

# Acknowledgements

I would like to thank all those who helped me during this study, especially, my supervisors, Dr. Ibrahim Mohamed Elhassan , Head Department of Parasitology, Institute of Endemic Diseases, University of Khartoum for his supervisions, training, supplement of reagents and continuous support during the field and laboratory work.

I would also like to express my deep thanks to Dr. Khalida Mirghani Hamza, the vice-dean of the faculty of sciences of medical laboratories, Sudan;s university and the co-advisor of this work for her continuous help and encouragement throughout the periods of this work.

Special thanks to Dr.. Hammodi Ahmad Saeed, Dean of faculty of Sciences of Medical Laboratories, Sudan University of Sciences and Technology, for his continuous help and encouragement during this study.

My thanks are extended to Dr.Malik Elfadni, head of department of hematology for paving financial obstacles in collaboration with the Dean of the faculty. A lot of thanks are extended to Dr Tarig Alfatih for his co-operation in solving administrative problems.

I would like to thank the group of Malaria Research in the institute of endemic diseases, faculty of medicine, University of Khartoum, specially Mr Mishaal, Miss Wigdan, Miss Reem, Miss Badria, Miss Suha, Miss Zianab, Madam Mirvat and Mr Mohammed Mohy for their generous technical help during preliminary trials of this work and data analysis.

Great thanks for Professor M. M. Mukhtar for supplying printer for ELISA, fruitful suggestions and technical help during ELISA work. My thanks are extended to my colleagues and all members

of faculty of Sciences of Medical Laboratories, University of  
Sudan, Khartoum, Sudan.

## Dedication

This work is dedicated

to those who care

about mankind welfares

specially

those working to combat endemic

diseases

my

family and friends.

# Abstract

A total of 570 samples of both sexes were collected from malaria-suspected patients who attended Medani Pediatric and adult teaching Hospital during August-December 2005. That period was the peak of malaria transmission season in the area. Out of these, 155 samples were detected to be positive for malaria and 140 cases (90%) out of the positive samples were due to Plasmodium falciparum and the rest, which were excluded from the study, (15 patients) were due to other plasmodium species. Based on the clinical data, the Plasmodium falciparum patients were classified as asymptomatic (7 patients) and symptomatic patients (133 patients). Among the symptomatic patients, 106 (75.7%) were detected as mild anemia, 24 (17.1%) as severe anemia and 10 (7.2%) as subject with normal hemoglobin level. Among the symptomatic patients also there are 22 cerebral malaria, 12 pregnant, 8 patient with splenomegally and two with hepatomegally. Patients are categorized into 4 age groups, group 1 has 71 (50.7%) individuals followed by groups 2,3, and 4 which have 44 (31.4%), 17 (12.1%), and 8 (5.8%) individuals respectively. One of the goals of this work was molecular characterization of the parasite populations circulating in Gazira, central part of Sudan. This work investigated the extent of genetic polymorphism in P. falciparum field isolates from Gazira. Allelic diversity was analyzed in the highly polymorphic parasite genes encoding the merozoite surface proteins-1 (MSP-1) and -2 (MSP-2) and the glutamate-rich protein (GLURP) by the polymerase chain reaction. Different size polymorphism was detected in all genes analyzed, with 13 and 8 variants for the MSP-1 and MSP-2 alleles, respectively, and 5 size variants for the GLURP. Moreover, based on the studied genetic markers, most infections consisted of more than one genetically distinct parasite clones. These results suggest that the *P. falciparum* parasite populations 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 high parasitemia and anemia on one hand and variants of K1 were found, MAD20, RO33 and FC allelic families on the other hand. In addition, some

