I also owe many thanks to the patients and patients guardians who participated in the study for their cooperation and patience during the study period.

Thanks to my family for the support they provided me through my entire life and for their sincere encouragement to pursue my interests.

Abstract

The aim of this work was to characterize the malaria parasite population in Central Sudan Sennar State. A total of 434 blood sample were collected from malaria suspected patients, who attend Abyay clinical center during September to October 2006 . 268 samples out of 434 samples (62%) were detected to be positive for malaria by microscopic examinatiot and rapid ICT test ,all the positive samples due to *P.falciparum* Prevalence of malaria was found to be (38%) . Based on the clinical data, the malaria patients were classified as mild symptoms 14 (5.2%), moderate symptoms 222 (82 %) and sever symptoms 32 (11.9%).

Among malaria patients 221 (82.3%) were detected as mild anemia , 8 (3.4%) as sever anemia and 39 (14.3%) as subject with normal hemoglobin level .Among malaria patients there are 3 patients with cerebral malaria .Patients were categorized in to five groups ≤ 5 years 54 patients (20.1%), 6-14 years 144 patients (53.7%), 15-40 years 59 patients (22.0%), 41-60 years 9 patients (3.4%), ≥ 61 years 2 patients (0.7%). Malaria prevalence is high in despite of control effort produced by the Roll back malaria program.

Allelic diversity was analyzed in the highly parasite polymorphic genes encoding the merozoite surface protein-1 and merozoite surface protein-2 by polymerase chain reaction. Different size polymorphism was detected in all genes analyzed with 10 & 9 variants for Msp1 & Msp2 alleles. Moreover based on the studied genetic markers, most infections consisted of more than one genetically distinct parasite colone. This results suggest that the parasite population circulating in this region are genetically homogeneous and point to an association between the extent of parasite genetic diversity and the intensity of malaria transmission.

Different genotypes were found to be associated with severity of disease. In this respect, association between parasitemia and anemia ($P=0.001$), parasitemia and age ($P=0.002$), and between parasitemia and polymorphism regions of Msp1 and Msp2 ($P=0.004$ & 0.001). In addition some variants of allelic families found to be associated with malaria in children (5-14 years of age.) Individuals living malaria endemic areas generally harbor multiple parasite strains which known by multiplicity of infection (MOI) and can be used as an indicator of immune status. One of goals of this study was re-examine the MOI in *P.falciparum* infected patient, and to relate in to severity of disease. Result of

genotyping reveal that MOI was significantly higher at the peak of transmission season and the majority of PCR positive subjects had multiple infections at that time points (64%). There was significant correlation between MOI and parasite density ($P=0.00$), as the higher parasite counts increases the probability of having multiple infections. Also significant correlation between MOI and variants of Mad20, K1, and RO33 ($P=0.000$).

P.falciparum isolates of this area were genotyped for detection of mutations in *P.falciparum* chloroquine transporter (*Pfcrt* 76T) and multidrug resistance (*Pfmdr1* 86Y) genes. High levels of Chloroquine resistance have been found in the study area, also there was strong significant association between the prevalence of *Pfcrt* 76T and *Pfmdr1* 86Y which are located into two different chromosomes and conferring resistance against chloroquine. Significant correlation was observed between *Pfcrt* 76T and anemia ($P=0.003$), Fc27 allele ($p=0.03$) and *Pfmdr1* ($P=0.00$).

