University of Ghana http://ugspace.ug.edu.gh

DEVELOPMENT OF A RAPID URINE-BASED DIPSTICK TEST FOR DIAGNOSIS OF MALARIA

BY

URI SELORM MARKAKPO

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFULLMENT OF THE REQUIREMENT FOR THE AWARD OF PHD PUBLIC HEALTH DEGREE

DEPARTMENT OF BIOLOGICAL, ENVIRONMENTAL AND OCCUPATIONAL HEALTH SCIENCES
SCHOOL OF PUBLIC HEALTH,
COLLEGE OF HEALTH SCIENCES
UNIVERSITY OF GHANA,
LEGON

JULY, 2014

University of Ghana http://ugspace.ug.edu.gh

DECLARATION

I hereby declare that this thesis is the result of my own original research, except for areas where specific references have been made and duly acknowledged. I also affirm that the studies reported in this document were carried out by me under the supervision of my team of academic advisors. Lastly, I declare that this work has not been submitted, either in part or in whole, to any other institution for an award of a degree.

Uri Selorm Markakpo (M.Phil.)
(10156003)

Prof. Isabella A Quakyi (Ph.D.)
(SUPERVISOR)

Prof. Kwabena M. Bosompem (Ph.D.)
(SUPERVISOR)

Dr. Mawuli Dzodzomenyo (Ph.D.)
(SUPERVISOR)

University of Ghana http://ugspace.ug.edu.gh

DEDICATION

This thesis is dedicated to my supervisors, my children and my wife for the moral and emotional support given me during the trying moments of the conduct of the studies herein.

University of Ghana http://ugspace.ug.edu.gh

ACKNOWLEDGEMENT

First, and foremost, I wish to express my sincere thanks to Professor Ernest Aryeetey, the current Vice Chancellor of University of Ghana (UG), Prof. Alexander K. Nyarko, the former Director of the Noguchi Memorial Institute for Medical Research (NMIMR), UG, and Prof. Richard Adanu, the current Dean of SPH, UG, for the various roles played towards the successful completion of this study. Secondly, I sincerely appreciate the efforts made by my supervisors, Prof. Isabella A. Quakyi, SPH, Prof. Kwabena M. Bosompem, NMIMR and Dr. Mawuli Dzodzomenyo, SPH, University of Ghana as well as my collaborating mentor, Prof. David Sullivan Jr. of the Malaria Research Institute, Department of Molecular Immunology and Microbiology, Johns Hopkins University School of Public Health, Baltimore, Maryland, USA, for their guidance and support towards the conduct of this study. I also like to thank Dr. William K. Anyan and Dr. Irene Ayi of the Parasitology Department, NMIMR, Dr. Michael F. Ofori and Prof. Ben Gyan of the Immunology Department, NMIMR, Mr. Alfred Dodoo of the Electron Microscopy and Histopathology Department, NMIMR and Dr. Daniel Boamah of the Parasitology Department, NMIMR and Centre for Scientific Research into Plant Medicine (CSRPM), Mampong, for the great contributions made towards the success of this thesis project.

Furthermore, I like to express my deep appreciation to the Bill and Melinda Gates Foundation' Grand Challenges Explorations, World Health Organization (WHO), the University of Ghana, the Johns Hopkins University School of Public Health and Prof. David Sullivan Jr for providing funds and/ or other logistic support required for the execution of this project. In addition, I am greatly indebted to the NMIMR and SPH all of the University of Ghana, Legon, and the Kpone Health Centre (KHC), Kpone-on-Sea, for providing me with the laboratory facilities where all the experiments involved in this thesis project were conducted. Also, my heartfelt gratitude goes to Dr. Mawuli Dzodzomenyo, and Prof. Julius Fobil, the past

University of Ghana http://ugspace.ug.edu.gh

and current Heads of the Department of Biological, Environmental and Occupational Health Sciences (BEOHS), SPH, and Dr. Mawuli K. Gyakobo, Dr. Isaac Anim-Baidoo and Mr. Richard H. Asmah for the encouragement, companionship and moral support given me during the course of this project work.

My special appreciation also goes to Mrs. Naa Adjeley Frempong, Mr. Joseph Otchere, Mr. Kwadwo K. Frempong and the late Mr. Osei A. Duah of the Department of Parasitology, NMIMR, Mr. Jonas Danquah of the Department of Biochemistry, Cell and Molecular Biology, UG, and Mr. Parnor Madjitey of the Department of BEOHS, SPH as well as my field Assistants Mr. Isaac Ankrah and Mr. Nicholas Amoah for the assistance given me during the collection of samples and the conduct of the experiments leading to the production of this thesis. The great assistance in data entry given me by Ms. Tina Ayeh of the Department of Epidemiology, NMIMR, Ms. Philomena Amankwah of the Kpone Malaria Project and Mr. Oliver K. Adotey of the Madina Central SDA Church, Madina, is also highly appreciated.

I am most grateful to Dr. Phyllis Addo and Dr. Samuel Adjei of the Animal Experimentations Department, NMIMR, for providing the facilities for the immunization experiments; the staff of Parasitology Department, NMIMR, particularly, Messrs. Jonas. R. K. Asigbee (deceased) and Joseph. Quartey, for their technical and moral support during the conduct of this research project; the Administrative Staff of SPH, especially, Ms. Hannah T. Addo of the Department of BEOHS; Staff of the College of Health Sciences, UG; my study participants and the Kpone study community as a whole for their contributions during the conduct of this research.

Ultimately, I thank the Lord God Almighty for the protection, support and special insights He provided me to ensure the successful completion of this research project.

University of Ghana http://ugspace.ug.edu.gh

TABLE OF CONTENTS

CONTENTS **PAGE**

DECLARATION i
DEDICATION ii
ACKNOWLEDGEMENT iii
TABLE OF CONTENTS v
LIST OF TABLES xii
LIST OF FIGURES xiii
LIST OF PLATES xv
ABBREVIATIONS xvi
SUMMARY xix
CHAPTER ONE 1
1.0 GENERAL INTRODUCTION 1
1.1 BACKGROUND AND RATIONALE 1
1.2 PROBLEM STATEMENT 4
1.3 OBJECTIVES 6
1.3.1 Main Objective 6
1.3.2 Specific Objectives 6
1.4 JUSTIFICATION 7
CHAPTER TWO 8
LITERATURE REVIEW 8
2.1.0 MALARIA 8
2.1.1 Economic Cost of Malaria 8
2.1.2 Geographic Distribution of Malaria and the *Plasmodium* Parasites 9
2.1.3 The Biology and Life-Cycle of the *Plasmodium* Parasite 13
2.1.4 Clinical Signs and Symptoms of Malaria 17
2.1.5 Pathogenesis of Malaria 18
2.1.6 Classification of the *Plasmodium* Parasite 21
2.2.0 ANTIBODIES 24
2.2.1 The Basic Structure of Antibodies 24
2.2.2 Classification of Antibodies 28

University of Ghana http://ugspace.ug.edu.gh

2.2.3 Classification of Antibodies According to Multiplicity of Clones 31
2.2.4 Uses and Applications of Monoclonal and Polyclonal Antibodies 37
2.3.0 IMMUNIZATION OF LABORATORY ANIMALS 40
2.3.1 Introduction 40
2.3.2 Route of Immunization of Laboratory Animals for Antibody Production 40
2.4.0 IMMUNITY TO MALARIA 48
2.4.1 Innate Immunity to Malaria 48
2.4.2 Acquired Immunity to Malaria 49
2.4.3 Vaccines against Malaria-1 50
2.5.0 DIAGNOSIS OF MALARIA 52
2.5.1 Clinical Diagnosis of Malaria 53
2.5.2 Microscopic Diagnosis of Malaria 54
2.5.3 Rapid Diagnostic Tests for Malaria 57
2.6.0 MALARIA TRANSMISSION AND ITS EFFECT ON AGE-DEPENDENT DISEASE BURDEN IN ENDEMIC POPULATIONS 58
2.6.1 Malaria Transmission at Kpone-on-Sea 59
CHARPTER 3 61
GENERAL MATERIALS AND METHODS 61
3.1.0 STUDY SITE 61
3.1.1 Kpone-on-Sea 61
3.2.0 STUDY DESIGN 62
3.2.1 Sample Size Determination 62
3.2.2 *In Vitro* Propagation of Laboratory (3D7) Strain of *Plasmodium falciparum* Parasites 64
3.2.3 Extraction of Soluble Crude *Plasmodium falciparum* Antigens from *In Vitro* Cultured 3D7 Parasites 65
3.2.4 Preparation of Non-Immune Sera and Antisera of Mice for Microplate ELISA 67
3.2.5 Coating of Microplates for ELISA 67
3.2.6 Screening of BALB/c Mice for Anti-*Plasmodium* Antibodies by Indirect ELISA 68
CHAPTER FOUR 70
IDENTIFICATION OF MALARIA INFECTED AND UNINFECTED SUBJECTS IN AN ENDEMIC POPULATION 70

University of Ghana http://ugspace.ug.edu.gh

4.1.1 Objectives 71
4.2.0 MATERIALS AND METHODS 71
4.2.1 Study Subject Clinical History and Baseline Characteristics 71
4.2.2 Urine Sample Collection and Analysis 72
4.2.3 Blood samples collection and analysis 72
4.3.0 RESULTS 76
4.3.1 Characterization of *Plasmodium falciparum* infected subjects from a malaria infected population 76
4.4.0 DISCUSSION 87
CHAPTER FIVE 95
EXTRACTION AND CHARACTERIZATION OF BIOMARKERS OF MALARIA FROM INFECTED HUMAN URINE 95
5.1.0 INTRODUCTION (1) 95
5.1.1 Aim 96
5.1.1 (b) Specific Objectives 96
5.2.0 MATERIALS AND METHODS 96
5.2.1 Characterization of the Sample Population for the Studies to Characterize Biomarkers of Malaria from Infected Urine 96
5.2.2 Determination of Malaria Infection Status of Study Participants 97
5.2.3 Determination of Blood Haemoglobin Concentration and the Presence or Absence of Anaemia 97
5.2.4 Extraction of *Plasmodium* Proteins from Malaria Positive Urine 97
5.2.5 Urine analysis by BioRad protein assay 100
5.2.6 Test Principle for Detection of *Plasmodium* Antigens in Samples by Different Commercial Rapid Diagnostic kits: 100
5.2.7 Detection of *Plasmodium* Antigens in Infected Urine, Blood and Cultured *P. falciparum Parasites* by Rapid Diagnostic Test 101
5.2.8 Detection of Malaria Proteins in Infected Urinary and Cultured Antigen Extracts by SDS-PAGE 102
5.2.9 Coomassie Brilliant Blue R-250 Staining of Gel 103
5.2.10 Identification of Biomarkers of Malaria by Western blotting Analysis 104
5.2.11 Characterization of urinary hepcidin from *Plasmodium* infected human urine 105

University of Ghana http://ugspace.ug.edu.gh

5.3.0 RESULTS 107
5.3.1 Detection of *P. falciparum* HRP2 Antigen in Infected Human Urine by Commercial Diagnostic Kits 107
5.3.2 Detection of *Plasmodium* LDH Antigens in Infected Human Urine by the CareStart™ Rapid Diagnostic Kit 108
5.3.3 Urine analysis by BioRad protein assay 108
5.3.4 Characterization of proteins in Different Urine and *Plasmodium* Antigens by SDS-PAGE 110
5.3.5 *Plasmodium* Proteins in Different Antigen Preparations by Western Blotting Analysis 112
5.3.6 Characterization of Hepcidin from *Plasmodium* Infected Human Urine 113
5.3.7 Association between urinary hepcidin level and parasitaemia, anaemia and haemoglobin level 114
5.4.0 DISCUSSION 117
CHAPTER SIX 124
GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST *PLASMODIUM falciparum* ANTIGENS using URINARY AND *in vitro* CULTURED parasites 124
6.1.0 INTRODUCTION 124
6.1.1.0 Aim 125
6.2.0 MATERIALS AND METHODS 125
6.2.1 Preparation of Immunogens for Immunization of BALB/c Mice 125
6.2.2 Immunization of BALB/c Mice 126
6.2.3 Anti-*Plasmodium* parasite Antibodies in Immunized Mouse Sera by Microplate ELISA 128
6.2.4 Feeder Cell Preparation and *In Vitro* Propagation of Myeloma Cells 128
6.2.5 Cell Fusion and Selection of Hybridomas 129
6.2.6 Screening, cloning and cryopreservation of hybridoma cells 131
6.2.7 Determination of cut-off point for ELISA negative reactivity results 131
6.2.8 Immunoglobulin Classes of Monoclonal Antibodies in Hybridoma Culture Secretions 132
6.3.0 RESULTS 132
6.3.1 Immune Response in BALB/c Mice to Urinary and Cultured *Plasmodium* Antigens 132

University of Ghana http://ugspace.ug.edu.gh

6.3.2 Detection of Anti-Urinary *Plasmodium* Antibodies in Immunized Mouse Sera by Microplate ELISA 133
6.3.3 Detection of Cultured *Plasmodium* Parasite Antibodies in Immunized Mouse Sera by Indirect ELISA 135
6.3.4 Characterization of Anti-*Plasmodium* Monoclonal Antibodies by Microplate ELISA 139
6.3.5 Immunoglobulin Class and Reactivity of Monoclonal Antibodies by Rapid Isotyping Kit (PRIK) 140
6.3.6 Cloning of Hybridoma Cells the Limiting Dilution Method 141
6.4.0 Discussion 142
CHAPTER SEVEN 148
REACTIVITY OF MOUSE ANTI-*PLASMODIUM* MONOCLONAL AND POLYCLONAL ANTIBODIES IN ELISA AND WESTERN BLOTTING Assay 148
7.1.0 INTRODUCTION 148
7.1.1 Overall Aim 149
7.2.0 MATERIALS AND METHODS 150
7.2.1 Optimum Conditions for ELISA 150
7.2.2 Reactivity of Anti-*Plasmodium* Monoclonal Antibodies by Microplate ELISA 150
7.2.3 Prevalence of Malaria in the Sample Population by MAb Microplate ELISA 151
7.2.4 Prevalence of Malaria in the Sample Population by anti-*Plasmodium* PAb Microplate ELISA 152
7.2.5 Reativity of Anti-*Plasmodium* PAbs to Different Antigen Preparations by Western Blotting Analysis 152
7.2.6 Reactivity of UCP4W7 MAb to different antigen preparations by SDS-PAGE and western blotting assay-1 153
7.2.7 Reactivity of UCP4W7 MAb to different antigen preparations by ..... SDS-PAGE and western blotting assay-2 154
7.3.0 RESULTS 156
7.3.1 Optimum Conditions for ELISA to Ascertain the Reactivity of Anti- *Plasmodium* PAbs and MAbs 156
7.3.2 Reactivity of mouse anti-*Plasmodium* MAbs to urinary and cultured parasite antigens by microplate ELISA-1 156
7.3.3 Reactivity of mouse anti-*Plasmodium* MAbs to urinary and

University of Ghana http://ugspace.ug.edu.gh

cultured parasite antigens by microplate ELISA-2 159
7.3.4 Summary of the reactivity of mouse anti-*Plasmodium* MAbs to urinary and cultured parasite antigens 161
7.3.5 Reactivity of UCP4W7 MAb to different antigen preparations by SDS-PAGE and western blotting assay-1 162
7.3.6 Separation of proteins in different antigen preparations by SDS-PAGE and reactivity of UCP4W7 by western blotting assay-2 164
7.3.7 Detection of *Plasmodium* Proteins in Different Fractions of Urine and *Plasmodium* Antigens by Western Blotting Analysis 167
7.3.8 Relationship between Molecular Weights of Standard Proteins and Their Mobility through the NuPAGE-MOPS Gel 168
7.3.9 Molecular Weights of Standard Proteins and Their Mobility through the NuPAGE-MOPS Gel 169
7.3.10 Estimation of Molecular Weights (MW1) of Unknown Proteins 170
7.3.11 Relative sensitivity and specificity of tests for different biomarkers of malaria 171
7.3.12 Relative sensitivity and specificity of UCP4W7 ELISA and quatitative buffy coat test for malaria 173
7.4.0 Discussion 174
CHAPTER EIGHT 183
GENERAL DISCUSSION AND CONCLUSIONS 183
REFERENCES 198
APPENDICES 224
APPENDIX 1: MAP OF KPONE-ON-SEA STUDY AREA 224
APPENDIX 2: ADDITIONAL DATA FROM CHAPTER FOUR 225
APPENDIX 3: SCHEMATIC REPRESENTATION OF THE RAPID DIAGNOSTIC TEST PROCEDURE AS SHOWN IN WHO (2000) 230
APPENDIX 4: RESULTS OF URINE ANALYSIS 231
APPENDIX 5: PUBLICATIONS 247
Appendix 5a A poster presented at the First Annual College of Health Sciences Conference, held on 26th to 28th September, 2007 247
Appendix 5b A publication on the relationship of parasitemia and anemia

University of Ghana http://ugspace.ug.edu.gh

among patients with Plasmodium falciparum malaria in Ghana 248
APPENDIX 6: ETHICAL CONSENT FORMS 252
Appendix 6a 252
Appendix 6b 254
Appendix 6c 256
APPENDIX 7: ETHICAL CLEARANCE CERTIFICATE 257
APPENDIX 8: TECHNICAL CHALLENGES ENCOUNTERED IN THE COURSE OF THE STUDY AND HOW THEY WERE CIRCUMVENTED 258

University of Ghana http://ugspace.ug.edu.gh

LIST OF TABLES

Table 1: Host and Geographic Distribution of some *Plasmodium* parasites 12
Table 2: Classification of antibodies based upon location, structure and function..... 30
Table 3: Study Subject Clinical History and Baseline Characteristics 78
Table 4: Association between Malaria and Sex 79
Table 5: Prevalence of Parasitaemia among *Plasmodium* Parasite Positive Subjects Relative to Age..... 81
Table 6: Association Between Malaria and Anaemia 84
Table 7: Association between malaria and fever 87
Table 8: Characterization of Hepcidin from *Plasmodium* Infected Human Urine and Study Subjects Characteristics 114
Table 9: Association between urinary hepcidin level and parasitaemia, haemoglobin level and anaemia 116
Table 10: Mode of grading of monoclonal antibody reactivity patterns..... 131
Table 11: Immunoglobulin classes of selected hybridoma culture secretions 140
Table 12: Reactivity of anti-*Plasmodium* MAbs to urinary and cultured parasite antigen extracts-1 158
Table 13: Reactivity of mouse anti-*Plasmodium* MAbs to urinary and cultured *Plasmodium* antigen extracts 160
Table 14: Summary of monoclonal antibody reactivity results 161
Table 15: Relationship between the molecular weights of standard proteins and their mobility through NuPAGE MOPS gel 168
Table 16: Estimation of molecular weights (MW1) of unknown proteins 170
Table 17: Estimation of molecular weights (MW2) of unknown proteins 171
Table 18: Mean molecular weights of unknown proteins 171
Table 19: Sensitivity and specificity of tests for different biomarkers of malaria..... 172
Table 20: Sensitivity and specificity of UCP4W7 ELISA and QBC tests..... 173

University of Ghana http://ugspace.ug.edu.gh

LIST OF FIGURES

Figure 1: Global distribution of malaria (Sachs and Malaney, 2002)..... 11
Figure 2: General and Ultra-structure of the P. falciparum merozoite 14
Figure 3: Life cycle of the *Plasmodium falciparum* malaria parasite in the human and the mosquito hosts (Bannister and Sherman, 2009)..... 15
Figure 4a and b: Basic structures of the immunoglobulin molecule..... 28
Figure 5 Morphological views of the various stages of the four (4) main human malaria parasite species 73
Figure 6: Analysis of blood by hematocrit system for haemoglobin concentration 75
Figure 7: Prevalence of Malaria among Study Subjects Relative to Age, Parasite Density and Grading 80
Figure 8: Prevalence of malaria among study participants in relation to age 82
Figure 9: Variation of Mean Parasite Density Among Malaria Positive Subjects in Relation to Age 83
Figure 10: Variation of Mean body (Axillary) Temperature of Study Subjects in Relation to Malaria Infection and Age..... 86
Figure 11: Flow chart for extraction of *Plasmodium* antigens from urine of infected individuals 99
Figure 12: Determination of Concentration of Proteins in *Plasmodium* Infected Urine by BioRad Assay 109
Figure 13: Coommassie blue gel electrophoresis results 111
Figure 14: Western blotting results 112
Figure 15: Immune Response of BALB/c Mice to Urinary Malaria Antigens 134
Figure 16: Immune Response in BABLB/c mice to Cultured *Plasmodium* Antigens..... 135
Figure 17: Reactivity of Anti- *Plasmodium* Monoclonal Antibodies by Microplate ELISA 139
Figure 18: Mode of arrangement of the western blotting apparatus and gel system for western blotting analysis..... 153
Figure 19: a) Analysis of proteins in different antigen preparations by SDS-PAGE-1 and b) UCP4W7 probed western blotting assay-1..... 163
Figure 20: a) Analysis of proteins in different antigen preparations by SDS-PAGE-2. and b): UCP4W7 probed western blotting assay-2. 166

University of Ghana http://ugspace.ug.edu.gh

Figure 21: Western blotting results 167
Figure 22: Molecular weights of standard proteins and their mobility through the NuPAGE-MOPS Gel 169

University of Ghana http://ugspace.ug.edu.gh

LIST OF PLATES

Plate 1: A commercial RDT cassette showing the presence of *P. falciparum* histidine rich protein II (pfHRP2) in crude antigens (PAgHU) extracted from malaria infected human urine 107

Plate 2: A commercial RDT cassette showing the presence of *P. falciparum* histidine .rich protein II (pfHRP2) in crude antigens (PAgHU) extracted from malaria infected human urine 108

Plate 3: Collecting blood from the tail of BALB/c mouse before and after immunization 127

Plate 4: Inoculating BALB/c mouse with PAgHU or CPfAg Immunogen.....127

Plate 5: A microtitre plate showing the response of mice 1 and 2 to urinary *Plasmodium* antigens as determined by microplate ELISA.....136

Plate 6: A microtitre plate showing response of mice 3 & 4 to urinary *Plasmodium* antigens as determined by microplate ELISA:.....137

Plate 7: A cell fusion product on day-3 post cell fusion, showing a well on a 24-well tissue culture plate containing a mixture of unfused myeloma cells, spleen cells and hybridoma cells resulting from cell fusion. 138

Plate 8: Hybridoma cell colonies that are actively dividing and are in various phases of growth on day 9 post cell fusion.and 14 post cell fusion. 138

Plate 9: Hybridoma cell colonies that are actively dividing and are in various phases of growth on days 14 post cell fusion. 138

University of Ghana http://ugspace.ug.edu.gh

ABBREVIATIONS

µg:	Microgram
µl:	Microlitres
µm:	Micrometer
ABTS:	2, 2-Azino-bis (3-ethylbenethiazoline-6-sulphonic acid)
ACT:	Ammonium chloride and tris (hydroxymethyl, aminomethane)
BCE	Before Common Era or, before the first period of the Gregorian calendar
BSA:	Bovine serum albumin
$C_7H_5O_2Na$:	Sodium benzoate
CBB	Carbonate-bicarbonate buffer
$Co(NO_3)_2.6H_2O$:	Cobalt nitrate, hexahydrate
CPfAg	Cultured *Plasmodium falciparum* antigen extract
CPR	Cytochrome P450 reductase
CSA:	Condroitin Sulfate A
CW:	Control wells
DAB:	3, 3'-Diaminobenzidine
DED:	Dangme East District
dH_2O:	Deionized water
ddH_2O:	Double deionized water
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
ELISA:	Enzyme-linked immunosorbent assay
FBS:	Fetal bovine serum
g:	Gram
Ga:	Gauge
GPCI:	Global Parasite Control Initiative
GST:	Glutathione *S*-transferase
HA:	Hyaluronic acid
HBMM:	Home-based management of malaria
H_2O_2:	Hydrogen peroxide
HAT:	Hypoxanthine aminopterin thymidine
HGPRT:	Hypoxanthine guanine phosphorybosyl transferase
HIV:	Human immunodeficiency virus
HRP:	Horseradish peroxidase
PAgHU	Human urinary *Plasmodium* parasite antigen extract
ICAM	Intracellular adhesion molecule
ICT:	Immuno-chromatographic Test
IFA:	Indirect immunofluorescent assay
IgA:	Immunoglobulin A
IgE:	Immunoglobulin E
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
IHA:	Indirect haemagglutination
IMDM:	Iscove's modified Dulbecco's medium
JICA:	Japan International Cooperation Agency
KCl:	Potassium chloride
KHC:	Kpone Health Centre
$KHPO_4$	Potassium hydrogen phosphate

University of Ghana http://ugspace.ug.edu.gh

KMP:	Kpone Malaria Project
KOS:	Kpone-on-Sea
mA:	Milliampere
MSS	Mass Spectrometry Sequencing
mg:	Milligram
min:	Minute
ml:	Millilitre
mm:	Millimeter
mM:	Millimolar
MAb:	Monoclonal antibody
MBT	Mean body temperature
MPD	Mean parasite density
M_r:	Molecular mass
MW	Molecular weight
MWCO:	Molecular weight cut off
Na_2HPO_4:	Disodium hydrogen phosphate
NaCl:	Sodium chloride
$NaCO_3$:	Sodium carbonate
Na_2-EDTA:	Disodium ethylene diamine tetra acetate
$NaHCO_3$:	Sodium hydrogen carbonate (sodium bicarbonate)
nm:	Nanometer
NMCP:	National Malaria Control Programme
NMIMR:	Noguchi Memorial Institute for Medical Research
PBS	Phosphate buffered saline
PDCD:	Parasitic Diseases Control District
PEG:	Polyethylene glycol
Pf (*P. falciparum*)	*Plasmodium falciparum*
*pf*EMP1:	*Plasmodium falciparum* erythrocyte membrane protein 1
PRIK	Pierce rapid isotyping kit
PVDF:	Polyvinylidene diflouride
RBC:	Red blood cell (erythrocyte)
RDT	Rapid diagnostic test
RIA:	Radioimmunoassay
RT:	Room temperature
RUBDA	Rapid urine-based dipstick assay
SASS:	Saturated ammonium sulphate solution
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second(s)
SPH:	School of Public Health
SWA:	Soluble worm antigens
TBS:	Tris-buffered saline
TEMED:	N, N, N'-N'-tetramethylethylenediamine
Th1:	T helper type 1
Th2:	T helper type 2
TK:	Thymidine kinase
TW:	Test wells
RUBDA:	Urine-Base Dipstick Test
v/v:	Volume per volume
w/v:	Weight per volume
WACIPAC	West African Centre for International Parasite control

University of Ghana http://ugspace.ug.edu.gh

WHO:	World Health Organization
3D7	*Plasmodium falciparum* clone 3D7
µg	Microgramme
µl	Microliter
µM	Micromolar
°C	Celsius
cm	Centimeter
Da	Dalton
kDa	kilodalton
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
MACS	Magnetic activated cell sorter
OD	Optical density
UCP4W7	Uri, culture plate-4, well-7

University of Ghana http://ugspace.ug.edu.gh

SUMMARY

Bloodsmear microscopy is currently the gold standard and the principal test for confirmatory diagnosis of malaria. However, microscopy is labour-intensive and is limited by inadequate sensitivity and specificity.