variants of the allelic families were found to be associated with children between 0-10 years of age. Individuals living in 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 the goals of this study was to examine the MOI in Plasmodium faciparum-infected samples and to relate it to severity of the disease. Results of genotyping reveal that MOI was significantly higher at the end of transmission season and the majority of PCR positive subjects had multiple infections at that time points (54%). No association between MOI and severity of disease was observed. MOI did not vary over age at any time points. There was a significant correlation between MOI and parasite density, as the higher parasite counts increases the probability of having multiple infections. One of the major goals of this work was the investigation of the immune response of glutamate rich protein among the study population as an indicator for acquisition of protective immunity. This is achieved by estimation of IgG, IgG1, IgG3 and IgM antibodies directed against GLURP-R0, GLURP-R1 and GLURP-R2. The results indicate comparatively high titres of these antibodies to the three fragments of GLURP, although R2 and R0 encountered with significantly higher concentrations of the antibodies. The results also showed that IgG3 was presented with high concentration followed by IgG1 suggesting their protective role and their major participation in acquisition of immunity to malaria. The results of association between immune response and different strain of Plasmodium population isolated in this work reveal that some strain are encountered with high immune response and severity of malaria. This association supports the speculation of the presence of strain-specific immunity and premunition among inhabitants of malaria endemic areas. The results also re-enforce the ongoing announcement that any future malaria vaccine should include GLURP fragments notably GLURP-R2 and GLURP-R0.

Ethical clearance for this work has been approved by the ethical committee of the institute of endemic diseases, faculty of medicine, University of Khartoum.

## الخلاصة

تم جمع 570 عينة من اشخاص من الجنسين مشتبه فى اصابتهم بالملايا وذلك من مستشفيات مدنى للاطفال و الكبار فى الفترة من سبتمبر وحتى ديسمير 2005 وهى فترة نهاية موسم انتقال الملايا فى هذه المنطقه. من هذه العيذات وجد ان 155 مصابة بالملايا وان 140 (90%) منها سببها طفيل الملايا من نوع فالسبرم. حسب الاعراض المرضية تم تقسيم ال 140 عينة الى غير عرضيه (7 عينات) و عرضيه (133 عينة) وان الفئات العرضيه كانت تشمل 84 حالة انيميا خفيفه 31 حالة انيميا خطيره و 18 حالة ملايا غير انيمييه. ووجد ان بين الحالات المرضيه هنالك 22 حالة ملايا دماغيه ، 12 حالة حمل و 8 مرضى بتضخم الطوخال و 2 بتضخم الكبد. تم وضع المرضى فى 4 مجموعات عمريه. الفئه الأولى 71 فرد (50,7%) والثانيه 44 فرد (31,4%) والثالثه 17 فرد (12,1%) والرابعة 8 افراد (5,8%). دراسة التباين الجيني أوضحت أن للبلازموديوم فالسبيرم العديد من الصور ال وراثيه فى المناطق الجغرافيه المختلفه وأن هذا التباين أدى لتعقيد الاصابه بالملايا وانه يمثل احد العقبات فى طريق تطوير سبل السيطرة على الملايا. ولما كان التوصيف الجزئي للطفيل فى منطقة الدراسه احد اهداف هذه الدراسه فقد تم فحص التباين الجيني للطفيل فى هذه المنطقه وذلك بدلالة الجينات التي تؤدي لتكوين بروتين سطح الميروزوبت 1 و 2 و البروتين الغنى بالقلوتاميت و اوصحت النتائج وجود (PCR) ذلك بواسطة تفاعل الانزيم مجمع السلسله عدة صور لاليات الجينات التي درست حيث ظهرت 13 صورة جينية للجين هذا و قد وجد ان GLURP و 5 صور للجين MSP2 و 8 صور للجين MSP1 معظم العينات (54%) مصابة باكثر من طفيل واحد فى نفس الوقت. و قد اشارت هذه النتائج الى ان انواع الطفيل غى هذه المنطقه متجانسة بالرغم من