يهدف هذا العمل إلى دراسة التصنيف الجزيئي لطفيلي الملاريا في ولاية سنار (وسط السودان). تم جمع (434) عينة من أشخاص يشتبه في إصابتهم بالملاريا وذلك في مركز صحي أبيي بولاية سنار، في الفترة من سبتمبر وحتى أكتوبر 2006م، وهي تمثل أعلى فترة لنقل الملاريا في هذه المنطقة. من هذه العينات وجد أن 268 عينة (62%) مصابة بالملاريا سببها طفيلي الملاريا من نوع فالسيبارام وذلك عند استخدام الفحص المجهرى والمناعى الكروماتوغرافى السريع، وبلغت نسبة انتشار المرض (38%). وحسب الأعراض المرضية تم تقسيم مرضى الملاريا إلى ذوي أعراض بسيطة 14 بنسبته (5.3%)، أعراض متوسطة 222 بنسبة (82.8%)، أعراض حادة 32 بنسبة (11.9%). وأن الفئات المرضية كانت تمثل 221 حالة أنيميا بسيطة بنسبة (82.5%)، و 8 حالات أنيميا حادة بنسبة (3.0%)، و 39 حالة ملاريا غير أنيميا بنسبة (14.5%). ووجد أن بين الحالات المرضية أن هنالك 3 حالات دماغية. تم وضع المرضى في 5 مجموعات عمرية، الفئة الأولى (>= 5 سنوات) 54 فرد بنسبة (20.2%)، والثانية (من 6-14 سنة) 144 فرد بنسبة (53.7%)، والثالثة (من 15-40 سنة) 89 فرد بنسبة (22.0%)، والرابعة (من 41-60 سنة) 9 أفراد بنسبة (3.4%)، الخامسة (61 سنة فأكثر) فردين بنسبة (0.7%). وبالرغم من الجهود المبذولة بواسطة برنامج دحر الملاريا فإن نسبة انتشار الملاريا في المنطقة عالى.

وقد أوضحت دراسة التباين الجيني للبلازموديوم فالسيبارام العديد من الصور الوراثية في المناطق الجغرافية المختلفة، وأن هذا التباين أدى لتعقيد الإصابة بالملاريا، وأنه يمثل أحد العقبات في طريق تطوير سبل السيطرة على الملاريا. ولما كان التوصيف الجزيئي لطفيلى في منطقة الدراسة أحد أهداف هذه الدراسة فقد تم فحص التباين الجيني لطفيلى في هذه المنطقة، وذلك بدلالة الجينات التي تؤدي لتكوين بروتين سطح الموروزويت 1 و 2، وذلك بواسطة تفاعل الإنزيم مجمع السلسلة (PCR). وقد أوضحت النتائج وجود عدة صور لآليات الجينات التي درست، حيث ظهرت 10 صور جينية للجين (MSP1) و 9 صور للجين (MSP2). هذا وقد وجد أن معظم العينات (64%) مصابة بأكثر من طفيل واحد في نفس الوقت. وقد أشارت هذه النتائج إلى أن أنواع الطفيل في هذه المنطقة متجانسة بالرغم من تميزها بتعدد الصور (Polymorphism)، وأنه يوجد ارتباط بين التباين الجيني لطفيلى ومدى الإصابة بالملاريا، هذا وقد وجد أن بعض الصور الجينية مرتبطة بشدة المرض، حيث وجد في هذا الإطار ارتباط بين حالات فقر الدم وكثافة الطفيل من جهه (P=0.001)، وصورة جينات (MSP1 & MSP2) P=0.004 and 0.001)) من جهة أخرى، هذا بالإضافة إلى صور محددة من آليات هذه الجينات خاصة في الأطفال عمر 6 وحتى 14 سنة، مما يدل على ارتباط هذه الصور الجينية بشدة المرض بمنطقة سنار. في هذه الدراسة أيضاً تم فحص حالة الإصابة بأكثر من طفيل في العينة الواحدة، حيث أن هذه الظاهرة تعتبر ميزة لمناطق

المالريا المستوطنة، وأن لها علاقة بشدة المرض والحالة المناعية للمريض. وأشارت الدراسة إلى أن هذه الظاهرة موجودة بكثرة في ذروة موسم انتقال المرض، حيث وجد أن معظم العينات مصابة بأكثر من طفيل. وقد لوحظ ارتباط زيادة هذه الظاهرة بكثافة الطفيل ($P=0.00$ ، إذا أنها تزيد كلما زاد تعداد الطفيل. كذلك يوجد ارتباط معنوي بين (MOI) و صورة الجينات (Mad20, K1, & RO33 ($P=0.00$)).