Apart from microscopy, rapid diagnostic tests (RDTs), which include the quantitative buffy coat test (QBC) and the immuno-chromatographic tests (ICTs) are alternative tests for confirmatory diagnosis of malaria. RDTs, particularly, ICTs are fast gaining momentum as alternative tests for routine diagnosis of the disease. These RDTs are fast and have yielded remarkable diagnostic outputs in certain localities, however, they are invasive, and sometimes, have limited sensitivity and/ or specificity.

The work reported in this thesis was performed in order to generate monoclonal antibodies (MAb) towards the development of rapid urine-based assay (RUBDA) for non-invasive diagnosis of malaria, through detection of parasite antigens in the urine of infected individuals. Such an antibody-based test, is hoped to serve as a promising alternative for diagnosis of the disease, especially, for home-based management of malaria, particularly, in the hard-to-reach, low-income communities where the morbidity and mortality are highest.

Urine and blood samples were collected from Kpone-on-Sea (KOS), a malaria endemic coastal fishing village in south-eastern Ghana. KOS is being developed as a model site for malaria intervention studies in a larger study titled the Kpone Malaria Project (KMP).

Blood smear microscopy, quantitative buffy coat (QBC) test and commercial rapid diagnostic test (RDT) kits were used to distinguish between malaria infected and uninfected blood and urine samples. This was necessary, partly to select infected urinary antigens for immunizations towards MAb generation. Also, it was necessary to select true negative control urine samples for ascertaining the specificity of the MAb.

University of Ghana http://ugspace.ug.edu.gh

Analysis of the results showed a high malaria prevalence of 46.5% among the study population. Also, the results showed that malaria was significantly associated with sex (P = 0.0001), age (P= 0.0001), and fever (P = 0.0001). The results however showed that malaria was not associated with anaemia (P = 0.173).

The observation of significantly higher prevalence of malaria and higher parasite density among children than adults in the studies were important findings that confirmed reports by other writers that children are more susceptible to the disease than adults. In addition, the occurrence of higher prevalence of malaria among females in this community, even though it confirmed what Ayele *et al*. (2012) reported, was suspected to be linked to behaviour more than to sex, as reported by other authors (Steketee *et al*., 2001). Immunological and biochemical analyses needed to ascertain the link between malaria and sex were not carried out so it was difficult to conclude that malaria was sex-linked.

Immunization of mice towards MAb generation showed that 30 µg of urinary antigen was more immunogenic than 15 µg. Mice immunized with 15 µg of the antigen showed weak immune response, with absorbance values ranging between 0.36 and 0.45, measured at 414 nm. A majority (80%) of these mice did not show any observable response at all. In contrast, almost all (93.3%) of the 15 mice immunized with 30 µg of immunogen, showed high immune response (absorbance = 2.75) with only one (6.7%) not showing any response at all. Also, the *Plasmodium*-infected human urinary antigen extract (PAgHU) was found to be more immunogenic than the cultured parasite antigen extract (CPfAg) in these experiments.

In all, 96 MAb clones were successfully generated against urinary and cultured *Plasmodium* parasite antigens. Determination of the immunoglobulin class of some of the MAb clones produced showed that 57.14% were of the IgM and 42.86% were of the IgG isotype. Characterization of these MAbs by microplate ELISA showed that they were all (100%) reactive to urinary *Plasmodium* parasite antigen extract (PAgHU). Also, 66 (68.8%)

University of Ghana http://ugspace.ug.edu.gh

of these clones were reactive to cultured parasite antigens (CPfAg), 30 (31.3%) reacted specifically to only PAgHU, and none reacted with CPfAg alone.

One of the MAb clones (UCP4W7), showed strong reactivity in ELISA and immunoglobulin class analysis. This UCP4W7 MAb was consequently selected and evaluated by microplate ELISA for sensitivity and specificity in detecting malaria antigens in the urine of a cohort of 420 randomly selected individuals from the study community. In this experiment, using microscopy as a gold standard test, the UCP4W7 MAb distinguished between infected and uninfected urine with a relative sentivity and specificity of 96.9 and 75.6%, respectively. Compared to the UCP4W7 MAb, the mouse anti-*Plasmodium* PAb even though it had a relatively higher sensitivity (97.4%) in microplate ELISA the specificity was extremely lower (21.3%).

Also, determination of the relative sensitivities and specificities of the tests for various biomarkers of malaria compared to microscopy showed that tests for ketones, microhematuria, leukocytes, nitrites, glucose, bilirubinuria urobilinogen as well as PAb ELISA, were highly specific but had very low sensitivities. Each of these tests had a combined reactivity (Relative sensitivity + specificity) being less than the 170% threshold required to qualify it as a promising biomarker for diagnosis of malaria.

Characterization of proteins in PAgHU, CPfAg and other antigen preparations by SDS-PAGE showed that PAgHU contained more protein bands in the profile than the rest of the antigen preparations. The results also showed that the number of bands in the profiles of various urine samples from infected individuals decreased with decreasing parasite density. These findings demonstrated the likelihood of association between proteinuria and parasite density, and therefore, suggested that perhaps resolution of urinary *Plasmodium* proteins by SDS-PAGE could be used to quantify parasite density in infected individuals.

University of Ghana http://ugspace.ug.edu.gh

Further characterization of the urinary malarial antigens specific to UCP4W7, using microplate ELISA, SDS-PAGE, western blotting assay, mass spectrometry sequencing (MSS) and commercial RDT kits, showed that some of these antigens comprised the *P. falciparum* species-specific histidine-reach protein 2 (HRP2), *Plasmodium* pan-specific lactate dehydrogenase (LDH) antigen, the anti-malaria human metabolite peptide hormone (hepcidin), as well as other malarial proteins whose identities are yet to be determined.

The western blotting assay also revealed that the selected UCP4W7 MAb could distinguish between *Plasmodium* infected and uninfected human urine. In addition, it was found that the MAb could differentiate between urinary and cultured *Plasmodium* parasite antigens by western blotting assay.

Furthermore, the western blotting analysis demonstrated that UCP4W7 could distinguish specifically between urinary *Plasmodium* antigens and antigens from *Schistosoma* parasites as well as poliomyelitis and measles vaccines. These results showed that species-specific MAbs against *Plasmodium* parasite proteins in urine would be promising for diagnosis of malaria and should therefore be explored further for development of accurate non-invasive tests for diagnosis of the disease.

The results, however, showed that the UCP4W7 MAb could not distinguish between malaria antigens in urine and yellow fever vaccine antigens because of non-specific reactivity.

On the other hand, cross-reactivity of the MAbs with yellow fever vaccine suggests that the *Plasmodium*-infected urine samples used in generating the MAbs, also had yellow fever antigens possibly originating from vaccination and/ or active infections with the virus. In this respect, it was thought that ruling out yellow fever vaccination or infections from malaria suspected urine samples, would enable the UCP4W7 MAb to be used in its current state to distinguish between malaria infected and uninfected persons.

University of Ghana http://ugspace.ug.edu.gh

Finally, examination of urine samples from malaria infected and uninfected individuals by MSS, demonstrated that urinary hepcidin was associated with malaria parasitaemia in infected individuals, but was not associated with anaemia. Hepcidin is a peptide hormone produced by chronic inflammation in affected individuals. Since malaria causes inflammation in infected persons, the results were thought to suggest that urinary hepcidin would be a promising biomarker for malaria. Ultimately, these results therefore suggested that urinary hepcidin could be used to develop a non-invasive test for diagnosis of malaria.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 BACKGROUND AND RATIONALE

Malaria is a global health problem caused by protozoan parasites of the genus *Plasmodium*. It exerts a heavy toll of illness and death (WHO fact sheet 2002). Four main species of *Plasmodium* were originally known to infect humans, namely, *P. falciparum*, *P. malariae*, *P. vivax*, and *P. ovale*, of which, *P. falciparum* is the most predominant and lethal (WHO fact sheet 2002; White, 1998; Bradley, 1996). However, currently, *P. knowlesi* which was originally known to parasitize macaque monkeys of Southeast Asia has been reported to infect humans by many Authors (Sermwittayawong *et al*., 2012; Van den Eede *et al*., 2009).

According to WHO, in 2013, about 198 million people were infected with malaria globally, 384,000 people died from the disease, 3.2 billion people, constituting about 40% of the world population was at risk of the disease (WHO 2014). It is sad to note however that albeit so much is being done to control the disease, malaria is still a major global health problem. This situation was corroborated by WHO World Malaria Report, 2008, which reported that in 2006, approximately 3.3 billion people were at risk of developing malaria each year, with at least 500 million cases, and a death toll of nearly 1.0 million annually. Sub-Saharan Africa is most affected (Snow *et al.*, 2005), where malaria accounts for up to a third of all hospital admissions (Phillips, 2001; WHO fact sheet 2002).

Children and pregnant women are at the greatest risk, due to lack of adequate immune protection against the disease (Schwartlander, 1997; WHO, 1996). In areas where *Plasmodium falciparum* is endemic, delay in treatment of uncomplicated malaria may result in severe disease in these most vulnerable groups, characterized by conditions such as, anaemia, weakness, cerebral malaria, obstetric complications and spontaneous abortion in

University of Ghana http://ugspace.ug.edu.gh

pregnancy malaria, impaired consciousness, and death (Brewster *et al*., 1990; WHO fact sheet 2002). Early diagnosis and treatment are therefore crucial in preventing complicated malaria.

In Ghana, malaria due to *P. falciparum* is a major cause of morbidity and mortality (Quakyi, 1980). It accounts for 42% of all outpatient clinic visits, 8% of all certified deaths and 20% of under-five mortality (GHS/MOH, 2004.). Furthermore, malaria is the commonest cause of death in children aged 0-4 years (Kofi, 1989).

To curtail this problem, various malaria control efforts were initiated. These include:

1. The Ghana National Malaria Control Programme (NMCP), which works in collaboration with the Roll Back Malaria (RBM) programme and focused on the reduction of malaria burden by half by the year 2010.
2. The development of a model School-based Parasitic Diseases Control District (PDCD) in West Africa by the West African Centre for International Parasite Control (WACIPAC) (GPCI report, 2002).
3. The multidisciplinary case-control study of malaria pathogenesis and immunity in Ghana (Quakyi *et al*., 2004).

It is interesting to note, however, that each of these projects had one important limitation, which was the reliance on microscopy as a standard diagnostic technique even though it is not rapid enough and may fail to detect some infections because of low sensitivity. There is therefore the need to develop new improved diagnostic techniques for better management of the disease.

The Global Malaria Control Strategy (WHO, 1993) acknowledges that, prompt, rapid and accurate diagnosis is required to develop effective management strategies for prevention of severe and complicated disease. Although clinical diagnosis of malaria is relatively inexpensive to perform, fast, and requires no special equipment or supplies it may not be accurate enough (WHO, 2000). This is because, it relies on identification of clinical signs

University of Ghana http://ugspace.ug.edu.gh

and symptoms which may also be caused by several other diseases, including, typhoid fever, influenza, meningitis, hepatitis, as well as haemorrhagic fevers (White, 1998; Bradley, 1996). Clinical diagnosis of malaria therefore, is based on a high level of presumption (WHO, 1991), following which the laboratory microscopic and/or other detective approaches are needed for confirmatory diagnosis.

The conventional light microscopy is presently the established and most acceptable method for the laboratory confirmation of malaria (Katzin *et al*., 1991; Payne, 1988). Careful examination of a well-prepared and well-stained blood film by an expert microscopist is at the moment, the "gold standard" for specific detection and identification of malaria parasites (Payne, 1988). The microscopic method for diagnosis of malaria has several advantages (WHO, 2000). It is relatively inexpensive (Palmer *et al*., 1998), very specific, and the smears can provide a permanent record of the diagnostic findings and also be subject to quality control (Payne, 1988). However, several drawbacks such as inadequate sensitivity associated with microscopy make it unattractive enough for malaria diagnosis.

There are already available rapid test kits {immunochromatographic tests (ICTs)} for alternative diagnosis of malaria. These techniques involve the detection of malaria parasite antigens in blood. They mostly employ the use of a dipstick or test strip, bearing monoclonal antibodies against malaria parasite antigens in immuno-chromatography. Immuno-chromatographic tests do not need any specialized expertise, and can be performed within 15 minutes. However, they are blood-based and therefore invasive.

The development of a simple accurate rapid urine-based dipstick assay (RUBDA) for diagnosis of malaria could be very useful in overcoming these limitations. RUBDA is field-applicable and does not require any technical skill to perform. Lastly, the use of urine as test

University of Ghana http://ugspace.ug.edu.gh

material makes the RUBDA a safer, more affordable, attractive and acceptable method for home-based management of malaria as compared to already available ones.

Experience was drawn from earlier work in our laboratory, which led to the development of a field-applicable urine-based monoclonal antibody (MAb) dipstick for diagnosis of urinary schistosomiasis (Bosompem *et al*., 1997).

The motivation for this study was drawn from reports that parasite antigens are excreted in the urine of patients of Chagas' disease (Katzin *et al*., 1989); schistosomiasis (Bosompem *et al*., 1996*a*); and malaria (Oguonu *et al*., 2014; Katzin *et al*., 1988). Rodriguez-del Valle *et al*. (1991), also detected *Plasmodium* antigens and anti-malarial antibodies, in the urine of infected individuals and suggested that a urine-based assay for diagnosis of malaria would be feasible. In this study, malaria-related proteins were detected in the urine at concentrations high enough for direct analysis, and in view of this, it was suggested that a diagnostic assay based on the detection of specific antigens or antibodies in patients' urine should be considered (Rodriguez-del Valle *et al*., 1991).

1.2 PROBLEM STATEMENT

Obstacles to malaria control include delayed and inaccurate diagnosis and treatment; resistance of *Plasmodium* parasites and *Anopheles* vector mosquitoes to anti-malarial drugs and insecticides respectively as well as human behaviour that promote the proliferation of vector mosquitoes. Even though various strategies are being used to control malaria such as increased coverage of insecticide treated bed nets and intermittent preventive treatment for pregnant women, the morbidity and mortality attributable to malaria have not changed significantly. This situation emphasizes the need for improved tools to enhance the control efforts.

University of Ghana http://ugspace.ug.edu.gh

For example, prompt, accurate, safe and affordable diagnosis is the first step towards effective management of malaria. However, the uncertain differential diagnosis associated with the routine use of clinical signs and symptoms of malaria necessitates the use of microscopy to confirm diagnosis even though the test is not sensitive enough. Furthermore, microscopy is labour-intensive, time consuming and requires well-trained technical staff for accurate diagnosis. Unfortunately, such highly trained technicians are usually not available at the peripheral levels of the health care delivery system (WHO, 2000) where most malaria-related deaths and morbidity occur. In view of this, Payne (1988) indicated that microscopic diagnosis of malaria using scarce resources could be doubtful.

Existing immunochromtographic tests for malaria also have the weaknesses of invasive tests including exposure of health-workers to blood (Rodriguez-del Valle *et al*., 1991) and poor acceptance by some communities. Indeed, currently, none of the major tests for diagnosis of malaria can function without blood as a test material. This characteristic makes all these major tests unsuitable for home-based management of malaria (HBMM) in most of the low-income rural communities where most malaria-related deaths and morbidities occur. Perhaps, this is partly because, blood is used for pacification of gods and other rituals in these communities and therefore considered fetishism to engage in any practices that have connections with blood (Otabil, 1992). Also, with the connection of HIV/AIDS to blood, most people, would, under normal circumstances, not want to have anything to do with blood. The lack of effective diagnosis on account of these, therefore, makes the HBMM not to be effective enough. A simple dipstick test involving the use of urine as a test material would therefore be a welcome alternative.

In addition, many pregnant women in rural, low-income communities do not report for antenatal healthcare (ANC), or report late, because of factors including inaccessibility and inability to meet service charges, thereby increasing their risk of malaria disease and death.

University of Ghana http://ugspace.ug.edu.gh

However, in communities with inadequate healthcare infrastructure, simple field-applicable rapid diagnostic tools for malaria in pregnancy would lead to early detection and treatment of pregnancy malaria at the community and home-based level timely enough to avoid complications and/or death that could result. Therefore, a diagnostic technique, which combines high specificity, sensitivity, and rapidity to perform with non-invasiveness and cost-effectiveness, would be a most welcome alternative.

It is in a bid to improve upon the limitations of the current diagnostic tools that this study proposed to produce an accurate rapid urine-based field-applicable dipstick test for diagnosis of malaria in infected individuals using *Plasmodium* antigens extracted from cultured parasites and urine of infected subjects.

1.3 OBJECTIVES

1.3.1 Main Objective

The project aimed, at developing an accurate urine-based monoclonal antibody dipstick assay (RUBDA) for diagnosis of malaria utilizing *Plasmodium* parasite antigens from the urine of infected individuals.

1.3.2 Specific Objectives

1. Identification of malaria infected and uninfected subjects in an endemic population.
2. Extraction and characterization of biomarkers of malaria from infected human urine.
3. Generation and characterization of monoclonal antibodies against *Plasmodium falciparum* antigens using urinary and *in vitro* cultured parasites.
4. Reactivity of mouse anti-*Plasmodium* monoclonal and polyclonal antibodies in urine-based ELISA and western blotting assay.

University of Ghana http://ugspace.ug.edu.gh

1.4 JUSTIFICATION

The development of a simple, accurate, fast and non-invasive rapid urine-based dipstick assay (RUBDA) for diagnosis of malaria would lead to timely diagnosis and treatment of the disease, and so eliminate the complications and death that could result from delayed treatment of the disease.

Finally, home-based management of malaria (HBMM) which is recognized as an important strategy for reducing the morbidity and mortality of the disease, thrives on accurate diagnosis outside the health facility, usually in the home by parents, caregivers and/ or by oneself. HBMM is, however, presently done using the presumptuous symptomatic diagnostic approach, because of lack of technical skills and logistics such as microscopes, which are required to perform the current blood-based tests. This situation, prevents the full utilization of the potential of the HBMM to combat the disease. The proposed urine-based test for malaria is simple, user friendly and lacks all the limitations mentioned above. Therefore, when developed and deployed commercially, application of the system would increase patronage of HBMM, and contribute greatly towards reduction of the alarming complications and death that are presently attributable to malaria.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER TWO

LITERATURE REVIEW

2.1.0 MALARIA

Malaria is a severe debilitating disease caused by obligate intracellular protozoan parasites of the genus *Plasmodium*. It exerts a heavy toll of illness and death (Ohalete *et al*., 2011; WHO fact sheet 2002), due to the immunological interactions with the human host as part of its life cycle.

2.1.1 Economic Cost of Malaria

Economically, the cost of malaria is high. Even though the tropical regions of the world are the most affected, the impact of malaria on humans extends as far as the temperate zones (Nayyar *et al*., 2012). In areas where the disease is widespread, it is said to have major adverse economic effects on the inhabitants (Nayyar *et al*., 2012). Indeed, according to some authors, malaria is not just a disease commonly associated with poverty but also, it is a cause of poverty itselt and a major drawback to economic development (Worrall *et al*., 2005). Malaria was reported to be a major factor in the slow economic development of the American southern states in the late 19th and early 20th centuries (Humphreys, 2001). Also, between 1996 and 2002 for example, the total annual cost of treatment in Africa alone was estimated to be $US1.8 billion (WHO fact sheet 2002; Bradley, 1996).

According to Nayyar *et al*. (2012), a comparison of average per capita gross domestic productivity (GDP) in 1995, adjusted for parity of purchasing power, between malaria endemic and non endemic countries gave a fivefold difference ($1,526 USD versus $8,268 USD). Also, in the period between 1965 and 1990, countries where malaria was common had

University of Ghana http://ugspace.ug.edu.gh

an average per capita GDP that increased only 0.4% per year, compared to 2.4% per year in other countries without malaria (Sachs and Maloney, 2002).

In Ghana, malaria mortality and morbidity have been reported to retard economic growth by reducing the capacity and efficiency of the labour force. In addition, a 10% reduction in malaria was associated with 0.3% growth in the economy; and for every increase in the morbidity rate of malaria, the GDP of Ghana is said to reduce by 0.41% (Asante and Asenso-Okyere, 2003). In monetary terms, this economic burden is said to transcend to US$1.92 million in the 2002 fiscal year alone.

In its entirety, the economic burden of malaria has been estimated to cost Africa $12 billion (USD) per annum (Nayyar *et al*., 2012). This figure comprises the costs of health care, working days lost due to sickness, days lost in education, decreased productivity due to brain damage from cerebral malaria, as well as loss of investment and tourism (Greenwood *et al*., 2005). The degree of burden varies from country to country and is heavier in some countries, where it may be responsible for 30–50% of hospital admissions, up to 50% of outpatient visits, and up to 40% of public health spending (Roll Back Malaria WHO Partnership, 2003).

2.1.2 Geographic Distribution of Malaria and the *Plasmodium* Parasites

Even though various reports have indicated different times of origin of malaria parasites, virtually all the writers believe that if not all, most of the present-day populations of the human malaria parasites originated from Africa (Conway *et al*., 2001).

It is however important to note that irrespective of the origin of malaria and its causative *Plasmodium* parasites, the disease has generally, occurred in areas where environmental conditions allow parasite multiplication in the vector. Malaria today, therefore, is usually restricted to tropical and subtropical areas and altitudes below 1,500 m. In the past,

University of Ghana http://ugspace.ug.edu.gh

however, malaria used to be endemic in much of North America, Europe and even parts of northern Asia, and today, it is still present on the Korean peninsula and in many other parts of Asia. This present pattern of geographic distribution of malaria could have been influenced by climatic changes and population movements.

Plasmodium falciparum is the predominant species in the world. *P. vivax* and *P. ovale* are traditionally thought to occupy complementary niches, with *P. ovale* predominating in Sub-Saharan Africa and *P. vivax* in the other areas; but their geographical ranges do overlap. These two species are not always distinguishable on the basis of morphologic characteristics alone, and the use of molecular tools helps to clarify their diagnosis and exact distribution. *P. malariae* has wide global distribution, being found in South America, Asia, and Africa, but it is less frequent than *P. falciparum* in terms of association with cases of infection. *P. knowlesi* is found in south-east Asia.

Climatic factors such as temperature, humidity and rainfall are the primary forces controlling the geographic distribution of malaria (Kelly-Hope *et al*., 2009; Data retrieved from http://www.cdc. gov/malaria/distribution_epi/distribution.htm on14th Jan 2010). This is because the survival and multiplication of the vector *Anopheles* mosquitoes, and the completion of the *Plasmodium* parasite life-cycle in the mosquito depend on favourable climatic conditions (Kelly-Hope *et al*., 2009). Malaria transmission therefore occurs in areas where climatic conditions are favourable. Environmental temperature is very important in the transmission and distribution of *Plasmodium* parasites. At temperatures below 20^{o}C, for example, it is known that *P. falciparum* cannot complete its growth cycle in the vector *Anopheles* mosquito and therefore cannot be transmitted (Samuel *et al*., 2011).