و انه يوجد ارتباط بين التباين الجينى (Polymorphism) تتميزها بتعدد الصور للطفيل و مدى الاصابة بالمalaria. هذا و قد وجد ان بعض الصور الجينية مرتبطة بشدة المرض. حيث وجد قى هذا الاطار ارتباط بين حالات فقر الدم و كثافة من FC و RO33 و MAD20 و K1 الطفيل فى الدم من جهة و صور جينات جهة اخرى. هذا بالاضافة الى صور محددة من اليلات هذه الجينات خاصة فى الاطفال من عمر يوم وحتى 10 سنوات مما يدل على ارتباط هذه الصور الجينية بشدة المرض فى منطقة الجزيرة. فى هذه الدراسة ايضا تم فحص حالات الاصابة باكثر من طفيل فى العينة الواحدة حيث ان هذه الظاهرة تعتبر ميزة لمناطق الملاريا المستوطنة. وان لها علاقة بشدة المرض و الحالة المناعية للمريض. و اشارت الدراسة الى ان هذه الظاهرة موجودة حتى فى نهاية فترة موسم انتقال المرض حيث وجد ان معظم العينات مصابة باكثر من طفيل. و قد لاحظت عدم ارتباط زيادة هذه الظاهرة بشدة المرض او فئة عمرية محددة. و وجد ان هناك ارتباط بين كثافة الطفيل فى الدم و هذه الظاهرة. احد اهم اهداف هذه الدراسة اختبار الاستجابة المناعية لثلاث اجزاء من البروتين الغنى بالقلوتاميت قى افراد هذه الدراسة. و تم ذلك بتقدير تركيز الاجسام ( R0 و R1 و R2 ) المضادة المنتجة استجابة لاجزاء البروتين الثلاث. اوضحت النتائج ان افراز الاجسام المضادة لهذه الاجزاء من البروتين اكثر عندما تقارن بالحالات قوبلا بافراز تراكيز اعلى مما R0 و R2 القياسية الغير مرضية وان الجزئين هي الاكبر IgG3 و ان تركيز الاجسام المضادة من نوع R1 قوبل به الجزء تراكيزها كانت طبيعیه كالحالات IgG4 و IgG2 و أن IgG1 تركيزا يليه القياسيه الغير مرضيه. وعلى هذا فان النتائج توضح دور هذه الاجسام المضاده فى المناعه المكتسبه ضد الملاريا. كذلك اوضحت نتائج دراسة علاقة الاستجابه المناعيه والصور الجينيه ان هناك بعض الصور الجينيه

للطفيل مسؤولة عن شدة المرض وتشير إلى وجود المناعة المكتسبة لدى الأفراد الذين قضوا وقتاً طويلاً في مناطق الملاريا المتوطنة وأن هذا الارتباط يشير إلى وجود مناعة خاصة ببعض الصور الجينية ويدعم نتائج الأبحاث القائلة في أي تركيبه للحصول على مصد  $R_0$  و  $R_2$  بضرورة إدخال جزيء البروتين فعال ضد الملاريا.



## List of Contents

Page	
Dedication	i
English Abstract	ii
Arabic Abstract	iv
Acknowledgment	vi
List of contents	vii
List of tables	xi
List of figures	xii
1- Introduction and literature review	1
1.1- Global distribution of malaria	1
1.2- Malaria parasite	4
1.3- Malaria parasite life cycle	4
1.4- Malaria vector	6
1.5- Malaria disease	7
1.5.1- Adhesion and invasion of plasmodium	7
1.5.2- Pathogenesis and pathology of malaria	7
1.6- Epidemiology of malaria	9
1.7- Seasonality	9
1.8- Malaria stability	9
1.9- Malaria endemicity	9
1.9.1 Degrees of endemicity	10
1.10 Malaria in Sudan	10
1.11- Clinical diagnosis of malaria	11
1.11.1- Microscopic diagnosis	11
1.11.2- Antigen detection	12
1.11.3- Molecular diagnosis	13
1.11.4- Other diagnostic techniques	13