وتهدف هذه الدراسة أيضاً إلى معرفة انتشار المالريا المقاومة للكلوروكوين، حيث وجد أن نسبة هذه الظاهرة مرتفعة جداً. حيث لوحظ وجود علاقة ذات دلالة معنوية بين وجود الطفرات الأليلية المرتبطة بمقاومة طفيل المالريا لعقار الكلوروكوين (Pfcrt76T) و(Pfmdr1 86Y) ($P=0.00$) والتي تقع في كروموسومين مختلفين من جهة، وبين (Pfcrt76T) والانيما ($P=0.003$)، و (Fc27) ($P=0.03$).

LIST OF CONTENTS

No.	Subject	Page
	Dedication	I
	Acknowledgments	II
	English Abstract	III
	Arabic Abstract	V
	List of Contents	VII
	List of Tables	XII
	List of Figures	XIII
	List of maps	XVI
	List of Abbreviations	XVII
CHABTER ONE: Introduction and Literature Review		
1.1	General introduction.	1
1.2	History.	1
1.3	Global distribution of malaria.	2
1.4	The malaria parasite.	5
1.4.1	Life cycle of <i>P.Falciparum</i> .	5
1.4.2	Malaria vectors.	7
1.4.3	Clinical features of malaria.	8
1.4.4	Main symptoms of malaria.	9
1.4.5	Pathology of malaria.	10
1.4.6	Genetics of <i>P.falciparum</i> .	11
1.4.7	Genetic diversity of <i>P.falciparum</i> .	12
1.4.8	Genotyping of <i>P.falciparum</i> infection.	13
1.4.8.1	Meroziote surface protein-1 (MSP-1).	13
1.4.8.2	Meroziote surface protein-2 (MSP-2).	14
1.4.9	Plasmodium genetic and severity of the disease.	15
1.5	Epidemiology of malaria	17
1.5.1	Malaria endemicity	18
1.5.2	Malaria stability	18
1.6	Malaria immunology	19

No.	Subject	Page
1.6.1	Innate immunity	19
1.6.2	Humoral Immunity	21
1.7	Malaria diagnosis	22
1.7.1	Clinical Diagnosis of Malaria	22
1.7.2	Microscopic examination of blood films	22
1.7.3	Rapid antigen tests	23
1.7.4	Molecular methods	24
1.8	Chemotherapy of Malaria	24
1.8.1	Chloroquine	25
1.8.2	Quinine	26
1.8.3	Antifolate combination drugs	26
1.8.4	Antibiotics	26
1.8.5	Miscellaneous compounds (not exhaustive)	27
1.8.6	Combination therapy	27
1.8.6.1	Definitions	27
1.8.6.2	Artemisinin-based combination therapy:	28
1.8.6.3	Artemisinin compounds	29
1.8.6.4	Artemether	29
1.8.6.5	Artesunate	29
1.9	Resistance to antimalarials	30
1.9.1	Definition of antimalarial drug resistance:	30
1.9.2	Malaria treatment failure	30
1.9.1.1	Malaria treatment failure	30
1.9.1.2	Factors contributing to the development and spread of resistance	31
1.9.1.3	Epidemiology of antimalarial resistance	34
1.9.1.4	Monitoring of antimalarial drug resistance	36
1.9.1.4.1	<i>In vivo</i> tests	36
1.9.1.4.2	In vitro resistance tests	39
1.9.1.4.3	Molecular markers as tools for detecting drug resistance	40
1.9.4	Chloroquine mode of action	42
1.9.5	Mechanism of chloroquine resistance	44
1.9.6	Transporters involved in resistance to antimalarial drugs	47