Also, it is generally known that in areas characterized by very high altitudes, cold seasons, hot, dry atmospheres, as found in some deserts (excluding the oases) where malaria parasites cannot survive and multiply, there is no malaria transmission. In warmer regions

University of Ghana http://ugspace.ug.edu.gh

near the equator where humidity is high, malaria transmission is more intense and perennial (throughout the year), and *P. falciparum* predominates. Malaria transmission is therefore highest in tropical regions south of the Sahara, and high in subtropical regions in Africa, southern Asia, northern parts of South America, Central America as well as Indonesia, where the surrounding environmental factors are favourable. Figure 1 (Sachs and Malaney, 2002) and Table 1 (Zilversmit and Perkins. 2008) below illustrate the geographic distribution of malaria, the causative *Plasmodium* parasite and the vertebrate host.

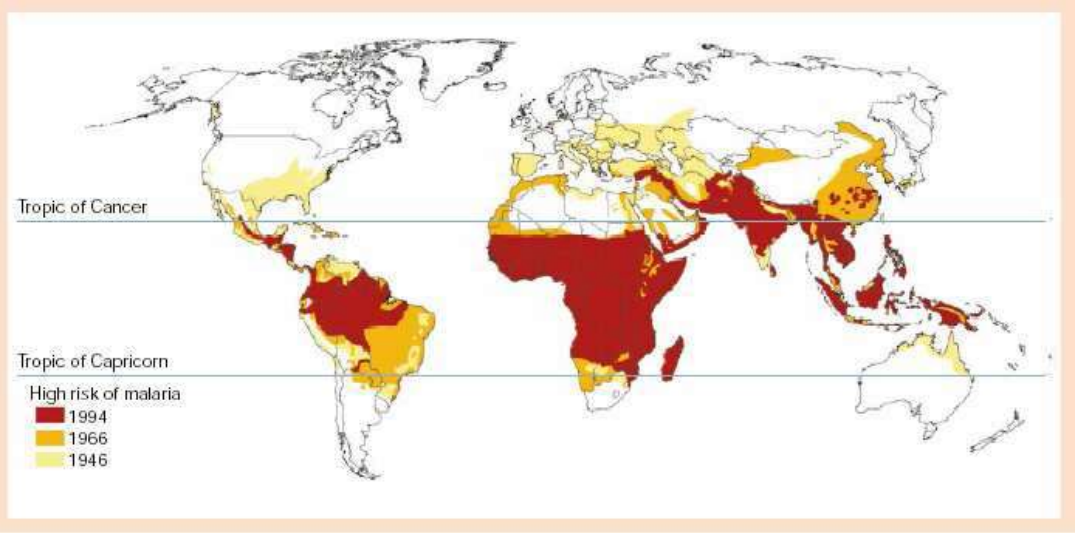


Figure 1: Global distribution of malaria (Sachs and Malaney, 2002)

University of Ghana http://ugspace.ug.edu.gh

Table 1: Host and Geographic Distribution of some *Plasmodium* parasites

Modified from Zilversmit and Perkins, 2008

Parasite	Host	Geographic Location
P. falciparum, P. vivax, P. malariae	Human	Africa, Asia, South and Central America
P. ovale	Human	Africa
P. reichenowi	Chimapanzee	
P. gonderi	Madrill	
P. atheruri, P. vinkei, P. chabaudi, P. berghei, P. yoelii	Rodent	
P. fieldi, P. simiovale, P. hylobati, P. inui, P. coatneyi, P. cynomolgi	Macaque	Southeast Asia
P. knowlesi	Macaque, Human	
P. gallinaceum	Bird	
P. elongatum, P. relictum	Bird	Worldwide
P. agamae, P. gigantum	Lizard	Africa
P. floridense, P. azurophilum	Lizard	Carribbean/Central America
P. mexicanum, P. chiricahuae	Lizard	North America
P. faichildi	Lizard	Central America
P. mackerassae	Lizard	Australia
P. simium	Spider Monkey	South America
P. brasilianum	Spider/Howler/ Night Monkey	
Hepatocystis sp.	Bat/Primate	Africa, Asia

Many temperate areas, such as Western Europe and the United States, used to be endemic for the disease, however, economic development and public health measures have led to the successful elimination of malaria even though most of these areas have *Anopheles* mosquitoes capable of transmitting the disease, and are therefore considered at risk of reintroduction of the disease (Kakkilaya, 2006).

In all, the World Malaria Report 2005 stated that malaria is endemic in 107 countries throughout the world. Several countries in North Africa, the Eastern Mediterranean and Central Asia that have recently made tremendous progress in reducing transmission and are now within reach of eliminating malaria have been included in the malaria-endemic countries.

University of Ghana http://ugspace.ug.edu.gh

However, countries that have only imported cases or occasional local transmission (introduced cases resulting from imported cases) are not included, although surveillance of malaria cases and provision of access to effective anti-malarial treatment remain important in these countries as well (WHO Malaria Report 2005).

2.1.3 The Biology and Life-Cycle of the *Plasmodium* Parasite

2.1.3.1 Structure of the Plasmodium

The genome of *Plasmodium falciparum*, the most common causative agent for human malaria, has been sequenced completely, yielding 14 chromosomes and 5,300 genes. A large number of these genes enable the parasite to evade the host's immune defense mechanisms. The mapping of this genome sequence provides new avenues for research on possible vaccines. The sporozoite, the stage infective to humans, is 10-15 µm in length and about 1 µm in diameter. Each has a thin outer membrane, a double inner membrane below which lies the sub-pellicular microtubules.

Figure 2 below illustrates the general morphology and ultrastructure of the *P. falciparum* parasite. The sporozoites, like the other motile invasive stages, the merozoites and ookinetes, are elongated and uni-nucleated. They all lack cilia and flagella except for the microgametes. However, they possess specialized secretory, locomotory and invasive organelles (rhoptries, micronemes and 3 polar rings) at their anterior end (Bannister and Sherman, 2009). These organelles constitute the apical complex required for penetration into host cells or tissues. The apical organelle complex gives these organisms the name Apicomplexa. The rhoptries are long, extending half the length of the body. The micronemes are convoluted elongated bodies which run into a common duct with the rhoptries at the anterior end of the body.

University of Ghana http://ugspace.ug.edu.gh

Also, *Plasmodium* parasites possess one or more mitochondria, and an elongated membranous organelle known as the apicoplast (Waller and McFadden, 2005; Williamson *et al*., 2002) which is located near the posterior end of the body. *Plasmodium* parasites move by a unique form of gliding locomotion. Evolutionarily, they are closely related to ciliates and dinoflagellates (Hoppenrath and Saldarriaga (2012).

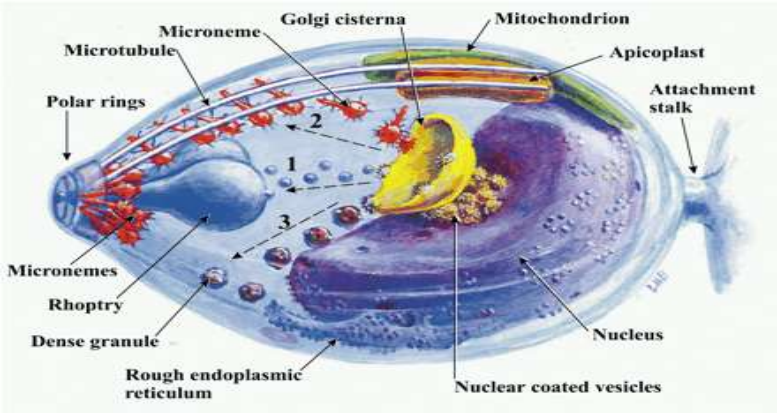


Figure 2: General and Ultra-structure of the P. falciparum merozoite {from Bannister and Sherman (2009) and Bannister *et al*., (2003), respectively}

2.1.3.2 Life Cycle of the Plasmodium Parasite

The *Plasmodium* genus of protozoan parasites has a life cycle, which is split between a vertebrate host and an insect vector (Bradley, 1996). The female *Anopheles* mosquito is the vector for transmission. Mosquitoes in general, feed on nectar or any edible sugar-containing fluid or solution found in the environment. The female species, however, require a protein of the vertebrate blood for development of their ovaries and maturation of the fertilized eggs, hence the need to feed on vertebrate blood (Brown *et al*., 2008). The schematic representation of the life cycle of the parasite is shown in figure 4 below.

The life cycle begins with the injection of spindle shaped infective sporozoites from the vector mosquito salivary gland into the human body (tissue or blood capillary) with mosquito anticoagulant saliva which serves to prevent clotting of blood in the mosquito proboscis and ensures an even flowing meal. It has been estimated by various methods

University of Ghana http://ugspace.ug.edu.gh

(Ponnudurai *et al*., 1991) that compared to the total number of sporozoites observed in the salivary gland, only about 10% (<50) sporozoites are injected into the vertebrate host during each blood meal.

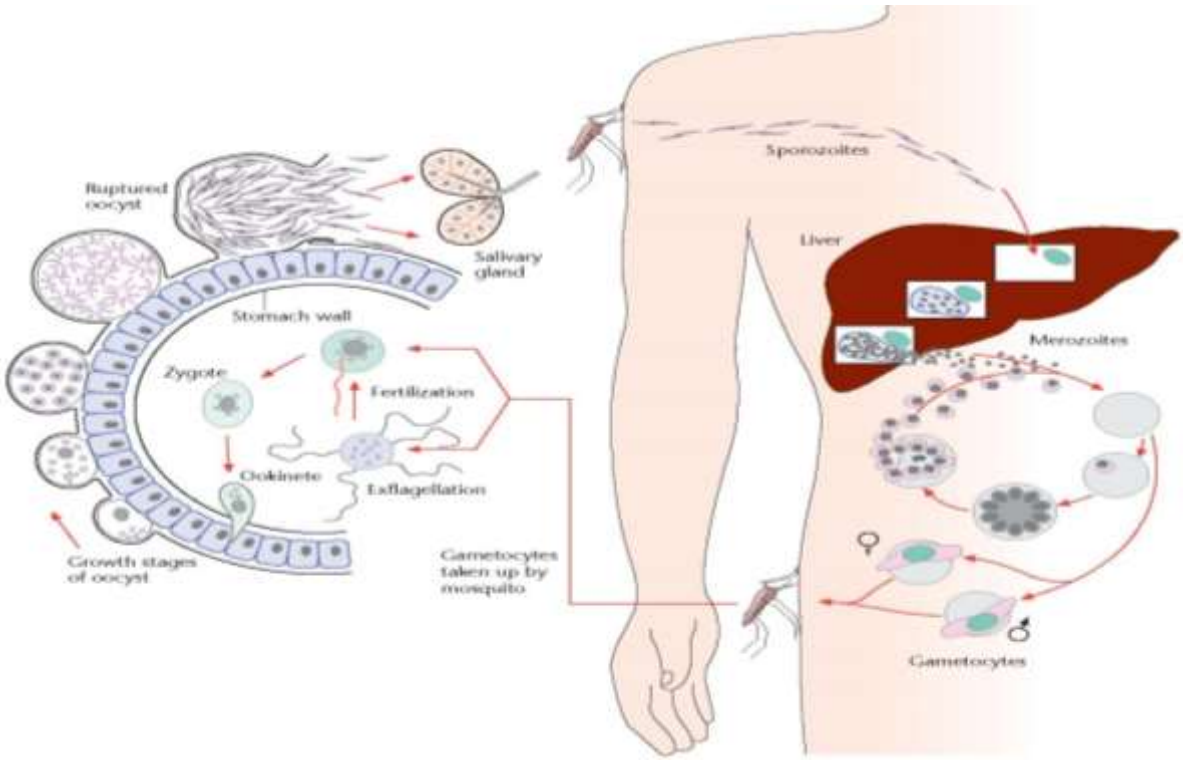


Figure 3: Life cycle of the *Plasmodium falciparum* malaria parasite in the human and the mosquito hosts (Bannister and Sherman, 2009)

The traditional perception has been that once the sporozoites have been injected into the peripheral circulation of the human host, they are carried into the liver, where they penetrate liver cells (hepatocytes), remain for 9-16 days, become ring shaped (schizonts) and multiply within the cells (Doolan *et al*., 2009). However, other studies on the kinetics of sporozoite migration (Yamauchi *et al*., 2007), have shown that sporozoites are first injected by mosquitoes into the skin, where they remain for up to 6 hours before trickling into the bloodstream, before migrating into the liver. Also, Amino *et al*. (2006), reported that approximately one-third of the sporozoites leaving the site of inoculation may enter lymphatic

University of Ghana http://ugspace.ug.edu.gh

vessels and finally end up in the regional lymph nodes. The findings of these studies therefore suggest that there are multiple potential sites for sporozoite-host interaction (Doolan *et al*., 2009).

In the liver, the sporozoites penetrate liver cells, become transformed into schizonts that multiply asexually. *P. falciparum* and *P. malariae* sporozoites trigger immediate schizogony whereas *P. ovale* and *P. vivax* sporozoites may either trigger immediate schizogony or have a delayed trigger, resulting in dormant hypnozoites, also referred to as merosomes (Bradley, 1996; Sturm *et al*., 2006; Liu and Tang, 2012). Some of the schizonts return into the blood stream in a form called merozoites and invade red blood cells (RBCs). Within the RBCs, they transform into feeding stage plasmodia called trophozoites that feed on the haemoglobin and multiply to produce more merozoites which re-infect the liver. It may take about 48 hours for *P. falciparum* to go through this erythrocytic schizogony.

On the other hand, the trophozoites may transform again into sexual stages called micro- (male) and macro- (female) gametocytes, which have no further activity within the human host. The *P. falciparum* parasite takes about 10-12 days to complete gametocytosis. The gametocytes may then be sucked up into the gut of a mosquito during another blood meal. Here, ex-flagellation of micro-gametocytes and transformation of macro-gametocytes occur, giving rise to micro (male) gametes and macro (female) gametes respectively. Subsequently, fertilization of the macro-gamete by the micro-gamete occurs, leading to the formation of a zygote.

The resulting zygote then develops into a motile ookinete, which penetrates the cell wall of the mosquito mid-gut lining, and develops into an oocyst. Sporogony within the oocyst inside the mosquito is governed by environmental temperature, as Anopheline mosquitoes are poikilotherms (Samuel *et al*., 2011). It produces many sporozoites, which are liberated when the oocyst ruptures. After rupturing out of the cyst, the sporozoites escape into

University of Ghana http://ugspace.ug.edu.gh

the haemocoele and migrate to penetrate salivary gland cells, where they lie in vacuoles for up to 59 days. Here, they mature to become up to 1000 times more infective and more antigenic, with the circumsporozoite antigen (CSA) being the predominant antigen. They then remain in this form until they are injected into the human, or another vertebrate host, during another blood meal to repeat the cycle.

2.1.4 Clinical Signs and Symptoms of Malaria

Generally, symptoms of uncomplicated malaria are flu-like (Bartoloni and Zammarchi, 2012) and include headache, fever and generalized body pains, chills, perspiration, anorexia, vomiting, malaise, arthralgia (joint pains) and sometimes, abdominal pains, diarrhoea and (White, 1998; Bartoloni and Zammarchi, 2012).

Clinically, the spleen and liver are often palpable, and may be misdiagnosed as influenza (Bartoloni and Zammarchi, 2012), and other conditions such as septicemia, gastroenteritis and viral diseases (Nadjm and Behrens, 2012) in non-endemic areas, and, unless treated promptly, the clinical picture can deteriorate rapidly (WHO, 1991). Severe and complicated malaria results, primarily, from delay in treatment of an uncomplicated *P. falciparum* attack (Bradley, 1996) although the most virulent of human malaria parasites are *P. falciparum* and *P. vivax* (White, 2003). *Plasmodium* parasites infect a variety of vertebrate hosts including primates, rodents, ungulates, birds, and lizards, however, they rarely cause severe disease in any vertebrate hosts apart from humans (Zilversmit and Perkins. 2008). A patient with severe and complicated malaria will often present with impaired consciousness, weakness and jaundice (Bartoloni and Zammarchi, 2012). Other complications include cerebral malaria (Bartoloni and Zammarchi, 2012; Brewster *et al*., 1990), generalized convulsions, normocytic anaemia, hemolytic anemia, retinal damage renal failure, hypoglycaemia, fluid-electrolyte and acid-base disturbances, pulmonary oedema, circulatory

University of Ghana http://ugspace.ug.edu.gh

collapse, shock, disseminated intravascular coagulation, hyperpyrexia, hyperparasitaemia, malaria haemoglobinurea and death (WHO, 1991; Bartoloni and Zammarchi, 2012).

2.1.5 Pathogenesis of Malaria

Under certain circumstances *Plasmodium* infection causes severe disease such as anemia or cerebral malaria (Horata *et al*., 2009). The expression of disease is influenced by both parasite and host factors (Van der Heyde *et al*., 2006), as exemplified by the worsening severity of disease during pregnancy. Pathogenesis relates to the various host (host immunological processes) and parasite factors that are responsible for injury/ damage to host cells/ tissues. The pathogenesis of severe malaria therefore involves a cascading interaction between parasite and host red cell membrane products, cytokines and endothelial receptors, leading to inflammation, activation of platelets, hemostasis, a procoagulant state, microcirculatory dysfunction and tissue hypoxia, resulting in various organ dysfunctions manifesting in severe malaria (Van der Heyde *et al*., 2006).

Understanding the pathogenesis of malaria is essential because, it enhances our ability to develop strategies to prevent the most severe forms of malaria. The liver stage of *Plasmodium* infection does not cause any disease, though liver cells are infected. The pathogenic process occurs only during the erythrocytic cycle. During this stage, there is a huge, periodic increase in parasite populations that may enhance the probability of differentiation to gametocytes, the stage infectious to mosquitoes. A peculiarity of *P. falciparum* is its ability to cause erythrocytes infected with maturing parasites to adhere to endothelium of venules (cyto-adherence).

Central to the interaction between the host's immune system and the parasite factors that leads to pathogenesis are cytokines which are released by immunocompetent cells in a highly regulated fashion (Clark *et al*., 2006; Van der Heyde *et al*., 2006).

University of Ghana http://ugspace.ug.edu.gh

The the pathological symptoms of malaria begin with the invasion of the erythrocytes by the merozoites and the rupture of erythrocytes releasing *Plasmodium* parasite soluble products (malarial toxins) which trigger systemic release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and Interleukins (IL1α, 1β and IL6) into the host blood stream (Clark *et al*., 2006; Van der Heyde *et al*., 2006). The presence of malaria toxins and antigens, and the release of TNF-α, IL platelets in the body cause inflammation marked by increase in body temperature above 37.5^{o}C (fever) and pain in the host.

The pro-inflammatory cytokines are also involved in cerebral malaria (Van der Heyde *et al*., 2006). During cerebral malaria, infected RBCs adhere to the inner wall of the blood vessels and block the blood capillaries of the brain (Van der Heyde, *et al*., 2006). Occlusion of the capillaries interrupt the flow of nutrient and oxygen, and also disrupts brain function that may lead to coma and death (Idro *et al*., 2010).

Also, owing to the cleaning behavior of the spleen which filter off infected RBCs, ruptured RBC membranes and malaria pigment from the blood, it may swell up (splenomegaly) and become brownish in colour. The architecture of the white pulp becomes markedly disorganized, with dissolution of the marginal zone and relative loss of B cells (Urban *et al*., 1999). Though rare, malarial splenomegaly may also lead to pathological splenic rupture (Imbert *et al*., 2009; Cinquetti *et al*., 2010).

Anemia is caused by the destruction of erythrocytes (RBCs) during asexual replication of the parasites. This process leads to tremendous multiplacation of parasites with densities that may reach 400,000 parasites/µl of blood per patient (Halbert *et al*., 2010). As these parasites multiply and feed on the RBCs, they degrade and rapture resulting to reduction in RBC concentration in the blood, a condition which is called anemia. Anemia may also be caused by the absorption of antigen on non-parasitized erythrocytes with complement-

University of Ghana http://ugspace.ug.edu.gh

mediated lysis and suppression of hematopoiesis by cytokines. In some cases, the therapy-associated hemolysis induced by medications in G6PD deficiency may also lead to anemia.

Hypoglycemia is common in malaria. Malaria parasitized erythrocytes utilize glucose 75 times faster than uninfected cells although there is no direct correlation between hypoglycemia and parasitaemia. In addition, treatment with quinine and quinidine stimulate insulin secretion, thereby reducing blood glucose in the brain.

Parasites sequester (cytoadhere) themselves also in the placenta, the lung, the liver, the kidney and subcutaneous tissues (Van der Heyde, *et al*., 2006). The cyto-adherence to many endothelial receptors in such tissues is mediated by variants of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family, which are anchored at the red cell membrane skeleton by the knob-associated histidine-rich protein and expressed at the surface of the infected erythrocytes (Maier *et al*., 2008; Miller *et al*., 2002). The presence of this protein on the surface of infected RBCs creates surface changes that allow parasite encoded adhesion and sequestration into host cells (Smith *et al.*, 2001)

PfEMP1 is the product of the *var* gene family. It undergoes antigenic variation (diversity) and thus plays a major role in the pathogenesis of malaria (Horata *et al*., 2009). The extracellular region of PfEMP1 possesses multiple adhesion domains which promotes binding to a broad range of endothelial cell receptors, including CSA, ICAM1, and CD36 for the placenta, the brain and the vascular endothelium.

The severity of malaria is observed mostly in pregnant women and children below 5 years of age (WHO Malaria Policy Meeting Report, 2012; Hviid, 2007) because of lack of adequate immune protection against the disease (Hviid, 2007). In areas where *P. falciparum* is endemic, women who normally have immune protections against malaria tend to lose this protection during pregnancy (Hviid, 2007). The supposed reduction in immunity during pregnancy is attributed to a transient depression of cell-mediated immunity to allow retention

University of Ghana http://ugspace.ug.edu.gh

of foetal allograft, which unfortunately, also causes a reduction in resistance to various infectious diseases, including malaria (Meeusen *et al*., 2001). This condition causes both the frequency and the severity of the disease to be higher in pregnant than non-pregnant women (WHO, 1983).

During pregnancy, especially, in primigravidae, *P. falciparum* infected RBCs coated with pfEMP1 of CSA-binding capacity, become attracted to and sequestered in CSA within the placenta. The aggregation of *P. falciparum* infected erythrocytes bearing pfEMP1 and uninfected erythrocytes in tissues occlude fine blood capillaries and ultimately blocks blood flow, limits the local oxygen and nutrient supply.

The aggregation of *P. falciparum* infected erythrocytes bearing pfEMP1 and uninfected erythrocytes in tissues occlude fine blood capillaries and ultimately blocks blood flow, limits the local oxygen and nutrient supply, hampers mitochondrial ATP synthesis, and stimulates cytokine production thereby causing abnormal functioning of host cells and tissues to which the pfEMP1 are attached. All of these factors contribute to the development of severe disease, hence the obstetric complications and death of pregnant women (Clark *et al*., 2006; Horata *et al*., 2009; Anstey *et al*., 2009), or intra-uterine growth retardation (Poovassery *et al*., 2009), leading to pregnancy failure, miscarriages or delivery of low birth-weight babies (Poovassery *et al*., 2009), which is an important risk factor for infant mortality (Steketee *et al*., 2001).

2.1.6 Classification of the *Plasmodium* Parasite

According to Bannister and Sherman (2009), *Plasmodium* is currently classified on the basis of morphological and molecular evidence as: Kingdom *Protozoa*, Subkingdom *Biciliata*, Infra kingdom *Alveolata*, Phylum *Myzozoa*, sub-phylum *Apicomplexa*. The Subphylum Apicomplexa, which are so named because of the possession of the apical

University of Ghana http://ugspace.ug.edu.gh

organelle complex at the anterior end of the parasite for locomotion and penetrating into host cells and tissues. It comprises nearly 5000 described species, all parasitic including several genera of medically and economically important organisms such as *Plasmodium*, *Babesia*, *Toxoplasma*, *Cryptosporidium*, *Theileria*, *Eimeria* and *Isospora*. Three Classes of organisms fall under Apicomplexa (Escalante and Ayala, 1995), with *Plasmodium* belonging to the class *Aconoidasida*, Order *Haemosporina*, and Genus *Plasmodium*.

The genus *Plasmodium* was first described by Ettore Marchiafava and Angelo Celli in 1885. Currently, with new species being continuously identified and described, it is said to comprise over 200 species (Chavatte *et al*., 2007) which are all parasites of higher order organisms. With increasing understanding of the evolution and systematics of malaria parasites over the past 20 years, Martinsen *et al*., 2008, indicated that the genus *Plasmodium* may not to be monophyletic, and includes parasites of other genera including *Hepatocystis*. The parasite is known to have two hosts in its life cycle which comprise a vector, usually a mosquito and a vertebrate host, ranging from humans to monkeys, rodents, bats, birds, and reptiles (Martinsen *et al.*, 2008; Vargas-Serrato *et al.*, 2003).