1.12- Immunity to malaria	14
1.12.1- Innate immunity	14
1.12.2- Humoral immunity	17
1.12.2.1- Malaria antigens	17
1.12.2.2- Antibodies	21
1.12.2.2.1- Antibody-dependent protection	22
1.12.3- Cell-mediated immunity	23
1.12.3.1- CD4+ and CD8+ T cells	23
1.12.4- Regulation of immunity to malarial infection	24
1.12.4.1- Immune evasion	25
1.12.5- Cytokine network	26
1.12.6- Nitric oxide	29
1.12.7- Immunity and memory in malaria	29
1.13- Immunogenicity of Plasmodium falciparum GLURP	31
1.14- Genetic diversity of Plasmodium falciparum	32
1.14.1- Multiplicity of infection	33
1.14.2- Plasmodium genetic diversity and severity of the disease	34
1.15- Genotyping of malaria antigens	35
1.16- Recrudescence and new infection	36
1.17- Microsatellite markers	37
1.18- Plasmodium genome structure and content	37
1.19- Malaria vaccine development	39
1.19.1- Immunization against malaria	39
1.20- Aim of the study	45
2- Material and methods	47
2.1- The study area	47
2.2- study population	47
2.3- Parasitological examinations	48
2.3.1- Thin and thick blood film	48
2.3.2- Staining techniques	48
2.3.3- Malaria parasite count	48

2.4- Processing of blood sample	48
2.5- PCR typing and amplification of MSP-1 and MSP-2 gene fragments	48
2.5.1- Preparing of DNA for PCR	48
2.5.1.1- Freeing parasite from erythrocytes	49
2.5.1.2- Protinase K digestion	49
2.5.1.3- DNA purification	49
2.5.2- PCR amplification and PCR product detection	50
2.5.2.1-Primers	50
2.5.2.2- PCR condition	50
2.5.2.3- Detection of PCR product	51
2.5.3- Estimation of allele frequencies	51
2.5.4- Multiplicity of infection	51
2.6- Enzyme-linked immunosobent assay (ELISA)	53
2.6.1- Antibody quantification	53
2.6.2- Negative controls	53
2.6.3- ELISA working procedure	53
2.7 Analysis of the data	54
3- The results	55
3.1- Patient characteristics	55
3.2- Parasitological results	56
3.2.1- Blood film and the PCR results	56
3.2.2- Parasitemia	57
3.2.2.1- Correlation between parasitemia ad study variants	59
3.3- Hematological results	60
3.3.1- Correlation between hemaglobin and some variant of study data	61
3.4- Genetic diversity of the parasite	61
3.4.1- Allelic families of MSP-1	62
3.4.2- Allelic families of MSP-2	64
3.4.3- GLURP genotyping	67
3.4.4- Correlation between alleles and symptoms	68
3.4.5- Multiplicity of infection	72

3.5- ELISA results	73
3.5.1- Prevalence and level of IgG directed against GLURP-R0	73
3.5.2- Prevalence and level of IgG directed against GLURP-R1	83
3.5.3- Prevalence and level of IgG directed against GLURP-R2	84
3.5.4- Prevalence and level of IgM directed against GLURP-R0	84
3.5.5- Prevalence and level of IgM directed against GLURP-R1	84
3.5.6- Prevalence and level of IgM directed against GLURP-R2	85
3.5.7- Prevalence and level of IgG1 directed against GLURP-R0	85
3.5.8- Prevalence and level of IgG1 directed against GLURP-R1	86
3.5.9- Prevalence and level of IgG1 directed against GLURP-R2	86
3.5.10- Prevalence and level of IgG3 directed against GLURP-R0	87
3.5.11- Prevalence and level of IgG3 directed against GLURP-R1	87
3.5.12- Association between age and anti-GLURP antibodies	88
3.5.13- Relation between anti-GLURP antibodies and anemia	92
3.5.14- Relation between anti-GLURP antibodies and parasitemia	94
3.5.15- Antibody responses to recombinant GLURP in relation to sex	94
3.5.16- Antibody responses to GLURP in patient with cerebral malaria	95
3.5.17- Antibody responses to GLURP in patient with splenomegally	96
3.5.18- Correlation between GLURP immune responses and genotypes	96
3.5.19- association between parasite strains and immune responses	96
4- Discussion	101
5- References	113
6- Appendices	129

## List of Tables

Table	page
1- Size range of MSP-1 allelic families.	50
2- Molecular sequences of different primes used for this study.	52
3- Stages of pregnancy.	56
4- Prevalence of different plasmodium strains for MOI in this study.	72
5- Numbers of responders and non-responders encountered with CM, Splenomegally, Hepatomegally and pregnancy states.	95