No.	Subject	Page
1.9.6.1	PfCRT	48
1.9.6.2	PfMDR1	51
1.10	Malaria vaccine	53
1.11	Malaria in Sudan	55
1.11.1	The disease	55
1.11.2	Malaria epidemiology in Sudan	55
1.11.3	Antimalarial drug resistance in Sudan	57
	Objectives of the study	61
CHAPTER TOW: Material and Methods		
2.1	Study area	62
2.2	Study population	64
2.3	Parasitological examinations	64
2.3.1	Thin and thick blood films	64
2.3.2	Staining techniques	65
2.3.3	Malaria parasite count	65
2.4	Processing of blood sample	65
2.5	Molecular diagnosis	65
2.5.1	Preparing of DNA for PCR	65
2.5.1.1	DNA extraction	65
2.5.1.2	DNA amplification for species detection	66
2.5.1.3	PCR reagents	66
2.5.1.4	Outer amplification (genus detection)	67
2.5.1.5	Inner amplification (species Detection)	67
2.5.1.6	DNA amplification program	67
2.6	Molecular typing and amplification of MSP1 , MSP2 gene fragments	67
2.6.1	PCR amplification and PCR product detection	67
2.6.2	Primers	68
2.6.3	PCR condition	68
2.6.4	Detection of PCR product	69
2.6.5	Estimation of alleles frequencies	69
2.6.6.	Multiplicity of infection (MOI)	69
2.7	Identification of alleles of drug resistance genes	71

No.	Subject	Page
2.7.1	<i>Pfcr1</i>	71
2.7.2	<i>Pfmdr1</i>	71
2.8	Statistical analysis	72
2.9	Ethical consideration	72
CHAPTER THREE:		
	Results	73
CHAPTER FOUR:		
	Discussion	105
CHAPTER FIVE: Conclusion and Recommendations		
	Conclusion	118
	Recommendations	119
	References	120
	Appendix	159

List of tables

No.	Subject	Page
Table (1-1)	<i>P.falciparum</i> resistance to anti-malarial, 1997-2003	58
Table (2-1)	Sequences of oligonucleotide primers used in detection of Plasmodium species and their corresponding amino acids sequences.	66
Table (2-2)	Approximate size range of amplification products of MSP1 block2.	68
Table (2-3)	Sequences of the oligonucleotide primers used to genotype <i>P.falciparum</i> MSP1 and MSP2 proteins used in this study.	70
Table (2-4)	Sequences of PCR primers used for amplification of <i>P. falciparum</i> drug resistance genes, <i>Pfcr1</i> and <i>Pfmdr1</i> .	72
Table (3-1)	Frequency of different strains those are responsible for MOI among study population.	88
Table (3-2)	<i>Pfcr1</i> 76 and <i>Pfmdr1</i> 86 polymorphisms	88
Table (3-3)	Frequency of different CQ. marker	92
Table (3-4)	Frequency of Polymorphism in <i>P.falciparum</i> Chloroquine resistance transporter and multi drug resistance genes (<i>Pfcr1</i> K76T and <i>Pfmdr1</i> -86Asn).	93
Table (3-5)	Frequency of <i>Pfmdr1</i> 86Y * <i>Pfcr1</i> K76T	94
Table (3-6)	Frequency of CQ. resistance genes of <i>Pfcr1</i> and <i>Pfmdr1</i> within age groups:	102