According to Bradley (1996), with the exception of *P. malariae*, which may also infect the higher orders of primates, four distinct species of *Plasmodium* parasites were originally known to infect humans, and included, *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. However, Kakkilaya (2006) reported that by 1966, 10 species of *Plasmodium*, naturally present in monkeys and apes had been shown to be capable of infecting humans. In corroboration of Kakkilaya (2006)'s report, molecular studies recently revealed the possible existence of additional parasite species or morphological variants of existing species that also infect humans (Sutherland *et al*., 2010). This caused the number of *Plasmodium* parasites now known to infect humans to be at least eleven (Sutherland *et al*., 2010). For example, sequencing of the gene for the *Plasmodium* circumsporozoite surface protein (CSP) showed

University of Ghana http://ugspace.ug.edu.gh

that some individuals initially thought to have been diagnosed with *P. vivax* infections were actually infected with a distinct species more closely related to *P. simiovale*, a simian malaria parasite which is morphologically identical to *P.* vivax. Consequently, this species was named the *P.* vivax-like.

In addition, molecular analysis has indicated that *P. ovale* consists of two clades that are as divergent as distinct species and has suggested that they should be designated as sub-species *P.o. curtisi* and *P.o. wallikeri* (Oguike *et al.*, 2011). Similarly, molecular analyses have indicated that some morphological variants of *P. malariae* that infect humans are distinct parasites related to *P. malariae* and *P. brasilianum*. *P. brasilianum* is a simian parasite of the South and Central America which is often speculated to have originated from humans as a result of colonization of the New World. Furthermore, molecular studies showed that nearly all the humans diagnosed to be naturally infected with *P. malariae* in Malaysia, were actually harbouring the simian malaria parasite, *P. knowlesi* (Cox-Singh and Singh, 2008). Also, four fatalities associated with *P. knowlesi* infection were reported in Malaysia, and this finding coupled with other naturally occurring human infections of *P. knowlesi* elsewhere therefore indicate that human infections with *P. knowlesi* may not be a rare occurrence but may be widespread in Malaysia and perhaps other parts of southeast Asia (Cox-Singh and Singh, 2008).

It is interesting to note however that while some of these current taxonomic names are repetitions or confirmation of earlier nomenclature, the molecular evidence has introduced new dimensions of taxonomic names which were not part of the above. This observation, in accordance with scientific discovery, points to the fact that as additional elucidation of the characteristics of organisms is obtained from further studies, the taxonomy of *Plasmodium*, like that of all other organisms will continue to change.

University of Ghana http://ugspace.ug.edu.gh

2.2.0 ANTIBODIES

Antibodies are large Y-shaped glycoprotein molecules produced and secreted by specialized white blood cells or plasma cells known as B lymphocytes (B cells) which form part of the humoral arm of the host immune system (Pier *et al.*, 2004; Lipman *et al.*, 2005).

They are very essential in immunity. They are one of the principal effectors of the adaptive immune system and are deployed by the immune system in response to the invasion of the host body by foreign objects such as bacteria and viruses (Lipman *et al.*, 2005). They are then used by the immune system to identify and destroy such foreign matter, thereby preventing them from causing disease or injury to the body (Lipman *et al.*, 2005).

Each of the bi-forked ends of the Y-shaped antibody has a structural receptor like a lock known as a paratope or antigen binding site (Figure 4a and b below) to which a specific epitope of an antigen is attached before destruction. By the 'lock and key' attachment or binding mechanism, antibodies mark infectious agents, microbes or infected body cells before they are neutralized or destroyed by other parts of the immune system. Antibodies may occur in two physical forms, one being a soluble form secreted by B-cells and found in the body circulation, and the other being membrane bound and attached on the surface of B-cells and is therefore known as the B-cell receptor (BCR).

2.2.1 The Basic Structure of Antibodies

Antibody molecules are heavy globular plasma glycoproteins comprising one or more basic structural units called immunoglobulin (Ig for short) monomers. Each Ig monomer is a "Y"-shaped molecule that consists of four polypeptide chains; two identical heavy (H) chains and two identical light (L) chains joined together by disulfide and non-covalent bonds (Lipman *et al.*, 2005). Each chain comprises structural domains called immunoglobulin

University of Ghana http://ugspace.ug.edu.gh

domains containing about 70-110 amino acids, some of which are attached to sugar chains thereby giving the antibody its glycoprotein properties.

Some of the structural or Ig domains at the amino acid end, show considerable variation in amino acid composition and are referred to as the variable (V) regions to distinguish them from the relatively constant (C) carboxylic acid regions.

One light chain has an approximate molecular weight of about 50 kDa. Each heavy chain on the other hand, has about twice the number of amino acids and molecular weight (~50-55,000) as each light chain (Janeway *et al.*, 2001; Lipman *et al.*, 2005). This gives the immunoglobulin monomer a molecular weight of approximately 150 kDa (150,000).

The amino acid sequence in the tips of the "Y" varies greatly among different antibodies. The variable region includes the ends of the light and heavy chains. Each variable region consists of 110-130 amino acids (Janeway *et al.*, 2001; Mayer, 2009) or 220 amino acids (110 for each of the heavy and light chains) (Lipman *et al.*, 2005). It gives the antibody its specificity for binding antigen. The tip of each arm of the Y, for example, has receptors that can bind to two identical antigens and, therefore, are specific to such antigens. This region of the antibody is called the antigen binding fragment (Fab region**)**. It consists of one constant and one variable domain from each heavy and light chain pair of the immunoglobulin molecule, which makes each antibody molecule at least bivalent. The structure of the paratope at the amino terminal is determined by the mode of arrangement of amino acids in the variable domains of each heavy and light chain pair. The antibody uses each of these antigen binding sites or paratopes to bind to an epitope on an antigen.

The base of the Y of the immunoglobulin molecule is called the crystallizable fragment (Fc region). It is composed of two heavy chains that may provide two or three constant domains depending on the class of the antibody (Janeway *et al.*, 2001; Mayer, 2009). It plays a role in modulating immune cell activity by ensuring that each antibody generates an

University of Ghana http://ugspace.ug.edu.gh

appropriate immune response for a given antigen. It achieves this by binding to a specific class of Fc receptors, and other immune molecules, such as complement proteins. Through this process, different biological effector functions are mediated. They include activation of natural killer cells and classical complement pathway, lysis of cells, phagocytosis, recognition of opsonized particles, and degranulation of mast cells, basophils and eosinophils. These functions therefore determines the mechanisms used in destroying the antigens.

2.2.1.1 Heavy chain immunoglobulin

There are five known types of mammalian Ig heavy chains or isotypes, which are denoted by the Greek letters: α, δ, ε, γ and μ (Janeway *et al.*, 2001). The type of heavy chain present defines the class of the antibody (Mayer, 2009). Consequently, the 5 heavy chain types lettered α, δ, ε, γ and μ have been given immunoglobulin class names that combine "Ig" with the initials of the names of the corresponding Greek letters as IgA, IgD, IgE, IgG, and IgM respectively. Distinct heavy chains differ in size and composition such that α and γ for example contain approximately 450 amino acids, while μ and ε have approximately 550 amino acids (Shimizu, 2004; Mayer, 2009).

Unlike mammals, birds, have only one known major serum antibody which is also found in the egg yolk and called IgY. Even though it has a partial resemblance to the mammalian IgG it is quite different from it. This partial resemblance caused the bird IgY to be called IgG in some older literature and even on some commercial life sciences product websites "IgG", which however, is incorrect and can be confusing.

Each heavy chain has two regions, the constant region and the variable region. The constant region is identical in all antibodies of the same isotype, but differs in antibodies of different isotypes. Heavy chains γ, α and δ have a constant region composed of 3 Ig domains in succession (in tandem), and a hinge region that confers additional. Heavy chains μ and ε,

University of Ghana http://ugspace.ug.edu.gh

on the other hand, have a constant region composed of 4 immunoglobulin domains (Janeway *et al.*, 2001). The variable region of the heavy chain differs in antibodies produced by different B cells, but is the same for all antibodies produced by a single B cell or B cell clone and is composed of a single Ig domain.

2.2.1.2 Light chain immunoglobulin

Two types of immunoglobulin light chain occur in mammals which are the lambda (λ) and kappa (κ) (Mayer, 2009). Each light chain has two domains in series, one being constant and the other one, variable. Each ligh chain is approximately 211 to 217 amino acids long (Janeway *et al.*, 2001; Shimizu, 2004; Mayer, 2009). As mentioned above, each mammalian immunoglobulin monomer contains two light chains that are always identical, and only one type of light chain, κ or λ, is present per antibody. Other types of light chains, such as the iota (ι) chain, have been reported in other vertebrates such as bony fishes (Teleostei) and sharks (Chondrichthyes).

The most significant region for antigen binding is the variable domain which is also referred to as the F_V region. Here, the actual receptors responsible for binding to antigen epitopes are 3 hyper-variable loops of β-strands, each comprising 5-10 amino acids in length also known as the complementarity determining regions (CDRs), which are located on each of the heavy (V_H) and light (V_L) chains (Lipman *et al.*, 2005).

University of Ghana http://ugspace.ug.edu.gh

Figure 4a

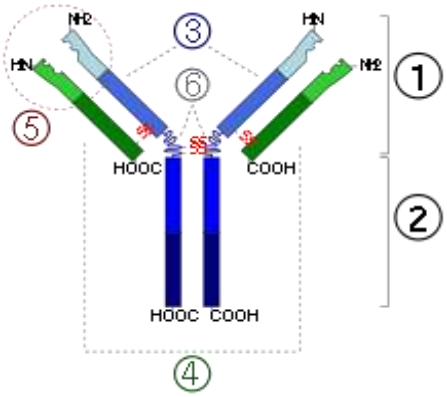


Figure 4b

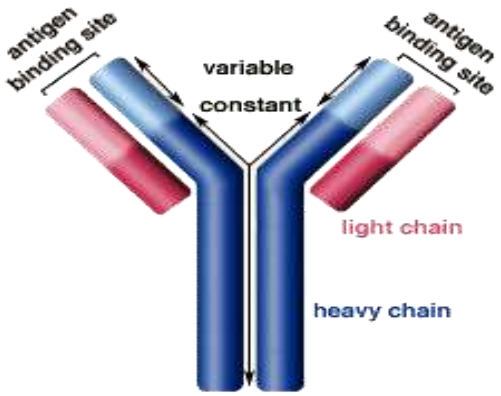


Figure 4a and b: Basic structures of the immunoglobulin molecule

(retrieved from "http://en.wikipedia.org_/wiki/File:Immunoglobulin_basic_unit.svg" and "http://www.biology.arizona.edu/immunology/tutorials/antibody/structure.html" respectively, on 2-2-2013.

1 is the Fab region of the antibody; **2** is the Fc region; **3** is the Heavy chain (long, bow-jointed units) with one variable (V_H) domain followed by a constant domain (C_H1), a hinge region, and two more constant (C_H2 and C_H3) domains; **4** is the Light chain (short unit) with one variable (V_L) and one constant (C_L) domain; **5** is the Antigen binding site (paratope) and **6** is the Hinge regions.

2.2.2 Classification of Antibodies

As stated above, 5 different primary antibody classes are known in mammals, which are IgG, IgM, IgA, IgD and IgE. They perform different roles, and help direct the appropriate immune response for each different type of foreign object encountered (Lipman *et al*., 2005). In avians, however, there are three classes which are IgY, IgM, and IgA (Lipman *et al*., 2005), which are distinguished by the type of heavy chain they possess. IgG molecules have heavy chains known as gamma-chains; IgMs have mu-chains; IgAs have alpha-chains; IgEs have epsilon-chains; and IgDs have delta-chains.

Differences in the constant region structure marked by differences in the amino acid sequence of the heavy chain polypeptides allow each immunoglobulin to function in a different type of immune response and at a particular stage of the immune response. The

University of Ghana http://ugspace.ug.edu.gh

polypeptide protein sequences responsible for these differences are found primarily in the Fc fragment.

Each of the five different mammalian primary antibody classes or heavy chains, in a selected group of mammals, IgG and IgA are further subdivided into subclasses, referred to as isotypes, due to polymorphisms in the conserved regions of the heavy chain (Lipman *et al*., 2005). Unlike the heavy chains, there are only two main types of light chains: kappa (κ) and lambda (λ). Antibody classes differ in valency as a result of different numbers of Y-like units (monomers) that join to form the complete protein. For example, in humans, functioning IgM antibodies have five Y-shaped units (pentamer) containing a total of ten light chains, ten heavy chains and ten antigen-binding sites (Table 2, below). The Ig class determines both the type and the temporal nature of the immune response (Lipman *et al*., 2005).

University of Ghana http://ugspace.ug.edu.gh

Table 2: Classification of antibodies based upon location, structure and function

Immunoglobulin Class	Types	Location and/ or Function	Antibody Complexes	Structure
IgA	2	Found in mucosal areas, such as the gut, respiratory tract and urogenital tract, and prevents colonization by pathogens (Schiff *et al*., 1986). Also found in saliva, tears, and breast milk.	Dimer (dimeric)	
IgD	1	Functions mainly as an antigen receptor on B cells that have not been exposed to antigens. It has been shown to activate basophils and mast cells to produce antimicrobial factors.	Monomer (monomeric)	
IgE	1	Binds to allergens and triggers histamine release from mast cells and basophils, and is involved in allergy. Also protects against parasitic worms (Pier *et al.*, 2004)	Monomer (monomeric)	
IgG	4	In its four forms, provides the majority of antibody-based immunity against invading pathogens (Pier *et al.*, 2004). The only antibody capable of crossing the placenta to give passive immunity to the fetus.	Monomer (monomeric)	
IgM	1	Expressed on the surface of B cells (monomer) and in a secreted form (pentamer) with very high avidity. Eliminates pathogens in the early stages of B cell mediated (humoral) immunity before there is sufficient IgG (Pier *et al.*, 2004).	Pentamer (Pentameric)	

University of Ghana http://ugspace.ug.edu.gh

2.2.3 Classification of Antibodies According to Multiplicity of Clones

Apart from the type of antibody classification presented above, antibodies may also be categorized into polyclonals or monoclonals based on the multiplicity of epitopes recognized, and regarding this mode of classification, the characteristics of antibodies are presented as follows.

2.2.3.1 Polyclonal Antibodies

Polyclonal antibodies are a collection of heterogeneous antibodies originating from multiple B cell clones of an immunocompetent animal that has been immunized with specific antigens (Nakazawa *et al*., 2010). As a result of their origin from B cell clones of different genetic conformation, they are the outcomes of various biochemical mechanisms of combination and recombination of heavy and light chains that give rise to different binding site characteristics with the ability to bind to a diverse spectrum of antigens, or multiple epitopes on the same antigen (Lipman *et al*., 2005; Nakazawa *et al*., 2010). Like MAbs, they are produced by inoculating an animal such as a goat, sheep, or rabbit with a specific antigen that elicits a primary immune response. Following this, a secondary and tertiary immunization produces higher titres of antibody against the particular immunogen. However, unlike the case for MAb production (Pandey, 2010; Yokoyama, 1999), the serum of the animal containing the polyclonal antibodies is harvested (Zhang *et al*., 2010; Sevier *et al*., 1981) and purified by affinity chromatography in order to enrich the antibodies generated (Zhang *et al*., 2010; Josic and Lim, 2001). This process ultimately leads to the production of high titre and high affinity polyclonal antibodies against the antigen of interest. A larger animal such as a goat or a sheep is preferred for polyclonal antibody production because the yield of antibodies per animal is high (Leenaars and Hendriksen, 2005).

University of Ghana http://ugspace.ug.edu.gh

Unlike monoclonal antibodies, polyclonal antibodies cannot be collected for an indefinite amount of time and often require multiple animals to be immunized with the same antigen. Antibodies are harvested from each animal; however, animals immunized with the same antigen develop differential immune responses and this could result in variability in polyclonal antibody production between batch preparations. Since each antibody preparation is collected from multiple B cell clones of different genetic characteristics, polyclonal antibodies are capable of recognizing different epitopes within the antigen and bind the antigen with varying affinities (Nakazawa *et al*., 2010). This can prove to be advantageous in many biological assays depending on the particular application. For example, polyclonal antibodies may cross-react with antigens that share high homology, which can be suitable when trying to detect known or unknown isoforms of an antigen (Lopata and Cleveland, 1987). In addition, they can be utilized to enhance the detection level of a particular antigen since multiple antibodies will bind the same antigen at epitope specific regions. This in turn can be valuable when attempting to detect low expressing antigens.

2.2.3.2 Monoclonal Antibodies

Monoclonal antibodies (MAbs) are a population of antibodies that recognize a single epitope of an antigen. They are produced from a single B lymphocyte (cell) clone of an immunized animal, thereby generating a clonal population of antibodies, identical to one another and all recognizing the same epitope of a specific antigen. MAbs were first detected in sera of patients with multiple myeloma in which clonal expansion of malignant plasma cells produce high levels of an identical antibody resulting in a monoclonal gammopathy (Lipman *et al*., 2005). The procedure for MAb production is in many ways similar to the isolation of a cDNA from a cDNA library.

University of Ghana http://ugspace.ug.edu.gh

However, in the case of MAbs, an immunized animal produces a library of B cells that are secreting antibodies of different specificities. The B cells can be isolated from the spleen of the animal and fused with myeloma cells to produce multi-specific antibody producing hybridoma cells. To obtain the MAbs therefore, a single B lymphocyte-myeloma hybrid cell (hybridoma cell) that is producing the antibody of required specificity is carefully selected and multiplied *in vitro* into a population of cells that can secrete a large amount of the desired antibody.

In practice, an animal such as a mouse or a rabbit is injected with a specific antigen to induce a primary immune response (Leenaars and Hendriksen, 2005), followed by a secondary and tertiary immunization to produce antigen-specific B cells that generate and secrete higher titres of antibody against the inoculated antigen (Leenaars and Hendriksen, 2005). Usually, the animal of choice is the BALB/c mouse because many of the myeloma cells available for cell fusion are of the BALB/c origin (Leenaars and Hendriksen, 2005). The spleen of the immunized laboratory animal is removed aseptically and minced to free the splenocytes (B lymphocytes).

Although antibody producing B cells alone can produce antibodies, characteristically, they have a short lifespan and cannot be easily cloned and expanded *in vitro* which eventually leads to their cessation of antibody production. Also, even though myeloma cells have the capacity to grow continuously *in vitro* they cannot produce antibodies. Therefore, by fusing specific antibody-producing B cells and myeloma cells together, a hybrid of the two (hybridoma) cells results which possesses both the ability to grow perpetually *in vitro* and also produce unique monoclonal antibodies, thereby causing the limited lifespan of the B cells to be prolonged indefinitely. The immortalized B lymphocyte-myeloma-hybrid cell (hybridoma cell) that results then provides a constant supply of highly specific monoclonal antibodies. Since monoclonal

University of Ghana http://ugspace.ug.edu.gh

antibodies only recognize one epitope (Deb *et al*., 2013), they generally have low cross reactivity with non-specific antigens. Their epitope specificity, limited cross reactivity, and long term yield make monoclonal antibodies attractive for use in many biological assays and applications.

This strategy for generating monoclonal antibodies of a desired specificity was invented by the two researchers, Georges J. F. Köhler and Cesar Milstein in the mid-1970s for which they were awarded the Nobel prize (Köhler and Milstein, 1975; Lipman *et al*., 2005; Deb *et al*., 2013). This technique was the first practical method for mass-production of monoclonal antibodies *in vitro* by inducing cells of the immune system to produce pure antibodies against a chosen antigen. Their breakthrough is considered one of the most important techniques of biotechnology as it led to the advancement of the use of antibodies to achieve various objectives.

2.2.3.2.1 The Hybridoma Principle and Mechanism of Reaction

Splenocytes are B-lymphocytes. They secrete antibodies but cannot survive *in vitro.* Myeloma cells are tumour-like or cancerous cells that have been conditioned to possess the following characteristics. First, they have been treated with 8-Azaguanine which aborts the Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene and inhibits HGPRT enzyme activity in the cells. HGPRT catalyzes the alternative/ salvage reaction pathway for cell metabolism and nucleotide synthesis. Its inhibition or absence therefore makes the cells susceptible to destruction by aminopterin which is a folic acid antagonist. Also, myeloma cells have been immortalized to make them survive *in vitro* perpetually. In addition, they are carefully selected to ensure that they do not secrete antibodies themselves (Leenaars and Hendriksen, 2005). Furthermore, they are treated with a marker that enables non fused parental cells to be

University of Ghana http://ugspace.ug.edu.gh

removed after the fusion is completed. Fusing B cells and myeloma cells together results in a hybrid cell, the hybridoma cell which can secret antibodies and survive *in vitro.*

The fusion solution contains HAT-selection medium (Pandey, 2010) which is prepared by mixing Hypoxanthine and Thymidine (precursors for DNA synthesis) with a normal culture medium, and later adding Aminopterin which is a protease inhibitor. Myeloma cells and B lymphocytes from the spleen of an immunized mouse are mixed together according to laid down procedures. Polyethylene glycol (PEG) is then added to concentrate and fuse the cells together (Pandey, 2010). Following this, HAT selection medium is added. The Aminopterin in the HAT medium blocks the synthesis of nucleotide precursors in both B-lymphocytes and myeloma cells, which adversely affect the cells, making them unable to make any purine nucleotides. The cells in this condition, are thus forced to use the salvage (alternative) pathway for protein synthesis (Pandey, 2010).

Also, the abortion of HGPRT activity in myeloma cells due to treatment with 8-Azaguanine prevents them from undergoing the alternative pathway and hence cell division leading to their death. Thus, addition of PEG causes the cell membranes of the B-lymphocytes and myeloma cells to fuse allowing the cytoplasmic contents to mix but not the nuclei. After cell fusion, cellular products containing only myeloma nuclei will abort because they cannot metabolize either by the forward or salvage reaction pathway for protein synthesis. Also, cells containing B-lymphocyte nuclei only, will abort because, by their nature, B cells cannot survive *in vitro* for long (Pandey, 2010).

Finally, only daughter (hybridoma) cells with both the myeloma and B cell characteristics can survive and multiply *in vitro* because, they have the myeloma cell nature of immortality as well as the B cell characteristics of being able to undergo the alternative pathway for protein

University of Ghana http://ugspace.ug.edu.gh

synthesis as a result of the presence of a functional HGPRT gene, and multiply *in vitro* (Pandey, 2010).

2.2.3.2.2 Hybridoma Cell Cloning by Limiting Dilutions Method

The next, and most experimentally challenging, line of activity is to design a method of selecting, isolating and multiplying (cloning) single (monoclonal) cells that produce antibodies of interest (Pandey, 2010). Usually, this is done by the method of limiting dilution (Pandey, 2010). In practice, the culture is divided into many individual smaller cultures, each containing a single cell which divides into a colony of several hundreds of cells. The supernatant from each colony of culture is then sampled and assayed for the antibody of interest.

The methods used are immunoassay techniques that allow antibodies which are monospecific and have very high sensitivity to the antigen of interest to be selected (Dewar *et al*., 2005; Lipman *et al*., 2005). The selected monoclonal hybridoma cell with qualities of interest is then up-scaled, multiplied or expanded (Leenaars and Hendriksen, 2005) to produce several cultures of hybridoma that generate large quantities of the desired antibodies.

2.2.3.2.3 Monoclonal Antibody production by Ascites Production Method

After a single hybridoma cell that secretes monoclonal antibodies of desired sensitivity and specificity has been obtained, large quantities of MAbs could be obtained through an *in vivo* alternative approach known as ascites production, rather than via the *in vitro* cell culture technique. In ascites production, the MAb-producing hybridoma cells of interest are injected into the abdominal cavity of laboratory mice and then after the next 7 to 14 days, the injected

University of Ghana http://ugspace.ug.edu.gh

hybridoma cells will secrete an increased quantity of highly concentrated MAbs known as ascites which can be collected.

This method of MAb production has some advantages. Firstly, this method is simpler and less time consuming. Also, the abdominal cavity provides an excellent environment for optimal growth of the hybridoma cells because it guarantees a constant temperature, an optimal nutrient and oxygen supply as well as the optimal removal of CO_2 and metabolic waste products (Leenaars and Hendriksen, 2005; Hendriksen and de Leeuw 1998). The hybridoma cells in this condition grow to high densities that secrete highly concentrated levels of MAbs (Leenaars and Hendriksen, 2005).