## List of figures

Figures	Page
1- Map of the world malaria distribution.	3
2- Life cycle of Plasmodium faciparum.	5
3- Blood film of P. falciparum..	12
4- Structure of MSP1.	20
5- Structure of MSP2.	20
6- Structure of GLURP.	21
7- Severity of anemia among study population.	55
8- Age groups of the population.	56
9- Species detection by PCR technique.	57
10- Parasitemia among age groups.	58
11- Hb level among study cohort.	59
12- Frequency of MSP1 allelic families.	60
13- Frequency of various copies of MSP1.	61
14- Frequency of MAD20 variants.	62
15- Prevalence of RO33 variants.	63
16- Frequency of MSP combinations.	63
17- MSP1 allelic families against MSP2 allelic families.	63
18- Frequency of IC variants.	64
19- Frequency of MSP2 combinations.	65
20- Determination of MOI.	65
21- Frequency of GLURP variants.	66
22- GLURP genotyping of the samples.	66
23- IgG antibodies directed against GLURP-RO.	66
24- IgG antibodies directed against GLURP-R1.	67
25- IgG antibodies directed against GLURP-R2.	68

26- Association between severity of malaria and alleles of K1	69
27- Association between severity of malaria and alleles of MAD20	70
28- Association between severity of malaria and alleles of R033	70
29- Association between severity of malaria and alleles of IC	71
30- Association between severity of malaria and alleles of FC	71
31- Association between severity of malaria and alleles of GLURP	72
32- IgG antibodies directed against GLURP-R0	74
33- IgG antibodies directed against GLURP-R1	74
34- IgG antibodies directed against GLURP-R2	74
35- IgM antibodies directed against GLURP-RO	75
36-IgM antibodies directed against GLURP-R1.	75
37- IgM antibodies directed against GLURP-R2	75.
38- IgGI antibodies directed against GLURP-RO.	76
39- IgG1 antibodies directed against GLURP-R1.	76
40- IgG1 antibodies directed against GLURP-R2.	76
41- IgG3 antibodies directed against GLURP-RO.	77
42- IgG3 antibodies directed against GLURP-R1.	77
43- Confidence interval (95%) for IgG antibodies against GLURP-R0.	78
44- Confidence interval (95%) for IgG antibodies against GLURP-R1.	78
45- Confidence interval (95%) for IgG antibodies against GLURP-R2.	79
46- Confidence interval (95%) for IgM antibodies against GLURP-R0.	79
47- Confidence interval (95%) for IgM antibodies against GLURP-R1.	80
48- Confidence interval (95%) for IgM antibodies against GLURP-R2.	80
49- Confidence interval (95%) for IgG1 antibodies against GLURP-R0.	81
50- Confidence interval (95%) for IgG1 antibodies against GLURP-R1	82.
51- Confidence interval (95%) for IgG1 antibodies against GLURP-R2 .	82
52- Confidence interval (95%) for IgG3 antibodies against GLURP-R0.	83
53- Confidence interval (95%) for IgG3 antibodies against GLURP-R1.	83
54-IgG/R0 level in different age of study population.	88
55- IgG/R1 level in different age of study population.	89
56- IgG/R2 level in different age of study population.	89

57- IgM/R0 level in different age of study population.	90
58- IgM/R1 level in different age of study population.	90
59- IgM/R2 level in different age of study population.	90
60- IgG/R0 level in different age of study population.	91
61- IgG1/R1 level in different age of study population.	91
62- IgG1/ R2 level in different age of study population.	91
63- IgG3/ R0 level in different age of study population.	92
64- IgG3/ R1 level in different age of study population.	92
65- Mean IgM/R1 among study population.	93
66- Mean IgG/R0 among study population.	94
67- Distribution of K1 allele among study population.	97
68- Distribution of MAD20 allele among study population.	98
69- Distribution of RO33 allele among study population.	98
70- Distribution of IC allele among study population.	99
71- Distribution of FC allele among study population.	99
72- Distribution of GLURP allele among study population.	100