List of Figures

No.	Subject	Page
Figure (1-1)	Global malaria world-wide distribution defined by annual parasite incidence, (top), temperature, and aridity (bottom) (adapted from Geura et al, 2008)	4
Figure (1-2)	Life cycle of <i>P. falciparum</i> parasite in both host and vector (Fujioka and Aikawa, 2002)	7
Figure (1-3)	Schematic diagram of structure of Merozoite surface protein 1 (MSP1	14
Figure (1-4)	Locations of <i>MSP2</i> oligonucleotide primers used in PCR of the <i>MSP2</i> gene from field isolates	16
Figure (1-5)	Predicted structure and representative heliotypes of <i>P. falciparum</i> chloroquine resistance transporter	50
Figure (1-6)	Predicted structure and genetic polymorphisms in <i>P. falciparum</i> multidrug resistance-1	53
Figure (3-1)	Frequency and percentage of gender among malaria patients.	73
Figure (3-2)	Frequency and percentage of malaria infection with in age groups among malaria patients.	74
Figure (3-3)	Degree of malaria severity among the study patients.	75
Figure (3-4)	Frequencies and percentages of mild and severe anemia among malaria patients.	75
Figure (3-5)	Frequency and percentage of Parasitemia among malaria patients.	77
Figure (3-6)	The hemoglobin values among the age groups.	78
Figure (3-7)	<i>Plasmodium falciparum</i> nested PCR. Lanes from right to left as follows: DNA molecular marker (100bp ladder), lane 1 negative Control, lane 2 positive control lane (3 - 8) samples.	78
Figure (3-8)	Frequency and percentages of MSP1, MSP2, and MSP1+MSP2 allelic families among malaria patients.	79
Figure (3-9)	Frequency and percentages of MSP1 allelic family among malaria patients.	80
Figure (3-10a)	Represent polymorphic gene MSP1 (K1). Lanes from right to left as follows: DNA molecular marker (100bp ladder), lane 13 negative Control, lane 12 positive control lane 1 – 11 and 14 -17 samples.	80
Figure (3-10b)	Frequency of various copies of K1 detected by PCR genotyping.	81

No.	Subject	Page
Figure (3-11a)	Represent polymorphic gene MSP1 (Mad20). Lanes from right to left as follows: DNA molecular marker (100bp ladder), lane 3 negative Control, and lane 4 positive control lanes 1,2,5 -8 samples.	81
Figure (3-11b)	Frequency of the variants detected for MAD20 in the PCR genotyping.	82
Figure (3-12a)	Represent polymorphic gene MSP1 (Ro33). Lanes from right to left as follows: DNA molecular marker (100bp ladder), lane 6 negative Control, 7 positive control 1 – 5 and 8 – 17 samples.	82
Figure (3-12b)	Frequency of the 3 variants of RO33 detected in the study samples by PCR genotyping.	83
Figure (3-13)	Frequency of genotypes of MSP1 block 1 as detected in the PCR genotyping.	83
Figure (3-14)	Frequency and percentage Of the allelic families of MSP2 obtained by the PCR genotyping of the study samples.	84
Figure (3-15a)	Represent polymorphic gene MSP2 (IC). Lanes from right to left as follows: DNA molecular marker (100bp ladder), lane 3 negative Control, 2 positive control lane 1, 4 – 17 samples.	84
Figure (3-15b)	Frequency of the four variant copies of IC allele.	85
Figure (3-16a)	Represent polymorphic gene MSP2 (Fc27). Lanes from right to left as follows: DNA molecular marker (100bp ladder), lane 13 negative Control, 12 positive control lane 1 – 11 samples.	85
Figure (3-16b)	Frequency of the five FC27 variants detected by the PCR in study samples.	86
Figure (3-17)	Frequency of genotypes of MSP2 combinations.	86
Figure (3-18)	Frequency of multiplicity of infection (MOI).	87
Figure (3-19)	MOI as can be observed in PCR genotyping of FC allelic family of MSP2, lane 2 positive control lane 11 negative control lanes 1,3,4 – 10 samples with different molecular weight, MW+ molecular ladder of 100bp lane 12.	87
Figure (3-20a)	<i>Pfcr76</i> nested PCR product. Lanes from right to left: 16 DNA molecular marker (100bp ladder) 1 negative control, 2 positive control 3 – 15 samples.	89
Figure (3-20b)	Frequency of <i>Pfcr76</i> K76T	89
Figure (3-20c)	Digestion of <i>Pfcr76</i> nested PCR product by Apo restriction enzyme.	90