In spite of these advantages, the ascites approach for MAb production has fallen into disfavour because of the following reasons. Firstly, the method is considered unethical because of the pain and distress that the mice are probably subjected to (Dewar *et al*., 2005). Secondly, high-quality *in vitro* MAb production systems and new production approaches are increasingly progressing and becoming available. In addition, there is substantial evidence of contamination with other immunologically active compounds from the murine peritoneum as well as the risk of contamination of the MAbs with viruses and other microorganisms in the mouse abdominal cavity (Dewar *et al*., 2005; Lipman *et al*., 2005). For these mitigating reasons, the studies described in this thesis write-up opted to develop the MAbs by the *in vitro* tissue culture method rather than via the ascites production approach.

2.2.4 Uses and Applications of Monoclonal and Polyclonal Antibodies

The importance of the capability of antibodies to selectively bind a specific epitope on a chemical, carbohydrate, protein, or nucleic acid has been demonstrated over the years by several

University of Ghana http://ugspace.ug.edu.gh

research and clinical applications in which antibodies have been utilized for specific purposes. Such applications include (1) simple qualitative and/or quantitative analyses to determine the presence of a particular antigen in a solution, cell, tissue, or organism; (2) methods to facilitate purification of the antigen, antigen associated molecules, or cells expressing the antigen; and (3) techniques that use antibodies to mediate and/or modulate physiological effects for research, diagnostic, or therapeutic purposes. Antibodies, both MAbs and PoAbs have a variety of academic, medical and commercial uses. It will be impossible to list all the instances in which antibodies have been explored for specific purposes in this discourse. Therefore, the various uses of antibodies which have been outlined below are by no means exhaustive but just to illustrate that antibodies are versatile, and their applicability is limited only by the imagination and determination of the user.

The following list therefore, is to indicate how wide the use of antibodies has become in biotechnology.

2.2.4.1 Uses and Application of Antibodies in the Diagnosis of Disease

Antibodies are used in several diagnostic tests to detect small amounts of drugs, toxins or hormones. For example, monoclonal antibodies (MAbs) to human chorionic gonadotropin (HCG) are used in pregnancy test kits. Secondly, MAbs and polyclonal antibodies (PoAbs) to *Plasmodium* antigens are used in rapid diagnostic kits for diagnosis of malaria (Azikiwe *et al*., 2012; Wongsrichanalai *et al*., 2007). Also, antibodies are used in ELISA for the diagnosis of AIDS. Finally, antibodies can be used in immuno-localization to detect a particular antigen in a tissue.

University of Ghana http://ugspace.ug.edu.gh

2.2.4.2 For treatment of disease

Antibodies are used in the radioimmunodetection and radioimmunotherapy of cancer, and some new methods can even target only the cell membranes of cancerous cells (Zola and Thomson, 2001). Also, Monoclonal antibodies can be used to treat viral diseases, traditionally considered "untreatable".

2.2.4.3 Uses and Application of Antibodies in the Classification of Organisms

Monoclonal antibodies are used in classification of strains of pathogens.

2.2.4.4 Application of Antibodies for Identification, Monitoring and/or Measurement of Biological Processes

Antibodies can be used in conjunction with gel-shift experiments to identify specific DNA-binding proteins (Carey, 2012; Ralston, 2008). Also, antibodies can have a biomarker effects in vivo. These effects enable them to be used in experiments to determine the importance of a particular molecule in vivo, such as the development of the sensory nervous system (Zhu *et al*., 2013. Finally, Antibodies can also be used in western blotting to detect and/ or identify a protein after separation by SDS-PAGE. With appropriate controls, this procedure can be used to measure the quantity of a protein in response to experimental manipulations.

2.2.4.5 Uses and Application of Antibodies For isolation and purification of proteins

Antibodies are used as affinity reagents in the purification and/ or precipitation of proteins from solution (Huse *et al*., 2002).

University of Ghana http://ugspace.ug.edu.gh

2.3.0 IMMUNIZATION OF LABORATORY ANIMALS

2.3.1 Introduction

Immunization is the introduction of pathogens (disease causing microorganisms) or their proteins into the body of immune-competent organisms with a view to strengthen their immune system so that the receipient organisms would become resistant to the related disease/ diseases.

Immunization may be carried out using various mechanisms/ techniques, of which the common approach is vaccination. Vaccination of the body against a particular disease helps to prepare the body's immune system so that it can fight and prevent the establishment of an infection or disease. For clinical and research purposes the term immunization and vaccination have been used interchangeably.

Laboratory (Lab) animals may be vaccinated in order to protect them against diseases. Immunization of lab animals may also be conducted in order to produce antivenoms or antisera for the cure of certain diseases or infections. Lastly, it is widely applied in research either to study the nature of certain infections or to generate antibodies required for serological research.

2.3.2 Route of Immunization of Laboratory Animals for Antibody Production

Recently, research on development of protective immunity has been directed toward determining strategies that specifically stimulate heightened and protective immune response. Extensive vaccine and immunization studies of infections with protozoan parasites such as *Plasmodium* and *Leishmania* in animal models have been carried out by different strategies involving variation of antigen preparations and the species of animal model used (Leenaars and Hendriksen, 2005; Mohanan *et al*., 2010). Of the many variables considered in immunization,

University of Ghana http://ugspace.ug.edu.gh

an important factor that was reckoned to influence the quality (type and strength) of immune response is the route of injection or delivery of the antigen preparation (Mohanan *et al*., 2010).

The route by which an antigen enters the body influences the tissues where immune responses are mounted as well as the magnitude of the immune response elicited (Boa-Amponsem *et al*., 2001). In line with this therefore, the route of antigen administration has been adjudged to be important in influencing immune responses at the initial site of pathogen invasion where protection is most effective.

Furthermore, the choice of injection route is, in turn, shaped to some extent by the choice of the animal species and adjuvant, as well as the nature, concentration, and volume of the antigen preparation to be inoculated (Leenaars and Hendriksen, 2005). For example, the intravenous (*IV*) route for inoculation of immunogens prepared by emulsifying aqueous antigens in oil-based, large particulate or viscous gel adjuvants such as aluminium salts, Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) is considered not advisable because of the high tendency (risk) of such antigen preparations to block blood vessels (cause embolism) and kill the animals (Hanly *et al*., 1995). Also, owing to the fact that injection into any closed space is very painful, the Canadian Council for Animal Care (CCAC, 2002) does not recommend the intradermal (*ID*) and intramuscular (*IM*) routes of antigen administration in small rodents like mouse, rat, and hamster, because these small animals do not have enough spaces in their muscle and skin (CCAC, 2002).

According CCAC (2002), therefore, the following four guidelines should be kept in mind when considering the route of antigen administration. They are:

- Inject the smallest possible volume per site.
- Use multiple injection sites when necessary.

University of Ghana http://ugspace.ug.edu.gh

- Space injection sites far enough apart to avoid coalescing of inflammatory lesions. Failure to do so may result in significant tissue necrosis.
- No single route of administration is ideal

In line with the above mentioned recommendations therefore, the most frequently used routes of injection of large animals such as rabbit, goat and sheep for PAb production are subcutaneous (*SC*), *ID*, *IM*, intraperitoneal (*IP*), and *IV* (Leenaars and Hendriksen, 2005). As all the routes of immunization have both advantages and demerits, no single route is considered perfect as far as the survival of the host organism and the elicitation of immune response are concerned (Shimizu, 2004).

Having satisfied all these conditions, the best route of antigen administration to adopt is the type that can yield heighten immune response and antibody production, with minimal or no inflammation and/ or death of the host animal (FSUIRAP, 2013). Through extensive immunization research, FSUIRAP (2013) has documented the merits and demerits of the various routes of antigen administration. This report and studies conducted by other researchers on the routes of antigen administration (Jerusalem and Eling 1969; Shimizu, 2004; Leenaars and Hendriksen, 2005; (FSUIRAP, 2013) have shown that the four major routes, *IV*, *IP*, *SC* and *IM* routes have been used most extensively in the immunization of mice towards the production of antibodies.

2.3.2.1 Intramuscular Route of Immunization

This route of immunization is often relied upon to provide a safer (safer than the intravenous route) rapid uptake into the bloodstream and lymphatic system. However, this is dependent upon the size of the antigen. It is a good site for small molecular weight drugs that are irritant as they

University of Ghana http://ugspace.ug.edu.gh

are rapidly absorbed into the blood and the inflammatory response distributed. Large molecules are more likely to be absorbed primarily by the lymphatic system which lie only in the fascial planes. The advantage in this route of inoculation is that larger amounts can be injected intramuscularly. The disadvantage is the more prolonged absorption and spread of antigen-adjuvant preparation along the fascial planes. Such spread (and accompanying inflammation) may cause problems especially where nerves are encountered. In addition, because of the prolonged assimilation of the immunogen, it takes a long time to elicit immune response which are normally poor (Jerusalem and Eling, 1969). Lastly, the use of this route for immunization of animals with small muscle masses is discouraged and therefore, not generally recommended in rodents because of their limited muscle mass (FSUIRAP, 2013; Leenaars and Hendriksen, 2005). However, the use of CFA via this route is controversial.

Intramuscular injections are usually made in the biceps femoris or quadriceps muscle mass. A great caution is required to avoid adjacent nerves and blood vessels as well as facial planes when injecting into a muscle bundle. The intramuscular route of immunization was not therefore used in this study partly because of the foregoing reasons. Also, the BALB/c mouse muscle does not have enough space to accommodate the amount of immunogen required to elicit the heighten degree of immune response necessary to produce antibodies with high sensitivities as required and was not therefore considered suitable for this study. In addition, considering the number of immunizations, both primary and secondary, that were carried out on each mouse, the intense pain and the tendency to immobilize the mice and prevent them from feeding normally, which is against CCAC (2002) recommendations, and would have also caused the premature death of the mice before immune response. Finally, in a study conducted by Jerusalem and Eling (1969) to investigate the effect of the three routes of antigen administration on immune response

University of Ghana http://ugspace.ug.edu.gh

of BALB/c mice to *Plasmodium* antigens, it was reported that mice immunized via the *IM* route elicited the lowest degree of immune response in terms of antibody titre, sensitivity, specificity and protection against challenge infection with live *Plasmodium* parasites, which is an indication that the use of the *IM* route could lead to the generation of antibodies with low sensitivity and specificity. It is for these reasons therefore, that the intramuscular route was not used for immunization of mice in this study.

2.3.2.2 Subcutaneous Route of Immunization

The ***subcutaneous*** (*SC*) route of antigen administration is probably the most frequently used route (FSUIRAP, 2013) because it is one of the easiest and rarely painful injection technique to carry out (Shimizu, 2004). Owing to its painless nature, it is conducted usually when the mice are conscious as for the intraperitoneal (*IP*) route (Shimizu, 2004). Up to 3ml of antigen preparation are recommended to be administered per site of this route of injection (Shimizu, 2004). As the antigen is introduced mainly via the lymphatic system (FSUIRAP, 2013), the rate of absorption is slower than the cases for the *IP* and *IM* injections (Simmons and Brick, 1970). The rate of absorption is dependent upon the blood flow in the area (dependent upon skin temperature), activity of underlying muscles and contact area (Shimizu, 2004). The loose skin areas will allow further spread of the antigen-adjuvant preparation, thereby increasing the contact area. It is a good choice for antigen administration. It is likely to induce anaphylactic reaction if absorbed into the bloodstream. The disadvantage associated with this technique is that inflammatory reactions are less likely to stay at the site of injection but may migrate with the development of fistulous tracts (FSUIRAP, 2013). Also, the relatively slower rate of absorption

University of Ghana http://ugspace.ug.edu.gh

of the immunogen via this route than the *IP* route make the latter superior in terms of the quality of immune response.

Subcutaneous administrations are made into the loose skin over the interscapular area as follows. The mouse is manually restrained and then placed on a clean towel or solid surface. The needle is inserted under the skin of the interscapular or inguinal area, and while being tented by the thumb and forefinger, the immunogen is then inoculated. The *SC* route was not employed for immunization of mice in this study because of its associated disadvantages of weaker immune response and migratory inflammatory reactions which could have a negative impacts on the survival of the mice through the immunization period and the sensitivity of the antibodies produced. Furthermore, the *SC* route was not used because, like the *IM* route it is not recommended for the immunization of rodents (FSUIRAP, 2013; CCAC, 2002).

2.3.2.3 Intravenous Route of Immunization

The ***intravenous*** (*IV*) route of administration delivers antigen to the spleen and secondarily, to the lymph nodes. It is a good choice for administration of soluble antigens without any adjuvant. It is not recommended for antigens prepared in adjuvants due to the risk of embolism, as mentioned above. Adjuvants that may be used intravenously include liposomes, properly prepared water-in-oil double emulsions and dispersed alum. The disadvantages of this route include 1) lack of antigen depot effect, 2) poorer response in antibody titre when used as the route for primary immunization, 3) increased risk of tolerance or anaphylactic reactions, 4) it is the most lethal of all the routes of immunization (Jerusalem and Eling, 1969), 5) it requires high technical expertise and skill to perform (FSUIRAP, 2013; Shimizu, 2004; Jerusalem and

University of Ghana http://ugspace.ug.edu.gh

Eling, 1969). It is often used for booster injections following primary immunization with an antigen-adjuvant given via another route.

In spite of the above, *IV* injection has certain advantages over other routes. Highly concentrated or irritating solutions, or solutions with high or low pH can be administered intravenously provided that the rate of injection is kept slow and precautions are taken to avoid getting the solution outside the vein. Compounds that are poorly absorbed by the digestive tract may be given intravenously. The intravenous (*IV*) route was not used in this study because 1) It is the most fatal (Jerusalem and Eling, 1969) among the four routes of immunization mentioned above; 2) The laboratory animal of choice for this study was the BALB/c mice, which were chosen based upon the cost of purchase and maintenance of the animal, scarcity of space and that the myeloma (NS1, P3X63Ag8.653 [Ag8],) cell lines available for cell fusion and production of the monoclonal antibodies originated from mice.

2.3.2.4 Intraperitoneal Route of Immunization

The ***intraperitoneal*** (*IP*) route is most frequently used for immunization of rodents, less often in other species (Shimizu, 2004; FSUIRAP, 2013). Antigen is taken up by the lymphatic system rapidly and transferred to draining nodes, the thoracic duct and hence the vascular system. The advantages of this route of immunization include the relatively larger volume of preparation that can be injected, several different types of adjuvants can be used and the antigen is widely distributed to lymphoid tissue. Also, unlike the intravenous route, injection through the (*IP*) route can lead to the elicitation of heighten immune response and protection which are slightly lower but comparable to that induced by the intravenous route (Bhowmick *et al*., 2009; Jerusalem and Eling, 1969). In addition it is relatively far safer than the inoculation via the intravenous route

University of Ghana http://ugspace.ug.edu.gh

(Jerusalem and Eling, 1969). The disadvantage of this route for administration of antigen, however, is the risk of anaphylactic shock if boosters are rapidly absorbed into the vascular system. Also, owing to the fact that the use of complete Freund's adjuvant (CFA) through the *IP* route to produce polyclonal antibodies induces inflammation, peritonitis, and behavioral changes (CCAC 2002) it is adviced that it should be carried out only after scientific justification and approval (CCAC, 2002). Finally, the *IP* route is not recommended in larger animals such as rabbit, goats and above (Hendriksen and Hau 2003).

The production of rodent peritoneal exudate by the intraperitoneal administration of antigen and adjuvant is a widely recognized valid scientific procedure for obtaining high-titer reagent. Undesirable side effects of painful abdominal distention and the resulting distress can be avoided by daily monitoring and relief of ascites pressure, or termination of the experiment.

This route of immunization is the most commonly applied because it is technically simple and easy. It allows quite faster but long enough periods of absorption from the repository site. The rate of absorption by this route is usually one-half to one-fourth as rapid as from the intravenous one (Woodard, 1965). The limitations of this route are few. Firstly, the affected tissues may react adversely to irritating substances. Also, the affected tissues are often less tolerant to solutions of non-physiological pH, as this route requires the use of isotonic solutions of which quite large volumes can be administered. This route of immunization was therefore used in this study because it is relatively safer, it results in the elicitation of a heighten immune response and that it is the most widely used method for immunization of BALB/c mice to generate antibodies.

University of Ghana http://ugspace.ug.edu.gh

2.4.0 IMMUNITY TO MALARIA

2.4.1 Innate Immunity to Malaria

In malaria endemic areas, immunity to malaria begins as natural defense or innate immunity, which is observed, right from birth, in populations continually exposed to malaria parasites. The first example is observed in people with inherited conditions such as sickle cell anaemia, beta-thalassaemia and glucose-6-phosphate dehydrogenase deficiency (retrieved from http://malaria.wellcome.ac.uk/ doc_WTD023885.html on April 2, 2007). These conditions are common in people from malaria endemic communities, and are known to cause deformities in red blood cells (RBCs), which make it difficult for the RBCs to be infected by malaria parasites and other pathogenic organisms.

The second example of innate immunity to malaria is demonstrated in people of west African origin, who are known to have RBCs that lack proteins called Duffy antigens on their surfaces (Weatherall *et al*., 2002;). These proteins act as receptors for *P. vivax* merozoites during infection of the RBCs, so in people without the Duffy antigens, the merozoites cannot bind onto the RBCs, and are cleared during the blood stage of infection, thus making these individuals resistant to infection by the *P. vivax* malaria parasite (Weatherall *et al*., 2002).

Lastly, infants from birth to about three months or even up to about 12 months of age have some protection against malaria which is conferred by immunoglobulin G (IgG) antibodies that crossed the placenta from mother to child during pregnancy (Moormann, 2009). This immunity, however, begins to decline from about the third month to about one year of age when it is completely lost as a result of loss of the maternally acquired IgG antibodies (Moormann, 2009).

University of Ghana http://ugspace.ug.edu.gh

2.4.2 Acquired Immunity to Malaria

The infection of immunocompetent humans by malaria leads to development of acquired immunity, such that individuals that are repeatedly exposed to *P. falciparum* infection acquire partial immunity.

The process of acquired immunity begins in babies and infants (with a few exceptions) who are exposed to *Plasmodium* parasite infection (malaria), on a regular basis, and is sustained into later life (Phillips, 2001). Most of these young people survive infection with *P. falciparum*, however, in about 1 to 2% of the infected individuals, severe malaria develops and could be fatal. Although children develop protective immunity against *P. falciparum*, such protection is not easily acquired (Phillips, 1994). In areas of stable or high malaria transmission and endemicity, the pattern is for non-immune children (babies and infants) up to 5 years of age to be frequently infected by *Plasmodium* parasites early in life (Weatherall *et al*., 2002).

Often, a number of such children do experience more severe malarial symptoms, and become at risk of dying from the disease, while adults suffer fewer clinical malaria episodes (Chattopadhyay *et al*., 2003). However, as immunity develops with repeated infection, parasite prevalence, density, number of clinical episodes and the severity of the disease decline progressively with increasing age (Weatherall *et al*., 2002).

In non-immune adults however, effective immunity probably develops more quickly after first infections (Baird, *et al*., 1991; Baird, 1995). For instance, it was observed in a malaria endemic area in Indonesia that after 2 years, there were no fatalities among adult residents, who were originally non-immune and had migrated from malaria non-endemic areas. This suggested that lifesaving immunity developed in 2 years in adults compared with 5 years in small children (Baird, 1995).

University of Ghana http://ugspace.ug.edu.gh

The common thinking is that natural acquired immunity to malaria is normally strain-specific, and appears to be lost if a person migrates from an endemic to a non endemic area for an extended period of time, suggesting that repeated exposure is necessary to maintain resistance (McGregor, 1986; retrieved on April 2, 2007, from http://malaria.wellcome.ac.uk/ doc_WTD023885.html). Immunity to malaria is never complete, even in adulthood and during the transmission season, clinical episodes do occur and parasites may be observed in blood films in the absence of clinical signs. Owing to the fact that immunity is apparently invariably incomplete, a vaccine that will confer total protection, therefore, requires a level of immunity never achieved by natural exposure. If natural immunity could be mimicked, then vaccination would prevent severe malaria and malaria-related deaths, but would not give complete protection (Weatherall *et al*., 2002).

2.4.3 Vaccines against Malaria-1

The unacceptable morbidity and mortality (Hill, 2011) caused by malaria to humans have led to the deployment of numerous control measures, including chemotherapy and destruction of vector mosquitoes by insecticides (Graham *et al*., 2004) as well as management of the environment to discourage the proliferation of vector mosquitoes (Randell *et al*., 2010). Nevertheless, the destructive toll of this disease has not seen any satisfactory improvement over the past decade. (WHO fact sheet 2002; WHO World Malaria Report, 2008; Snow *et al*., 2005). Acknowledging the phenomenal success of vaccines against diseases such as poliomyelitis, measles, diphtheria, tetanus, rabies, and more particularly, their ability to completely eradicate smallpox (Sallusto *et al*., 2010), that used to kill and disfigure a substantial population of the world (Breman and Arita, 1980), coupled with the belief in the potential of vaccines to reduce the global burden of infectious

University of Ghana http://ugspace.ug.edu.gh

diseases, the foregoing hopeless nature of malaria and its existing control strategies, mainly in endemic populations, led to the conclusion that vaccination against *P. falciparum* and *P. vivax* is the method of intervention with the greatest promise to reduce the morbidity and mortality associated with severe malaria in areas of intense transmission (Miller and Hoffman**,** 1998) and even would contribute to eradicating the disease with a very high probability (Greenwood, 1997). In addition, with the success of early vector control programs suggesting that it is reasonable to hold an optimistic view (Phillips, 2001), the belief in the ability of vaccines to eradicate malaria resulted to a variety of sustained efforts to develop effective malaria vaccines (Hill, 2011) to control the disease.

Immunological memory is the ability of the immune system to respond with greater intensity to infection when the causative pathogen is encountered again, and constitutes the basis for vaccination (Ahmed and Gray, 1996). To date, vaccination remains the most effective method of preventing infectious diseases, and represents the most relevant contribution of immunology to human health (Plotkin and Plotkin, 2008; Siegrist, 2008). Notwithstanding the tremendous achievement of vaccines against the viral and bacterial diseases mentioned above, and the remarkable optimism that they could be used to eradicate vector bone diseases, especially malaria, development of vaccine against this disease represents a major challenge, even with unlimited resources to devote to the task because, firstly, it would be a very tall order to produce a vaccine against a parasite for which limited natural exposure does not stimulate a protective immune response (Phillips, 2001). Also, there are a number of other features of malaria parasites which make the development of vaccine against the disease particularly difficult (Phillips, 2001).

For example, according to Phillips (2001) Protozoa are relatively complex, and in malaria parasites, it is estimated that there are 5,000 to 7,000 proteins. Immunity to each stage of the life

University of Ghana http://ugspace.ug.edu.gh

cycle is largely specific, probably because each stage produces its own novel antigens. Therefore, selecting targets for vaccine-induced immunity and the corresponding peptides with which to induce that immunity has been extremely difficult. Also, Phillips (2001) reported that unlike most viral vaccines, malaria vaccines must virtually be subunit vaccines because, although it is possible to grow all stages of the parasite life cycle *in vitro* to produce a whole-organism vaccine it is presently difficult to generate large quantities of sterile, efficacious protein material that is required for mass production of whole-parasite vaccines (Phillips, 2001; Draper, 2012).

In spite of the the suggestion by Phillips (2001) that malaria vaccines must be subunit vaccines, Wykes and Good (2007) stated that evidence from in-depth studies of the homologues of the *Plasmodium* vaccine candidate antigens show that these individual proteins on their own have various inherent immunological characteristics that make it difficult, if not impossible to use them in development of effective sub-unit vaccines against malaria.

2.5.0 DIAGNOSIS OF MALARIA

Diagnosis is a medical term used to indicate the act of demonstrating the presence of disease. It is probably the most important component of the healthcare delivery system, and has various ramifications depending on the nature of the disease as well as the geographic, social, cultural and the economic factors that influence its development and application.

Prompt, accurate and safe diagnosis is the key to effective disease management, a major intervention measure of the Global Malaria Control Strategy (WHO, 1993). This is probably because early and accurate diagnosis is crucial in the development of management strategies for the prevention of diseases. Also, at the onset of severe and complicated malaria, timely and

University of Ghana http://ugspace.ug.edu.gh

accurate diagnosis is essential to prevent death. Nevertheless, it is of great concern that poor diagnosis continues to hinder effective control of malaria (WHO, 2000).

Several approaches to the diagnosis of malaria are currently in adoption. Each approach has characteristics, including costs, availability, and ease of performance and accuracy that determines its applicability to different situations (WHO, 2000).

2.5.1 Clinical Diagnosis of Malaria

The clinical method is presently the first-line and the most widely used approach to malaria diagnosis. It is based on the observation of clinical signs, the most prominent being fever {persistent abnormal increase in body temperature (temp.), i.e. axillary temp. ≥37.3^{o}C; oral temp. ≥37.8^{o}C; rectal/ ear temp. ≥38.3^{o}C} accompanied by headache, chills, perspiration and anorexia (loss of appetite). It is inexpensive and requires no special equipment or supplies. It has been the only feasible diagnostic approach that residents, particularly, of rural endemic areas are familiar with, and frequently utilize in self-diagnosis of malaria (WHO, 2000). Nevertheless, clinical diagnosis is presumptive and unreliable because, malaria presents non-specific symptoms, which overlap with those of other febrile diseases such as meningitis, typhoid fever, influenza, septicaemia, hepatitis, scrub, gastro-enteritis viral encephalitis as well as haemorrhagic fevers (WHO, 2000; White, 1998; Bradley, 1996). Also, when chloroquine and other low-cost and less toxic drugs were highly effective, especially, in areas of high malaria transmission where microscopy is not readily available, treatment of malaria based on clinical signs alone was generally considered cost-effective and justifiable (WHO, 2000; Azikiwe *et al*., 2012). In areas of low-malaria transmission, however, indiscriminate dispensing of anti-malarial drugs due to unconfirmed clinical diagnosis has led to the emergence of multi-drug resistant malaria which

University of Ghana http://ugspace.ug.edu.gh

requires more expensive and toxic mefloquine or artemisinin-based combination therapies. It is therefore important to minimize anti-malarial drug use based on presumptive clinical diagnosis alone.

2.5.2 Microscopic Diagnosis of Malaria

Conventional light microscopy is presently the established and most acceptable method for confirmation of *Plasmodium* infections (Katzin *et al*., 1991; Payne, 1988). Careful examination of Giemsa, Wright's or Field's -stained thick and thin blood smears, well prepared from finger-prick or venous blood samples by an expert microscopist is presently the "gold standard" for specific detection and identification of malaria parasites (WHO, 2000).

Microscopy is also considered the gold standard for determining the efficacy of antimalaria drugs or vaccines (Ohrt *et al*., 2002), and the yardstick for evaluating the sensitivity and specificity of new diagnostic methods (Steenkeste *et al*., 2010; Okell *et al*., 2009).

Microscopic diagnosis of malaria has several advantages. It is relatively sensitive and can detect 5-10 parasites per a microlitre of blood when carefully done by a skilled technician (WHO, 1990). However, under general field conditions the detective capability might be more realistically placed at 100 parasites per a microlitre of blood (WHO, 1988). Microscopy is very specific and highly informative. It allows parasites to be distinguished into species such as *P. falciparum, P. malariae, P. vivax, P. ovale* or *P. knowlesi* and circulating stages such as trophozoites, schizonts or gametocytes (WHO, 2000). Also, microscopy enables quantification of parasites per leukocytes or erythrocytes that is needed for determination of parasitaemia or assessment of parasitological response to chemotherapy (WHO, 2000). The blood smear preparations can provide a relatively permanent record of diagnostic findings and be subject to

University of Ghana http://ugspace.ug.edu.gh

quality control (Payne, 1988). Furthermore, microscopy is relatively inexpensive, with cost estimates for endemic countries ranging from US$0.12 to US$0.40 per slide examined (Palmer *et al*., 1998). The cost however decreases when large numbers of samples are examined, and also when shared with other disease control programmes (WHO, 2000).

Nevertheless, microscopy has several disadvantages. It is labour-intensive and time-consuming, normally requiring at least 45 minutes to yield results (WHO, 2000). It is exacting and requires highly motivated skilled or well-trained and supervised technicians with good microscopes, reagents and electricity. Unfortunately, these conditions are usually not available at the peripheral levels of the health care delivery system (WHO, 2000) where most deaths and morbidity occur. In addition, long delays in communicating the microscopic results to clinicians, make decision on treatment to be taken without the benefits of the results. In view of this, Payne (1988) indicated that microscopic diagnosis of malaria using scarce resources could be highly doubtful and unreliable. Furthermore, microscopic method of diagnosing malaria is not safe enough because it is invasive and exposes healthcare workers to blood.

Blood is used in traditional societies for atonement and regarded as the essence of life. It is used to appease the angry spirits, ancestors and gods, or offered to thank the gods and ancestors for their protection in the past, present and future. The Efutus of Winneba in Ghana, for example, offer blood to their god "Penyi Otu" during Aboakyir festival as a thanksgiving sacrifice (Otabil 1992). Blood is also seen as an important ingredient in the preparation of very potent traditional medicine and magic. On account of these, blood collection in most of the highly malaria endemic traditional African societies is associated with fetishism, and therefore, making it difficult to encourage the use of diagnostic tests that involve invasive procedures at the community and

University of Ghana http://ugspace.ug.edu.gh

household levels, which is an important strategy for reduction of malaria morbidity and mortality in traditional African societies(WHO, 2004).

2.5.2.1 The Roles of Thick and Thin Blood Smears in Diagnosis of Malaria

Blood obtained by pricking a finger or an earlobe is an ideal sample, because these tissues are rich in capillaries, which have very high concentrations of developed trophozoites or schizonts (Moody, 2002). Alternatively, blood obtained by venipuncture collected in heparin or sequestrine (EDTA) anticoagulant-coated tubes may be suitable if processed immediately, to prevent alteration in the morphology of malaria parasites and white blood cells (Moody, 2002). The thick blood film concentrates red blood cell (RBC) layers over a small surface area by 20 to 30 fold to be stained as an unfixed preparation using Field's stain or diluted Giemsa or Wrights stain (Moody, 2002). This characteristic enhances the sensitivity of the blood film technique for detection of low-level parasitaemia and reappearance of circulating parasites during infection, recrudescence or relapse. Lysis of the RBCs during staining can make examination for parasites more difficult until experience is gained in finding the parasites among the white blood cells (WBCs) and platelets. The thin blood film on the other hand, comprises a monolayer of RBC that is fixed with methanol and stained with diluted Giemsa or Wright's stain using buffered water at pH 7.2 to distinguish parasite inclusions in the RBC. The fixed monolayer of RBC in this procedure makes it more specific because, morphological identification of parasites to the species level is much easier than the thick blood film examination. Also, the thin blood film makes it easier to see and count parasites so it is mostly the choice for routine estimation of parasitaemia. The ability to count parasites in sequential blood films is required in monitoring the response to therapy, especially, for *Plasmodium falciparum* infections.

University of Ghana http://ugspace.ug.edu.gh

2.5.3 Rapid Diagnostic Tests for Malaria

Conventional diagnosis still uses the skilled but laborious and time-consuming microscopic examination of thin and thick blood films stained with Giemsa's or Field's stain. Newly developed tests include the quantitative buffy coat method (Becton Dickinson, Sparks, Md.) for the fluorescent staining of parasites after concentration (reported to be as good as thick films for *P. falciparum* but not for the other species (Baird *et al*., 1992); the CareStat Malaria™ (AccessBio Inc., New Jersey, USA) (Eibach *et al.*, 2013) and the Paracheck (Orchid Pharmaceuticals) (Reyburn, *et al*., 2007), which are based on the immunological capture of *P. falciparum* histidine-rich protein 2 and/ or parasite pan-specific lactate dehydrogenase or aldolase enzymes in whole blood (Azikiwe *et al.*, 2012; Moody, 2002).

These antibody-based dipstick tests are still being evaluated. PCR-based diagnostic tests for human malarias have been developed (Morgan *et al*., 1998; Eibach *et al*., 2013), but these are more applicable to large-scale surveys than to clinical diagnosis. PCR has been especially effective at detecting submicroscopic levels of parasitemia (Alemu *et al.*, 2014; Mahajan *et al*., 2012; Okell *et al*., 2009; WHO, 2000). The quatitative buffy coat (QBC) test is a microscopy-based test that employs a fluorescent dye to enhance the visibility of parasites in microcapillary collected blood samples. Even though it is rapid and takes about 15 mins to perform it is invasive and requires blood to perform. Also, the QBC system comprising, the special micro capillary tubes with stoppers, microcentrifuge, the QBC reader and fluorescent microscope which must be powered by electricity are very expensive and cannot be afforded by hard to reach rural communities in which morbidity and mortality are highest. In view of this very few specialized health centers have and utilize the QBC system in the diagnosis of the disease.

University of Ghana http://ugspace.ug.edu.gh

2.6.0 MALARIA TRANSMISSION AND ITS EFFECT ON AGE-DEPENDENT DISEASE BURDEN IN ENDEMIC POPULATIONS

The pathogenesis of malaria is such that individuals with no or limited resistance to the disease, tend to be at the greatest risk of infection, harbour the greatest parasite load and suffer from the severest outcomes of the disease (Stauffer and Fischer, 2003).

Humans, right from birth to about 6 months after birth, have maternally transmitted anti-malaria IgG antibodies within them which give them immune protection against malaria (Doolan *et al*, 2009). After 6 months of age and onwards, the maternally acquired antibodies and the corresponding immunity to diseases begin to wane, while the body builds up its own acquired immunity for protection against subsequent infections (Doolan *et al*, 2009). During the time of development of adequate acquired immunity, it is common for unimmune individuals to suffer from the worst outcomes of infection, including heavy parasite density, heavy disease burden and high rate of mortality, which decline as the individual grows older and continuously build up adequate protective immunity (Doolan *et al*, 2009).

In an area of high transmission intensity, it takes about 5 years on the average (Doolan *et al*, 2009), for unimmune children to develop the theoretical immunity necessary to confer partial protection against the worst outcomes of malaria infection (Doolan *et al*, 2009), so the severest outcomes of the disease including heavy parasite density, is concentrated in children under 5 years of age (Doolan *et al*, 2009). However, in an area of low transmission intensity, the gestation period for acquisition of adequate protective immunity against malaria takes longer than 5 years (Wipasa *et al.*, 2002), and could even extend into adulthood, because the amount of infectious *Plasmodium* antigens that characterize low transmission of parasites is not enough to induce development of immune protection within 5 years.

University of Ghana http://ugspace.ug.edu.gh

2.6.1 Malaria Transmission at Kpone-on-Sea

Owing to the devastating nature of malaria, efforts are being made worldwide to control the disease (Reviewed by Tchouassi *et al*., 2012). As part of this worldwide malaria control effort, Kpone-on-Sea (KOS), a malaria endemic coastal fishing village in southeastern Ghana, is being developed as a model site for malaria intervention studies with a view to control the disease in the endemic population. The strategies being adopted include improved vector control, chemotherapy, use of insecticide treated bed net (ITN) and possibly, vaccination in the foreseeable future (Tchouassi *et al*., 2012). For the vector control strategy to be effective there was the need to characterize the malaria transmission pattern of the area. In a study to characterize malaria transmission at KOS (Quakyi *et al*., 2004), and at about the same period that data for the studies reported in this thesis was collected, the intensity of malaria transmission at KOS was estimated to be variable with an entomological parasite inoculation rate, EIR = 62.1 (Tchouassi *et al*., 2012).

A variable or an unstable malaria transmission intensity for KOS, implies that children in this community, after birth, can take 5 years or longer to acquire adequate protective immunity necessary to mitigate some of the adverse outcomes of malaria infection. Also, between 2004 and 2008, a WHO funded project, the KOS Malaria Project, carried out a multidisciplinary case control study of malaria pathogensis and immunity in souththern Ghana, during which artesunate-amodiaquine was used to treat infected members of the Kpone community (Quakyi *et al*., 2004). Furthermore, the Ghana Health Service embarked on the distribution of insecticide treated bednets (ITN) for use by pregnant women and nursing mothers in a bid to control malaria transmission at KOS. It is highly probable therefore, that these intervention programmes together, would lead to a considerable reduction in malaria transmission at KOS, especially, among

University of Ghana http://ugspace.ug.edu.gh

children under age 5 who shared the ITNs with their parents and had less exposure to infectious mosquito bites. The overall effect conceivable, is that the period for development of protective immunity would be shifted from 5 years on the average to a longer one, probably, 10 years, 15 years (Wipasa *et al*., 2002) or even longer, which in turn would lead to the shifting of the risk of the severest malarial outcomes into the 6 to 10 years age group of individuals (Griffin *et al*., 2014; Schellenberg *et al*., 2004). In view of this, after age 5, when the children are no longer allowed to sleep under ITN, and are exposed to infectious mosquito bites, it is probable that they would be incapable to offer any resistance to malaria infection and consequently would experience the worst outcomes of the disease including harboring of the heaviest load of parasites.

University of Ghana http://ugspace.ug.edu.gh

CHARPTER 3

GENERAL MATERIALS AND METHODS

3.1.0 STUDY SITE

3.1.1 Kpone-on-Sea

Kpone-on-Sea is located in the Dangme West District within the Greater Accra Region of Ghana (Map 1). It is situated on 5º69'N, 0º06'E within the coastal savanna belt of West Africa. The village is surrounded by coastal scrub. It is bordered, on the east by Prampram, on the west by Tema, on the North by the industrial free zone, and on the south by the gulf of Guinea which is 139 metres away.

Kpone stands at an altitude of 50m to 100m above sea level and has an equatorial climate. Temperatures range from 24.4ºC to 27.8ºC with a mean of 26.1ºC. Over the years maximal transmission of malaria occurs during the major rains (May to July) and after the minor rains (November). Mean annual rainfall averages between 1,133 and 3,606 mm with an average relative humidity index ranging from 78% to 85%.

The land formation and the drainage patterns of the 4 sectors of the village were such that all water from the village drains into a stream that lies on the outskirts of Kpone village. A lagoon also on the outskirts of the village separates Kpone from Prampram. Kpone has limited standing pools of stagnant water to promote mosquito breeding. The estimated population of Kpone was 9,300 in 2004 (Quakyi *et al*., 2004). Majority of the residents (80%) were of the Ga and Ga-Dangbe ethnic groups. Most of the inhabitants were fisherman of whom many were also involved in farming. The Dangbe West District Health Directorate manages health service delivery at Kpone.

University of Ghana http://ugspace.ug.edu.gh

3.2.0 STUDY DESIGN

The proposed research involved subjects from KOS. Finger-prick blood samples from study subjects were examined microscopically for *Plasmodium* infection and parasitaemia. Urine samples were also collected from study subjects and examined for biomarkers of malaria such as glucose, ketones, *Plasmodium falciparum* histidine rich protein II (*pf*HRPII) and hepcidine. Malaria antigens were then extracted from the urine of malaria positive individuals for monoclonal antibody (MAbs) generation.

Plasmodium falciparum parasites were cultured *in vitro* and used to prepare crude parasite antigens. These antigens together with the urinary *Plasmodium* proteins emulsified in Freund's complete adjuvant (FCA), were used to initiate immunization of laboratory bred BALB/c mice to select antibodies that target *P. falciparum* antigens. After the third booster post primary immunization, the *P. falciparum* antigens prepared from urine of positive subjects and cultured parasites emulsified with Freund's incomplete adjuvant (FIA), were then used to do the final booster of immunizations prior to cell fusion.

Spleen cells from immunized BALB/c mice were used to generate anti- *P. falciparum* species-specific MAbs, and explored to develop a urine-based membrane-based dipstick and micro-plate ELISAs for detection of parasite antigens in the urine of infected individuals. The accuracy of the developed urine-based dipstick-ELISAs was evaluated in the study areas and hospitals by comparing its sensitivity and specificity with those of ICT and microscopy.

3.2.1 Sample Size Determination

For accuracy, the developed urine-based dipstick assay has to be evaluated for sensitivity and specificity which requires collection of adequate number of samples. The following therefore

University of Ghana http://ugspace.ug.edu.gh

describes the estimation of the number of samples required for evaluation of the developed urine-based dipstick test.

In 2004, the estimated population size of Kpone-on-Sea (KOS) was 9300 (Quakyi *et al*., 2004). In 2005, the prevalence of malaria among patients visiting the Kpone Health Centre as determined by a pilot study was 52.0% (0.52), McKakpo *et al*. (2006, *data unpublished*). For the proposed urine based dipstick test (RUBDA) to be as accurate as required, it was expected to have a sensitivity of at least 96% (0.96). Considering that the lower 95% confidence limit for the test should not fall below 85%, then from the sample size determination chart for diagnostic assays provided by Flahault *et al*.(2005), 625 controls were required for evaluation of accuracy of the RUBDA.

However, the number of controls required for a diagnostic test in a population of disease prevalence greater than 50% (Prev > 0.50) is related to the number of cases and prevalence by the equation:

$N_{eon} = N_{cas}$ [(1-Prev) / Prev)] (1);

Where, "N_{con}" and "N_{cas}" are the required number of controls and cases, respectively, and "Prev" is the prevalence of the disease in the community.

Therefore from equation (1), 625 estimated controls will require 677 cases or a total of 1302 subjects for evaluation of the diagnostic test.

Convenient sampling was used to recruit patients for the study. This was necessary to obtain adequate number of infected samples before the end of the rainy season. The rainy season is the most favourable period for obtainment of the maximum number of *Plasmodium*-infected individuals from the Kpone study community. The development of the urine-based dipstick test for malaria required *Plasmodium* proteins isolated from malaria infected urine samples which are

University of Ghana http://ugspace.ug.edu.gh

obtainable mostly at the peak of the rainy season. Since house to house collection of urine samples would have taken a longer time which would have extended far beyond the rainy season into the dry season, sample collection was done only at the health centre instead.

3.2.2 *In Vitro* Propagation of Laboratory (3D7) Strain of *Plasmodium falciparum* Parasites

Citrate Phosphate Dextrose (CPD) anti-coagulated venous blood was collected from healthy individuals and kept for 24 hrs at 4^{o}C. The anti-coagulated blood samples were then washed 5 times in prewarmed (37^{o}C) washing medium and at each washing step samples were centrifuged for 7 minutes at 738 x g (2000rpm) to obtain fresh RBCs. Buffy coats were removed after each centrifugation.The 3D7 laboratory strain parasites were cultured in canted neck flasks until their maturation to schizont stage, using the Trager and Jensen protocols (Trager and Jensen, 1976 and 2005) for continuous culture of human malaria parasites with slight modification.

Cryopreserved 3D7 strain parasites in RBCs in vials retrieved from a liquid nitrogen tank were thawed in water bath at 37^{o}C. The cells were centrifuged at 738 x g (2000 rpm) for 5 minutes and the supernatant discarded. Equal volume of thawing mix (3.5% NaCl in distilled water) was added to lyse the RBCs and release parasites from the erythrocytes. The parasites were washed twice with 1ml of complete culture medium (RPMI-1640, supplemented with gentamycin, Albumax and L-Glutamine) and then added to a culture flask (25ml canted neck flask) containing 5ml complete parasite medium and 200µl of packed uninfected O^{+}, sickling negative erythrocytes. The flask was flushed for 30 sec with gas mixture (2.0% O_2, 5.5% CO_2 and 92.5% N_2) at 1.5-2.0 bar pressure and incubated at 37^{o}C to provide a suitable condition for parasite growth. The culture medium was changed daily to maintain the culture until a high yield of parasite growth at the schizont stage was obtained.

University of Ghana http://ugspace.ug.edu.gh

The parasitaemia of the culture was obtained using microscopic examination of geimsa stained thin films, and the percentage parasitaemia was determined using the formula indicated below;

$$\frac{\text{Number of infected erythrocytes X 100}}{\text{Total number of erythrocytes (500 erythrocytes)}}$$

When percentage parasiteamia exceeded 5%, the culture was transferred into a bigger flask (75ml canted neck flask). Added to the flask was 25ml culture medium and 800µl of fresh RBC's (to reduce the total percentage parasiteamia to 1%), gassed and then kept in the incubator at 37^{o}C. The culture was continuously maintained until percentage parasiteamia of between 8-10% was obtained with the parasites mostly in their schizont stage. At this point, the culture was used for the preparation of crude malaria antigen by first harvesting the schizonts using the magnetic activated cell sorting technique and then extracting the antigen, using the method explained below.

3.2.3 Extraction of Soluble Crude *Plasmodium falciparum* Antigens from *In Vitro* Cultured 3D7 Parasites

The schizont stage parasites were isolated and antigens prepared. In this process, RBCs infected with the schizont stage parasites were separated from the uninfected RBCs and ring stage RBCs using Magnetic-Activated Cell Sorting (MACS) technique.

The MACS column was mounted within the strong magnetic field which was suspended from a glass platform. The column was run with a PBS buffer to remove cellular debris. A 21G (0.8 x 0.4mm) needle was then fixed to the base of the column to regulate flow

University of Ghana http://ugspace.ug.edu.gh

rate from the column. The column was then washed twice with 2.5ml washing medium (RPMI-1640, L-gluthamine and gentamycin)

The parasite cultures were diluted with phosphate buffered saline (PBS, pH 7.2) to cause disintegration between both the infected and uninfected RBCs for a successful purification resulting in a good yield. The diluted parasite culture was then passed through the column and thereafter washed with a washing medium until colour of the eluent changed from red (culture medium) to colourless. As a result of their high haemozoin content which is paramagnetic, parasites in the late developmental stages (mainly schizonts) bound to iron fillings in the column and were removed by running the column again with buffer (2% FBS in PBS) and collecting the eluent into a sterile tube (50ml tube). The harvested buffer-containing parasite was centrifuged at 738 x g (2000rpm) for 10 minutes and the supernatant discarded. Pellets of schizont stage infected RBCs were diluted in the ratio 1:100 in PBS. The infected RBCs were counted in a haemocytometer under a light microscope and the concentration of the infected RBCs was obtained using the formula indicated below;

Number of cells/ml = n (number of cells)/4 x dilution factor $x10^4$

Number of cells/ml = n/4 x100 x 10^4

The schizont rich erythrocytes were then quickly subjected to three cycles of 'Freeze-thawing' process in liquid nitrogen at a temperature of -196^{o}C and in a water bath at 37^{o}C respectively to lyse up the cells to release parasite antigens into solution. The solution was then quickly centrifuged at a rate of 26564 x g for 30 minutes using a micro centrifuge. The obtained lysate containing the crude parasite antigens was aliquoted into small cryo-tubes and kept at -80^{o}C until ready to be used.

University of Ghana http://ugspace.ug.edu.gh

3.2.4 Preparation of Non-Immune Sera and Antisera of Mice for Microplate ELISA

The tail of each immunized BALB/c mouse was cleaned with a piece of cotton wool partially soaked in 70% ethanol. Measuring from the tip, about 3mm length of the tail was cut with a clean pair of dissecting scissors. Blood was drawn from the tail veins by applying gentle pressure from the base of the tail toward the tip (Plate 4) and a few drops of blood collected on clean parafilm. Twenty microlitres (20 µl) of blood was then fetched into an eppendorf tube containing 1000 µl of PBS and spun at 13,200 g for 5 min at 4^{o}C to obtain the serum. The serum of each mouse was collected into a new sterilized labeled eppendorf tube and stored at -20^{o}C until it was used for microplate ELISA

3.2.5 Coating of Microplates for ELISA

Microplate ELISA was used to investigate the production of anti-*Plasmodium* antibodies in laboratory bred BALB/c mice. Ninety-six well (96-well) flat bottom polystyrene microtitre plates (Immulon 2HB, Part. No. 3455, Thermo Scientific, NY, USA) were coated with 50 µl/ well of *Plasmodium* antigen solutions (either isolated from the urine of infected individuals or extracted from cultured parasites), comprising 0.1 µg/µl protein in Dulbecco's phosphate buffered saline (DPBS, pH 7.4; Sigma-Aldrich, Cat. No. D5773-10L). The microplates were covered with aluminium foil and incubated overnight at 4^{o}C.

University of Ghana http://ugspace.ug.edu.gh

3.2.6 Screening of BALB/c Mice for Anti-*Plasmodium* Antibodies by Indirect ELISA

Microplate ELISA was conducted to test the reactivity of sera from the BALB/c mice with PAgHU or CPfAg before immunization. This experiment was carried out to determine any background reactivity in the BALB/c mice, which is required for determination of true negative and positive samples and standardization of the ELISA. Microtitre plates previously coated with cultured *Plasmodium* parasite or human urinary *Plasmodium* proteins, PAgHU or CPfAg, respectively, were retrieved from the refrigerator at 4ºC. The plates were flipped empty to remove excess unbound antigen and then banged on blotting paper to remove droplets of antigen solution. The plates were rinsed briefly with DPBS washing buffer [DPBS and 0.5% (v/v) Tween 20, pH 7.4] to remove excess unbound *Plasmodium* antigens and then banged on blotting paper as before. Test sera were diluted serially from 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 up to 1:3200 in PBS and 50 µl applied to duplicate wells and incubated at room temperature 25ºC for 2hrs. DPBS and DPBS washing buffer were used as blanks and negative controls respectively. The plates were flipped empty and then washed two times by carefully filling the wells with DPBS washing buffer and then flipping empty after 10 min interval each. Each well of the microtitre plates was then incubated for 1hr at 25ºC with 50 µl peroxidase labelled goat anti-mouse IgGAM antibody conjugate (Abcam, Cambridge, UK; Cat No. ab6006), diluted 1:2000 in DPBS Blocking buffer {DPBS and 5% w/v casein (Wako Pure Chemical Industries Ltd., Japan, Cat. No. 030-01505)}. The plates were flipped empty as before banged and washed 5 times, to remove excess unbound enzyme conjugate. The presence of bound conjugate was revealed by addition of 100 µl/well substrate solution [40 mM 2, 2-azino-bis (3-ethylbenethiazoline-6-sulphonic acid) diammonium salt (ABTS) and 0.01% (v/v) hydrogen perioxide (H_2O_2) in 50 mM citrate buffer, pH 4.0] to each

University of Ghana http://ugspace.ug.edu.gh

well. The plate was shaken briefly on a plate shaker and kept covered in the dark at room temperature to allow colour development. The colourless substrate solution changed to green in wells with bound enzyme conjugate complex, which indicates positive reaction. Wells with negative reaction remained colourless. The optical densities (OD) were read at 414 nm using micro-plate reader (Thermo Labsystems Multiskan Ascent, Model No. 354).