No.	Subject	Page
	Lanes 2, 4, 6, and 7 represent the mutant allele. Lanes 1, 3 and 5 represent the wild type of allele.	
Figure (3-21a)	<i>Pfmdr1</i> 86 nested PCR product. Lanes from right to left: DNA molecular marker (100bp ladder) 1 positive control,6 negative control 2,3,4,5and 7 – 11 samples	90
Figure (3-21b)	Frequency of <i>Pfmdr1-86Asn</i>	91
Figure (3-21c)	Digestion of <i>Pfmdr1</i> 86nested PCR product by Apo restriction enzyme. Lanes 2, 9, 12, 13 and 14 represents wild allele. Lanes 3 and 11 represent mutant allele, and lanes 4, 5, 6, 7 and 10 represent mixed allele type, while lanes 8, and 15 represent DNA molecular marker (100pb ladder).	91
Figure (3-22)	Frequency of Hb with Parasitemia.	94
Figure (3-23)	Frequency of parasitemia distributed among age groups,(95% CI was considered).	95
Figure (3-24)	Association between parasitemia and MAD20 variants, (95% CI was considered).	96
Figure (3-25)	Association between parasitemia and IC variants, (95% CI was considered).	96
Figure (3-26)	Association between parasitemia and allelic family of Msp1 and Msp2.	97
Figure (3-27)	Association between age groups and allelic family of Msp1 and Msp2	98
Figure (3-28)	Association between severity of malaria disease and alleles of K1.	99
Figure (3-29)	Association between severity of malaria disease and alleles of MAD20.	99
Figure (3-30)	Association between severity of malaria disease and alleles of RO33.	100
Figure (3-31)	Association between severity of malaria disease and alleles of IC (3D7).	100
Figure (3-32)	Association between severity of malaria disease and alleles of FC27.	101
Figure (3-33)	Association between CQ. resistance genes (<i>Pfcr</i> t) and alleles of Msp1.	103
Figure (3-34)	Association between CQ. resistance genes (<i>Pfcr</i> t) and alleles of Msp2.	103
Figure (3-35)	Association between CQ. resistance genes (<i>Pfmdr</i>) and alleles of Msp1.	104
Figure (3-36)	Association between CQ. resistance genes (<i>Pfmdr</i>) and alleles of Msp2.	104

List of maps

No.	Subject	Page
Map (1-1)	Malaria endemicity level in Sudan, 2001.	59
Map (1-2)	Sudan major malaria strata, 2001.	60
Map (1-3)	Epidemic-prone areas (red color).	60
Map (2-1)	Sennar State location within the map of Sudan	63
Map (2-2)	A satellite image for Sennar State showing approximate locations Rahhal areas.	64

List of Abbreviations

ACTS	Artemisin based combination therapy
AMA-1	Apical Membrane Antigen - 1
CQ	Chloroquine
CQR	Chloroquine resistance
CSP	Circum sporozoite protein
CT	Combination therapy
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphate
EBA	Erythrocyte-Binding Antigen
EDTA	Ethylenediaminetetracetic acid
G.6.P.D	Glucose-6- Phosphate Dehydrogenase
HLA	Human Leukocyte Antigen
ICAM-1	Intracellular Adhesion Molecule One
MSP-1	Merozoite Surface Protein - 1
Msp2	Merozoite surface protein2
Na ⁺ ___H ⁺	Sodium hydrogen ions
P.	Plasmodium
PCR	Polymersae Chain Reaction
<i>Pfcr</i>	<i>P. falciparum</i> chloroquine resistant
<i>Pfdhfr</i>	<i>P. falciparum</i> dihydrofolatereductase
<i>Pfdhps</i>	<i>P. falciparum</i> dihydropetronatesynthase
<i>PfEMP1</i>	<i>P. falciparum</i> erythrocyte membrane protein 1
<i>Pfmdr</i>	<i>P. falciparum</i> multidrug resistant
PGtuDH	Plasmodium Glutamate Dehydrogenase
pLDH	Plasmodium Lactate Dehydrogenase
PNG	Papa New Gunea
RFLP	Restriction fraction length polymorphism
SE	South East
SNPs	single nucleotide polymorphism
SP	Sulfadoxine-pyrimethamine
TBE	Tris Boric EDTA
TNF	Necrosis Factor
VSA	Variant surface antigens
WBC	White blood cell
WHO	World health organization