University of Ghana http://ugspace.ug.edu.gh

CHAPTER FOUR

IDENTIFICATION OF MALARIA INFECTED AND UNINFECTED SUBJECTS IN AN ENDEMIC POPULATION

Even though various efforts have been made to control malaria in developing countries, the reduction in morbidity and mortality caused by the disease is less than expected (Wogu *et al*., 2013). Limitations of current major diagnostic tests are a major drawback to the control efforts. For example, most of the tests are invasive, requiring the use of blood, and are not userfriendly for home-based management of malaria.

Today, the application of MAbs in immunochromatographic tests to detect parasite antigens in tissue fluids, is fast gaining grounds in improved diagnostic tools for malaria, however, these tools are all invasive because they are blood-based. Even though *Plasmodium* antigens and anti-malaria proteins have been detected in the urine of infected individuals and are considered promising for development of diagnostic tools for malaria (Rodriguez-del Valle *et al*., 1991; Katzin *et al*., 1988; Howard *et al*., 2007), they are yet to be employed in a MAb-based immunochromatographic tests (ICTs) for diagnosis of the disease. In view of this, this chapter of the thesis research initiated the studies towards the development of MAbs for diagnosis of malaria, using *Plasmodium* parasite antigens extracted from the urine of infected individuals.

In order to identify and select infected individuals from whom to obtain antigen containing urine samples for extraction of antigen and development of the malaria-diagnostic MAbs therefore, the experiments reported in this chapter were conducted firstly with the aim of identifying malaria infected individuals in the endemic population.

University of Ghana http://ugspace.ug.edu.gh

Secondly, true negative control samples are required for ascertaining the reactivity and accuracy of the MAb-based test to be developed. To achieve this goal, therefore, this study was also conducted to identify malaria uninfected subjects in the endemic population in order to obtain true negative urine samples for ascertaining the accuracy (specificity and sensitivity) of the test and characterize its range of applicability in different geographic settings.

4.1.1 Objectives

1 Recruit study subjects and identify malaria infected and noninfected individuals by clinical diagnosis.

2 Examine blood and urine samples from study subjects to differentiate between malaria infected and non infected individuals

4.2.0 MATERIALS AND METHODS

4.2.1 Study Subject Clinical History and Baseline Characteristics

The study site Kpone-on-Sea, is described in Chapter 3, section 3.1.1. The clinical history of each study participant together with age, sex and body temperature were taken before sample collection. Briefly, subjects were asked if they had taken any antimalarial chemotherapy and those who responded yes were exempted from the study. Following this, the body (axillary) temperature of subjects was taken using a digital thermometer to ascertain their febrile statuses before urine sample collection. With respect to this, the digital thermometer was placed in the ampit of each participant until temperature reading had become stable. The thermometer was then withdrawn, and the temperature reading was noted and recorded. Finally, parameters such as age and sex of participants were also obtained and duly recorded.

University of Ghana http://ugspace.ug.edu.gh

4.2.2 Urine Sample Collection and Analysis

4.2.2.1 Urine analysis by Multistix 10 SG reagent strips for biochemical characteristics

About 20-150 ml of fresh clean catch urine was collected from each study participant in the period between 08:00 and 14:00 hours Greenwich Mean Time (GMT) into a 150 ml urine container before administration of anti-malarial chemotherapy. Immediately after collection, a preliminary urine analysis was carried out on the samples using the Multistix 10 SG reagent strips (Miles Diagnostics, Elkhart, Ind.). Briefly, the urine was mixed and a urine test strip was dipped into each sample according to the manufacturer's specification. The strip was then marched with a measuring chart on the strip container to determine the presence and amounts of malaria biomarkers, such as glucose, ketones and bilirubin. The samples were then transported to the laboratory at the Kpone Health Centre within 30 min on ice in ice-chest and then stored in a deep freezer at -20^{o}C for further examination and analysis. The urine samples were later retrieved from the freezer, and then transported again on ice in-chest to the main laboratories at the School of Public Health and the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, where part of each sample was aliquoted into a 14 ml Falcon centrifuge tube and then stored frozen at -20^{o}C till used.

4.2.3 Blood samples collection and analysis

4.2.3.1 Examination of blood samples by blood smear microscopy

The fourth finger of each subject was pricked with a lancet to let a drop of blood onto a glass microscope slide at each end of a glass microscope slide. The blood drop was spread into a thin film on one end, and a thick film on the other end of the slide, using the edge of a second

University of Ghana http://ugspace.ug.edu.gh

microscope slide. The blood smear was allowed to air dry and then dipped briefly in absolute methanol to fix it onto the slide. The slide was placed in a glass staining jar containing 5% giemsa stain for 30 min and then washed gently in tap water. Each slide was air dried for at least 30 min and a drop of immersion oil was dispensed on the stained blood smear preparation to enhance visibility. The thin blood film preparation was then examined under the ordinary light microscope for parasite shape and size to ascertain parasite species. The thick blood film preparation however was examined to determine parasite density/ load. Also, in low parasite density cases, the thick blood smear was used in microscopic diagnosis to enhance sensitivity.

Plasmodium parasite density was estimated as the number of parasites per unit volume of blood (e.g. 6,000 paras./µl). In this respect, parasites on a stained thick film were counted per 200 leukocytes, and then multiplied by an assumed standard count of 8000 leukocytes/µl (WHO, 2010).

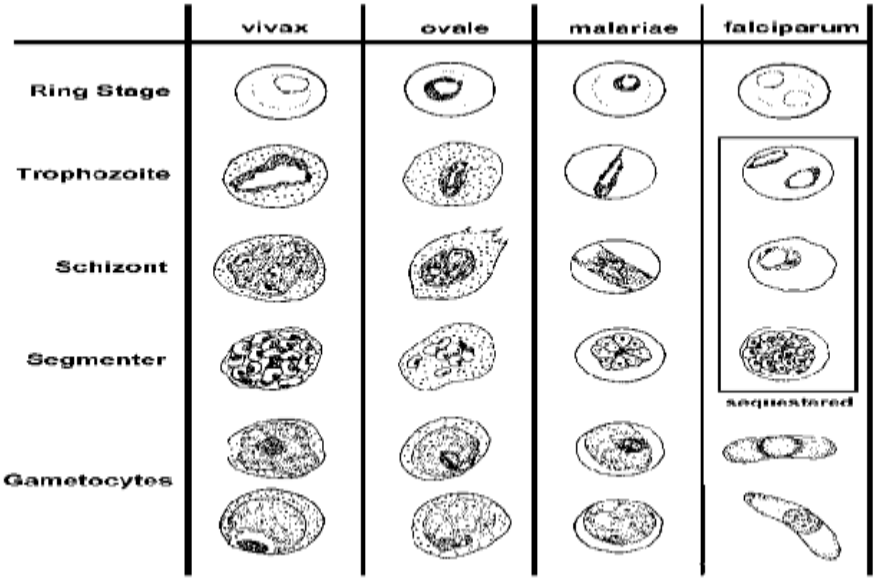


Figure 5 Morphological views of the various stages of the four (4) main human malaria parasite species

University of Ghana http://ugspace.ug.edu.gh

4.2.3.2 Analysis of blood samples by micro hematocrit centrifugation

Finger prick blood from each individual was also analyzed by the hematocrit system to determine hemoglobin concentration as an index of anemia as described by (Klee *et al*., 2000) with modification. Briefly, finger pricked blood from each subject was sampled into an anticoagulant coated (heparinized) hematocrit micro-capillary tube until it was 100 mm in height. The end of the tube that has not been contaminated by blood was then sealed with sealing wax. Each tube was inverted 10 times to allow the anticoagulant to mix with the blood sample thoroughly. The blood was then centrifuged using the The Kubota Model 3100 micro-hematocrit centrifuge (Kubota Corporation, Tokyo, Japan), at 15 000 x g for 5 min to obtain a 3 layered column of cells. The bottom opaque red layer of blood cells, representing the packed cell volume, a thin middle layer representing the buffy coat which comprised white blood cells and platelets, with the topmost transparent layer being blood plasma as represented in the Figure 6a and b below. The height of the packed red blood cell volume (PCV) was then read as a percentage of the total of 100 mm and the value obtained then divided by 3 to obtain the hemoglobin concentration of each blood sample.

University of Ghana http://ugspace.ug.edu.gh

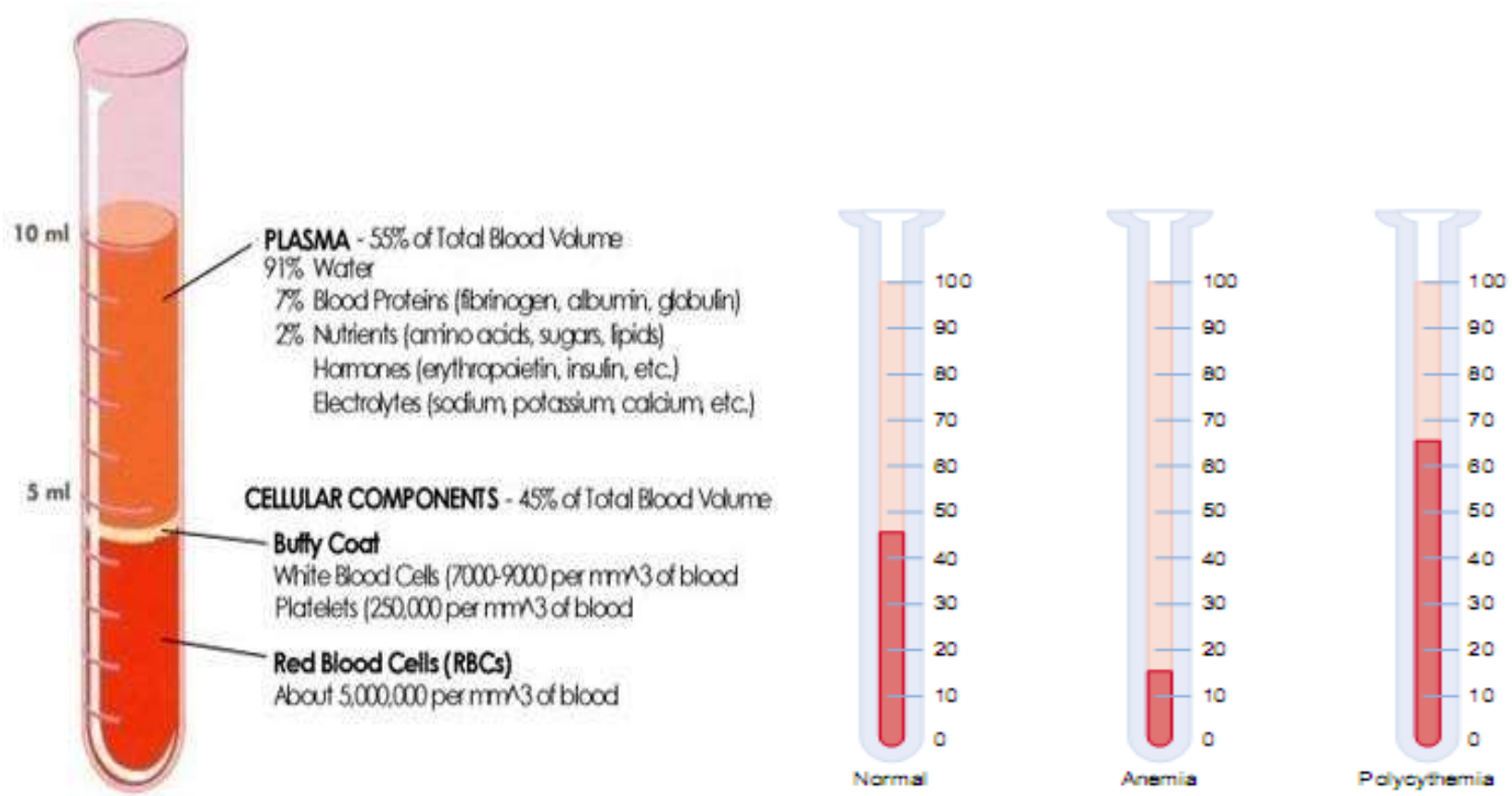


Figure 6: Analysis of blood by hematocrit system for haemoglobin concentration

- Children: 11 to 13 gm/dL
- Adult males: 14 to 18 gm/dL
- Adult women: 12 to 16 gm/dL

University of Ghana http://ugspace.ug.edu.gh

4.2.3.3 Analysis of blood samples by the quantitative buffy coat (QBC) method

To ascertain the accuracy of microscopic examination (quality control), finger prick blood samples were collected from 143 patients during blood smear preparation into QBC capillary tubes pre-coated with a fluorescent dye (FD). Tubes were inverted 5 times to allow FD to mix with the blood and stain any *Plasmodium* parasites present. Tubes were centrifuged at 10, 000 x g for 5 min and then placed in a QBC reader to measure the level of the component hemoglobin, platelets, leukocytes, etc. Each of the tubes was finally examined under a fluorescent microscope for *Plasmodium* parasites, which if present, were visible as light green fluorescent grains.

The Parasite-negative QBC capillary blood samples on the other hand, appeared dark under fluorescent microscope.

4.3.0 RESULTS

4.3.1 Characterization of *Plasmodium falciparum* infected subjects from a malaria infected population

In order to select *Plasmodium* infected subjects to be included in the study from the total population, the clinical history and some demographic characteristics of individuals from the community were taken to ascertain their suitability for the study. Also, blood samples from these individuals from the community were examined by blood smear microscopy, Quantitative Buffy Coat (QBC) test and hematocrit centrifugation for determination of their status of infection and blood haemoglobin concentration. Furthermore, aliquots of urine samples from study subjects were examined by the BioRad protein assay for protein concentration determination as described in section 5.2.5. The data from the various experiments and measurements performed were analyzed individually and in combination to ascertain their overall impact on malaria infection outcomes and suitability of inclusion in the study. The following tables and figures therefore

University of Ghana http://ugspace.ug.edu.gh

summarize the results of the analyses of the findings made in the various experiments conducted in this chapter.

4.3.1.1 Study Subject Clinical History and Baseline Characteristics

Table 3 and Figure 7 below, summarizes the results of the analyses of the data collected from the clinical history, hematocrit centrifugation and malaria microscopy. As shown, 1262 individuals were recruited in the study. This comprised 775 (61.41%) females and 487 (38.59%) males. In all, 587/1262 (46.51%) of the participants recruited were malaria positive, of whom 321/1262 (25.44%) were females. Also, of the 587 infected population, 321 (54.68%) were females.

In all, 479 individuals representing 37.96% of the total sample population had fever (Axillary temperature ≥ 37.5^{o}C). A significantly higher proportion (358/479) of the febrile study participants, representing 74.74% or 28.37% (358/1262) were malaria positive (P = 0.0001) by microscopy, thus indicating that malaria is associated with fever among the study participants. Furthermore, 655 of the study participants representing 51.90% of the total sample population had anaemia. Out of this, 291 individuals representing 23.06% (291/1262) or 44.42% (291/655) had malaria while the simple majority (364/1262) representing 28.84% or 55.58% (364/655) were negative by microscopy. Pearson Chi-Square analysis of this results indicated that anaemia in the subjects is not associated with malaria (P = 0.173). Also, generally, relative to age, a majority, 618 (48.97%) of the study participants were older than 15 years old, while the 11-15 years age group of individuals formed the minority of 156 (12.36%).

University of Ghana http://ugspace.ug.edu.gh

Table 3: Study Subject Clinical History and Baseline Characteristics

Total Number of Individuals that have the Given Condition or Parameter	Sex		Total no (%)
	Male {No (%)}	Female {No (%)}	1262 (100)
No. of individuals recruited	487 (38.59)	775 (61.41)	1262 (100)
Malaria positive	266 (21.08)	321 (25.44)	587 (46.51)
Malaria negative	221 (17.51)	454 (35.97)	675 (53.47)
Individuals with fever (**Axil temp ≥ 37.5**)	222 (17.59)	257 (20.36)	479 (37.96)
Individuals without fever (**Axil temp < 37.5**)	255 (20.21)	528 (41.84)	783 (62.04)
Malaria positives with fever	174 (13.79)	184 (14.58)	358 (28.37)
Malaria negatives with fever	48 (3.80)	73 (5.78)	121 (9.59)
Individuals with anaemia	244 (19.33)	411 (32.57)	655 (51.90)
Individuals without anaemia	243 (19.26)	364 (28.84)	607 (48.09)
Malaria positives with anaemia	128 (10.14)	163 (12.92)	291 (23.06)
Malaria negatives with anaemia	112 (8.87)	242 (19.18)	364 (28.84)
Individuals < 6 years of age	118 (9.35)	108 (8.56)	226 (17.91)
Individuals from 6–10 years old	116 (9.19)	146 (11.57)	262 (20.76)
Individuals from 11-15 years old	86 (6.81)	70 (5.55)	156 (12.36)
Individuals >15 years of age	167 (13.23)	451 (35.74)	618 (48.97)
Malaria positives <6 years old	64 (5.07)	56 (4.44)	120 (9.51)
Malaria positives 6-10 years old	77 (6.10)	81 (6.42)	158 (12.52)
Malaria positives 11-15 years old	55 (4.36)	33 (2.61)	88 (6.97)
Malaria positives > 15 years old	70 (5.55)	151 (11.97)	221 (17.51)

4.3.1.2 Association between Malaria and Sex of Study Participants

Table 4 is a summary of the result of the determination of the association between malaria and sex of study participants. According to the results, 54.7% (321) out of the 587 individuals with malaria parasite infection are females. Pearson Chi-Square analysis of the results indicated that malaria is strongly associated with sex among the study participants (P = 0.0001).

University of Ghana http://ugspace.ug.edu.gh

Table 4: Association between Malaria and Sex

Malaria Infection Rates		Sex		Total
		Female (F)	Male (M)	
Malaria Negative Individuals (0)	Count	454	221	675
	% within Malaria Infection	67.3%	32.7%	100.0%
	% within Same Sex	58.6%	45.4%	53.5%
Malaria Positive Individuals (1)	Count	321	266	587
	% within Malaria Infection	54.7%	45.3%	100.0%
	% within Same Sex	41.4%	54.6%	46.5%
Total (0 + 1)	Count	775	487	1262
	% within Malaria Infection	61.4%	38.6%	100.0%
	% within Both Sex	100.0%	100.0%	100.0%

P-value = 0.0001

4.3.1.3 Prevalence of Malaria among Study Subjects Relative to Age, Parasite Density and Grading

Figure 7 below describes the prevalence of malaria parasite infection among study subjects in relation to age, parasite density and grade of parasitemia. According to the results, when parasite density in the subjects is ranging from low to moderate (0 to 49,000 paras/µl) and grade of parasitemia is ranging from negative (-) to 2+, subjects of age above 15 yrs had the highest prevalence of infection (i.e., 59.1%, 49.8%, and 35.6%). However, when parasite density increases beyond 50,000 paras/µl of blood, with a corresponding grade of parasitemia raging from 3+ and above in the subjects, children of ages below 6 yrs and those within the 6 to 10 yrs age group had the highest prevalence of infection.

University of Ghana http://ugspace.ug.edu.gh

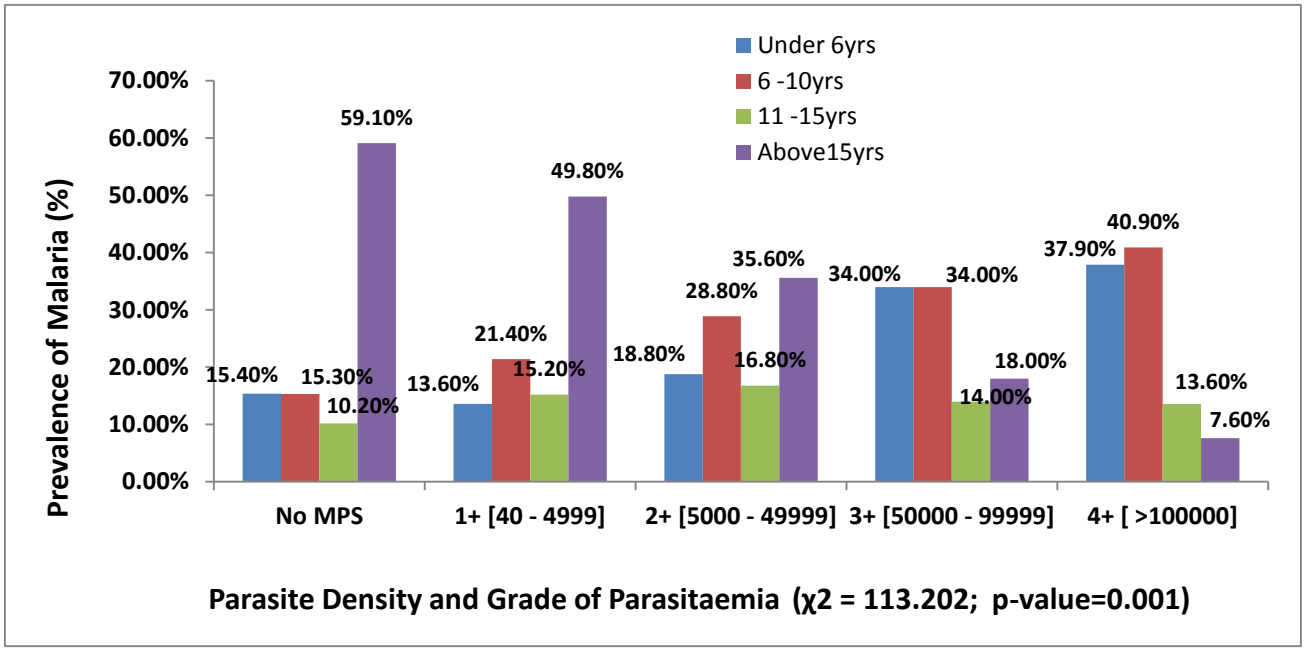


Figure 7: Prevalence of Malaria among Study Subjects Relative to Age, Parasite Density and Grading

4.3.1.4 Prevalence of Parasitaemia among Plasmodium Parasite Positive Subjects Relative of Age

Table 5 below summarizes the prevalence of *Plasmodium* parasite load among infected study participants in relation to age. According to the results severe (50,000 – 99,999 paras./ µl) and very severe (≥ 100,000 paras./ µl) malaria are more predominant in the youngest study participants than the older counterparts. The results show that, generally, *Plasmodium* parasite density is very heavy among study participants of ages from below 6 to 10 years of age and reduces as they grow older from 11 years and beyond. In respect of this therefore, children within the under 6 and 6-10 years of age have the highest prevalence, 37.90 and 40.90% respectively, of

University of Ghana http://ugspace.ug.edu.gh

heaviest parasite density. Furthermore, the results show that study participants of age above 15 years have the highest prevalence (49.80%) of low grade parasitaemia as compared to their counterparts within the younger age groups. In all, the results show that the prevalence of *Plasmodium* parasite infection among the study subjects is associated with age ($P = 0.001$).

Table 5: Prevalence of Parasitaemia among *Plasmodium* Parasite Positive Subjects Relative to Age

Parasite density Parasites/ul	**The number of individuals harbouring parasites (Parasite Prevalence) in relation to age**			
	below 6 years	**6-10 years**	**11-15 years**	**Above 15 years**
40 – 4,999	13.60%	21.40%	15.20%	49.80%
5,000 – 49,999	18.80%	28.80%	16.80%	35.60%
50,000 – 99,999	34.00%	34.00%	14.00%	18.00%
≥ 100,000	37.90%	40.90%	13.60%	7.60%

University of Ghana http://ugspace.ug.edu.gh

4.3.1.5 Prevalence of Malaria in Relation to Age of Study Participants

The results (Figure 8), showed that the prevalence of *Plasmodium* parasite infection was 53.10% in children below 6 years. It then increased to the highest peak of 60.31% in the 6-10 years age group, before reducing with increasing age of participants to 56.41% among individuals within the 11-15 age category, and finally, to 35.76% in individuals of age above 15 years. The results also showed that the prevalence of the disease among participants of age from 6 to 15 years was higher than that in children below 6 years.

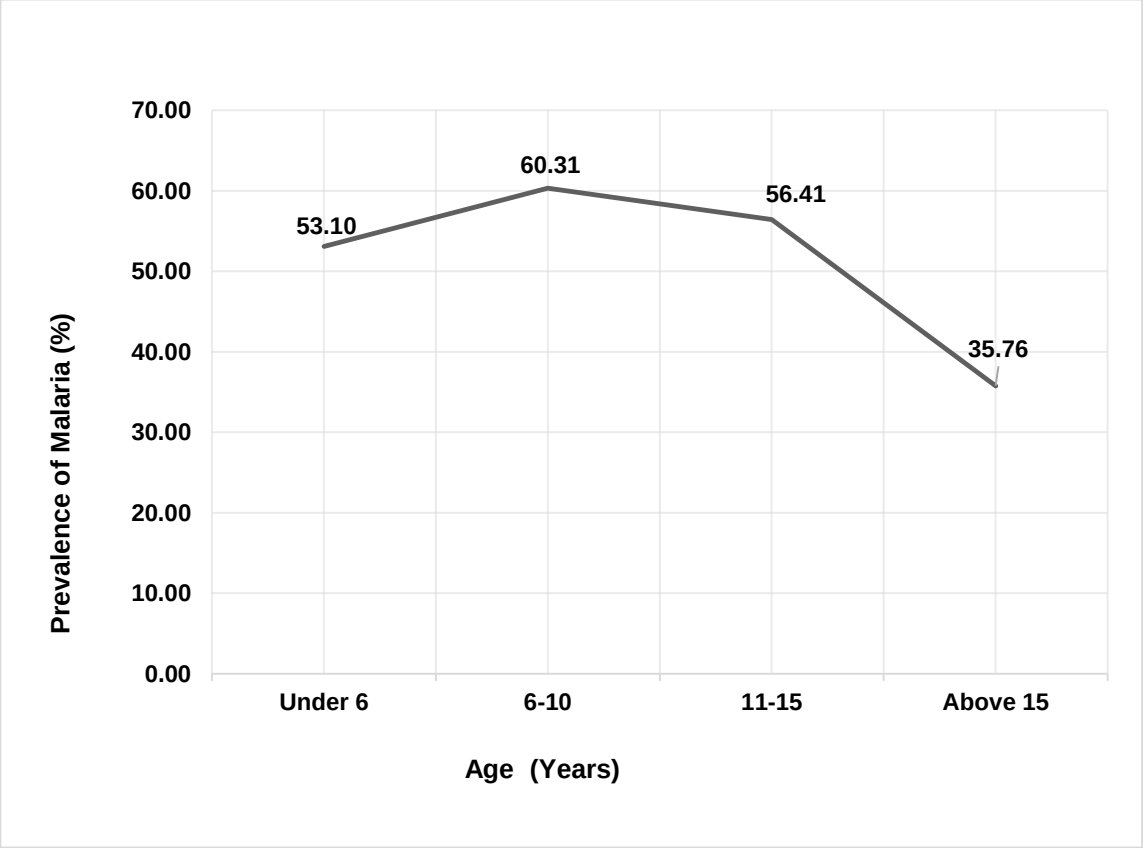


Figure 8: Prevalence of malaria among study participants in relation to age

University of Ghana http://ugspace.ug.edu.gh

4.3.1.6 Variation in Mean Parasite Density Among Malaria Positive Subjects in Relation to Age

Figure 9 below summarizes the variation in mean parasite density in malaria positive individuals in relation to age. As shown, the mean parasite load in malaria positive subjects reduces with increase in age of subjects from below 6 years to 15 yrs and above. Also, the reduction in mean parasite density among study subjects relative to age is linear. Furthermore, in conformity to the normal kinetics of malaria infection, children below 6 yrs harbor the highest mean parasite load of 52,064 paras/µl of blood whilst individuals of age above 15 yrs have the least parasite density of 9,600 paras/µl of blood.

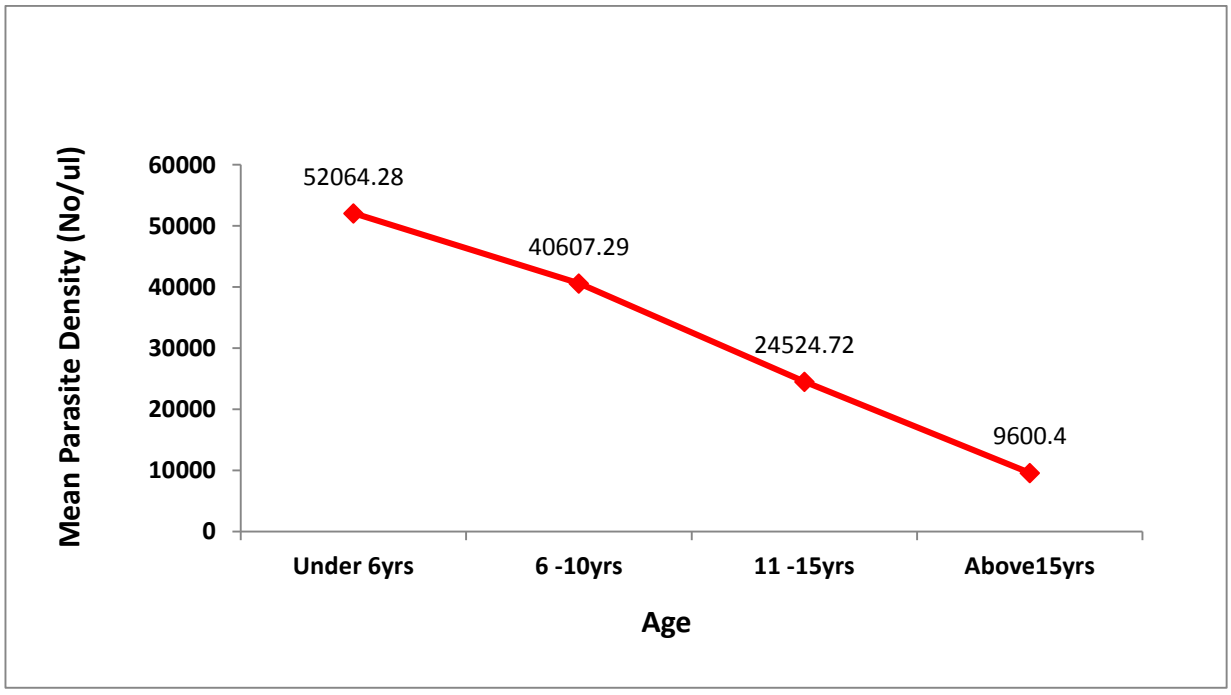


Figure 9: Variation of Mean Parasite Density Among Malaria Positive Subjects in Relation to Age

University of Ghana http://ugspace.ug.edu.gh

4.3.1.7 Association between Malaria and Anaemia Among Study Participants

Table 6 below summarizes the association between the prevalence of malaria and anaemia among the study participants. The results show that 53.9% (364/675) of the malaria negative individuals have anaemia while a considerably lower percentage (49.6%) of the malaria positive subjects (291/587) than expected have anaemia. A Pearson's Chi-Square test of significance of the relationships of these conditions shows that malaria is not associated with anaemia among the study subjects (P = 0.173).

Table 6: Association Between Malaria and Anaemia

Prevalence Rates		Anaemia		Total
		Negative (0)	Positive (1)	
Malaria Negative Individuals (0)	Count	311	364	675
	% within Malaria Infection	46.1%	53.9%	100.0%
	% within Anaemia	51.2%	55.6%	53.5%
Malaria Positive Individuals (1)	Count	296	291	587
	% within Malaria Infection	50.4%	49.6%	100.0%
	% within Anaemia	48.8%	44.4%	46.5%
Total (0 + 1)	Count	607	655	1262
	% within Malaria Infection	48.1%	51.9%	100.0%
	% within Anaemia	100.0%	100.0%	100.0%

P-value = 0.173

University of Ghana http://ugspace.ug.edu.gh

4.3.1.8 Variation of body temperature in different age categories of malaria infected and uninfected study subjects

As shown in Figure 10, the mean body temperature of malaria positive subjects in different age groups was generally above 37.5^{o}C. The temperatures ranged from 37.9^{o}C in subjects below 6 years of age to 37.5 ^{o}C in subjects above 15 years of age. Fever in malaria positive individuals rose sharply from 37.9^{o}C in children under 6 yrs age group, to the maximum value of 38.2^{o}C in children within the 6-10 years age group before decreasing gradually to the minimum value in subjects above 15 years of age.

The results as indicated in the figure, also showed that whereas all malaria positive subjects had fever (mean body temp > 37.5 ^{o}C) their malaria negative counterparts had normal body temperatures, ranging from a maximum of 37.11^{o}C in children under 6 years to a minimum of 36.97^{o}C in subjects above 15 years of age. Furthermore, the variation of fever among the study participants in relation to age, as shown in Figure 10, is similar to that in the prevalence of malaria among study participants in relation to age (Figure 8). A Pearson's Chi-Square test of significance of the relationship between malaria and fever (Table 7), showed that malaria was significantly associated with fever among the study participants (P = 0.0001).

University of Ghana http://ugspace.ug.edu.gh

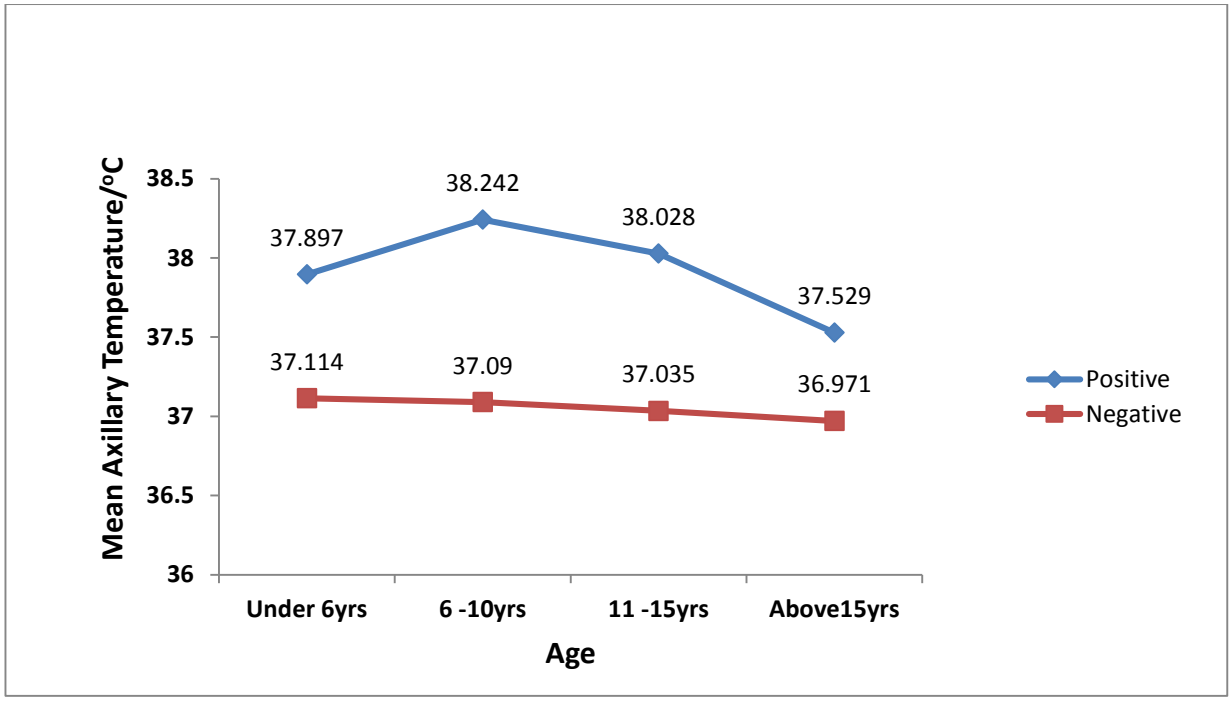


Figure 10: Variation of Mean body (Axillary) Temperature of Study Subjects in Relation to Malaria Infection and Age

University of Ghana http://ugspace.ug.edu.gh

4.3.1.9 Association between Malaria and Fever Among Study Participants

The association between malaria and fever among the study participants has been cross-tabulated in Table 7 below. Analysis of the results by Pearson's Chi-Square test of significance indicated that malaria is significantly associated with fever (*P-value* = 0.0001).

Table 7: Association between malaria and fever

Prevalence Rates		Fever		Total
		Negative (0)	Positive (1)	
Malaria Negative Individuals (0)	Count	554	121	675
	% within Malaria Infection	82.1%	17.9%	100.0%
	% within Fever	70.8%	25.3%	53.5%
Malaria Positive Individuals (1)	Count	229	358	587
	% within Malaria Infection	39.0%	61.0%	100.0%
	% within Fever	29.2%	74.7%	46.5%
Total (0 + 1)	Count	783	479	1262
	% within Malaria Infection	62.0%	38.0%	100.0%
	% within Fever	100.0%	100.0%	100.0%

4.4.0 DISCUSSION

The studies conducted in this chapter aimed at characterizing the prevalence of malaria in an endemic population using *Plasmodium* infection outcomes with a view to identifying and selecting infected and uninfected human urine samples for generation of monoclonal antibodies towards development of rapid urine-based test for diagnosis of malaria.

Prevalence of malaria among study participants

In the studies described in this chapter, the prevalence of malaria was determined in the study population to be 46.51%. Even though this current prevalence was lower than that

University of Ghana http://ugspace.ug.edu.gh

obtained in a pilot study (52.0%) conducted 9 years earlier (McKakpo *et al*., 2006; *data unpublished*) the difference was not statistically significant (*P*= 1.000).

There was significantly (*P* = 0.001) higher prevalence (25.44%) of malaria among females in the study community than in their male counterparts (21.08%). A similar observation was made by other researchers like (Ayele *et al*., 2012), who stated that "generally, malaria parasite prevalence differed between age and gender with the highest prevalence occurring in children and females".

In spite of these observations, other research findings, have suggested that when given equal exposure, both males and females are equally susceptible to malaria infection, except for pregnant women who are more vulnerable because of the reduction in immunity that the body undergoes in order to retain pregnancy (Steketee *et al*., 2001). In view of this, it is worth stating that the significantly higher prevalence of malaria obtained among females within the study population could be due to factors whose determination was beyond the scope of the study.

Association between malaria and age among study participants

Understanding the relationship between malaria burden and age is important in selecting high-risk age groups of individuals in whom targeted intervention measures should be focused in order to avoid malaria epidemics (O'Meara *et al*., 2008). In line with this observation, the association of malaria with age was determined among the study population from the Kpone-on-Sea (KOS) endemic area, as part of the efforts to identify infected and uninfected urine samples for antigen extraction.

Analysis of the results (Figure 7; Table 5) showed that the prevalence of malaria parasite density among the participants was significantly associated with age (*P* = 0.0001). According to the results, as parasite density reduced from >100,000 to 40 parasites/ µl, the

University of Ghana http://ugspace.ug.edu.gh

prevalence of younger aged participants harbouring malaria parasites also reduced from 37.9% to 13.6% for children under 6 years age, and from 40.9 to 21.4% for children within the 6-10 years old age group. Conversely, as parasite density reduced, the percentage of older study participants harbouring parasites increased from 13.6 to 15.2% among the 11-15 year old individuals and from 7.6 to 49.8% in individuals of age above 15 years.

This observation confirmed what had been reported earlier among individuals living in other endemic areas that malaria parasite infection is severest in young children, and reduces with increase in age (Doolan *et al*., 2009). The observation may be due to the fact that the participants from 5 months, to about 6 years of age, probably lacked adequate immune protection against malaria, which therefore, led to the high prevalence of parasitaemia among them.

This trend was repeated, among participants within the 6-10 year old age group probably because, the reduction in malaria transmission following intervention programmes, prevented development of adequate immunity in these children before 10 years (Doolan *et al*., 2009). However, beyond 10 years of age, the participants probably started developing protective immunity against the disease. It was therefore normal for the prevalence of parasitaemia to start reducing in these individuals as they advanced in age from 11 years and beyond.

The results (Figure 8), also showed that the prevalence of malaria was 53.10% in children below 6 years, increased to 60.31% in the 6-10 years age group, before finally reducing with increasing age of participants to 35.76% in individuals of age above 15 years. These findings were similar to that obtained by other authors who stated that higher risks of malaria infection were observed in children, with peak prevalence occurring among children of ages between 6 and 10 years (Ayele *et al*., 2012). The results confirmed what other authors (Doolan *et al*., 2009) have reported that in areas where intervention measures are used to

University of Ghana http://ugspace.ug.edu.gh

reduce transmission, the development of immunity against infection is delayed, causing the peak prevalence of the disease to shift to a higher age group of individuals, until immunity is developed, when the prevalence and other infection outcomes begin to fall with increasing age of the endemic population.

As reviewed earlier in the literature (Tchouassi *et al*., 2012), KOS experienced a considerable malaria intervention using ITN, and IPT for pregnant women just before and during the time of data collection. It is highly likely that these intervention programmes caused the peak of prevalence to shift to the 6-10 year age group.

Finally, the results (Figure 7) showed that mean parasite density (MPD) among the study participants was associated with age. The MPD was highest (52,064 parasites/ µl) among the children under age 6 and decreased lineally with increasing age of participants to the lowest density of 9,600 parasites/ µl in participants of age above 15 years. These trend of age-dependent variation in parasitaemia could be because the children under 6 years of age had the lowest adaptive immunity against malaria parasite infections and therefore had the least ability to reduce parasite load (Stauffer and Fischer, 2003; Doolan *et al*., 2009). The older aged participants on the other hand, had probably developed a more heightened immunity against the disease which increased as they grew older and older and therefore were better able to resist the multiplication of parasites within them, hence the reducing MPD with increasing age as shown by the results.

Association between malaria and anaemia among study participants

The association of malaria with anaemia in the study participants was determined. As reviewed by Douglas *et al*. (2013), acute *Plasmodium* parasite infection causes the removal of parasitized and unparasitized RBCs from the blood circulation through various mechanisms which result in anaemia (Douglas *et al*., 2013).

University of Ghana http://ugspace.ug.edu.gh

In respect of this, anaemia is regarded as a common manifestation of *Plasmodium* parasite infection. Also, the World Health Organization (WHO) and the Roll Back Malaria (RBM) partnership recommended that anaemia should be used as an additional indicator for monitoring malaria burden at the community level and interventions on the national scale (Reviewed by Santana-Morales *et al*., 2013).

Following these reports, the association of malaria with anaemia among the study participants was determined to ascertain whether or not the prevalence of anaemia in the study community could be used to characterize malaria.

According to the results (Table 3), the overall prevalence of anaemia in the study population was 51.90% (655/1262). Out of this, 291 individuals representing 23.06% (291/1262) had malaria and 364 (28.84%) were malaria negative as determined by blood smear microscopy. With the common knowledge that malaria leads to anaemia in infected individuals, it was expected that the prevalence of anaemia among the malaria positive individuals as recorded in this study would be significantly higher than that among the malaria negative counterparts, however, this was not so. A chi-square test of significance showed that there was no association between malaria and anaemia in this study ($P = 0.173$). Also, contrary to what was expected, a higher percentage, 52.40% (354/675) of malaria negative individuals as determined by microscopy had anaemia instead of the converse.

A similar observation was made in Ethiopia, sub-Saharan Africa, where Santana-Morales *et al*. (2013) reviewed that a numerically higher number (50.20%) of the study participants with anaemia did not have malaria.

However, recognizing the fact that anaemia is a complex condition which is not caused by only malaria, it would be explained that anaemia among the study participants would probably be multifactorial, and might have been partly due to factors such as hookworm infection and malnutrition, all of which are endemic in the study population. If this latter

University of Ghana http://ugspace.ug.edu.gh

explanation were right, then this finding only suggested that in the absence of malaria the prevalence of anaemia in the study population could reduce by about 50%, and where there is malaria, the number of people suffering from anaemia could double (Santana-Morales *et al*., 2013). Indeed, although it has been recommended that anaemia could be used as an additional indicator for the presence of malaria (WHO Report, 2005) it was also advised that in order to accurately establish this, the baseline haemoglobin levels in each population should be known while taking into consideration the nutritional status as well as the infection by other parasites such as hookworms which also depletes RBCs and cause anaemia and other haemoglobin disorders in endemic populations (Santana-Morales *et al*., 2013).

Furthermore, several studies conducted by (Steenkeste *et al*., 2010; Okell *et al*., 2009) have shown that microscopy is not sensitive enough, and often leads to underestimation of malaria prevalence.. Indeed, in a more recent study, using the improved PCR technique that enables detection of sub-microscopic *Plasmodium* parasite infection, Mahajan *et al*. (2012) reported that approximately 21% of the 101 blood samples that tested negative by blood smear microscopy, tested positive by the improved PCR-technique. It is however interesting to note that the 101 samples being referred to above, were collected from our KOS study community, prepared and examined microscopically by the same qualified technicians who assisted in the processing and analysis of the 1262 blood samples whose results are being discussed.

In view of the foregoing, it would be tenable to suggest that the observation made above that there was no association between malaria and anaemia was also probably because the prevalence of malaria as determined in the sample population might have been underestimated by blood smear microscopy. The polymerase chain reaction (PCR) technique was, however, not used to ascertain the association between malaria and anaemia, because of lack of logistics.

University of Ghana http://ugspace.ug.edu.gh

Association between malaria and fever among study participants

The use of fever, that is, increase in body (axillary) temperature from 37.5ºC and above, as a marker for diagnosis of malaria parasite infection forms part of the proposed recommendations of the Integrated Management of Childhood Illness (IMCI) (*Reviewed by* Okiro and Snow, 2010). To determine whether fever among the study participants was caused by malaria parasite infection or not, therefore, the association of malaria parasite infection with fever was estimated.

Fever, pyrexia or increase in body (axillary) temperature of humans above the normal range (36.5 - 37.5ºC), is said to be caused by inflammation (Laupland, 2009; Wikipedia, 2014). Inflammation is a complex innate immunological reaction mounted by the body in response to injury or tissue damage by disease causing organisms, including malaria parasites.

According to the results (Figure 10 and Table 7), the prevalence of fever among the study participants was significantly associated with *Plasmodium* parasite infection (P = 0.0001). Also, the results (Figure 10), showed that the mean body temperature of all the malaria positive subjects across all the various age groups was greater than 37.5ºC. In these malaria positive subjects, the prevalence of the disease was 61.0% (358/587). Since malaria leads to increase in body temperature, it is highly probable that fever among the study participants was due to malaria parasite infections as observed from the results. Further studies are however required to rule out the possible involvement of other concomitant infections like those caused by bacteria, viruses and measles (ECDPC Report 2013; CDC and WHO, 1998) which can also contribute to fever in the participants. Such studies were however not carried out because they were not part of the study objectives.

The results (Figure 10) also showed that the variation in body temperature among the study participants with respect to age, was similar to the variation in the prevalence of malaria with respect to age (Figure 8).

University of Ghana http://ugspace.ug.edu.gh

Since malaria was suspected as the most likely cause of fever among the study participants, variation in the age-dependent prevalence of malaria could be the only conceivable factor influencing the age-dependent severity of fever among the study participants. Therefore, the similarity of the trend in the age-dependent variation of fever to that of the age-dependent variation of malaria prevalence, is another factor to support the statement that fever among the study subjects could most probably have been caused by malaria parasite infection.

Furthermore, the comparably lower and normal MBT for all malaria negative subjects (Figure 10), is once again, a factor to support the view that fever was caused by malaria parasite infection among the study participants. Since the malaria negative subjects did not have the disease, there was no expectation for fever among them.

The results (Figure 10) also showed that in both malaria infected and uninfected individuals generally, the MBT was higher for children and decreased with increasing age to adulthood. This observation is similar to that reported in other literature (Antranik, 2012) where it was explained that children have higher body temperatures because of their improperly developed thermoregulatory mechanisms of the body, as well as immunity against inflammatory diseases. Therefore, as the children grow older, they develop better ability to regulate body temperature and better immunity against inflammatory infections and diseases hence, their body temperarture drops.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER FIVE

EXTRACTION AND CHARACTERIZATION OF BIOMARKERS OF MALARIA FROM INFECTED HUMAN URINE

5.1.0 INTRODUCTION (1)

Early diagnosis of malaria is important for prompt treatment needed to prevent complications and death, particularly, among pregnant women, children below 5 years of age and non-immune travelers (Ishengoma *et al*., 2011; Stauga *et al*., 2013). Accurate diagnosis of malaria could benefit from prior identification of specific biomarkers of the disease.

Biomarkers are measurable molecular, cellular or biochemical agents or alterations in tissues or body fluids that indicate the presence and or progression of disease, or exposure to harmful substances (Kim *et al*., 2010; Stauga *et al*., 2013).

Owing to their remarkable diagnostic potential, biomarkers are very useful in many aspects of health to detect infections; track the progression of disease; monitor drug delivery or metabolism; and also, to monitor drug efficacy, outcome of treatment or exposure to chemicals (Kim *et al*., 2010; Stauga *et al*., 2013). In respect of this, therefore, the development of tools for diagnosis of diseases has been largely dependent on the discovery of biomarkers of such diseases.

Efforts to disvover biomarkers of diseases, have largely involved the use of blood plasma esterases (Kim *et al*., 2010). Also, for malaria, several biomarkers have been discovered in blood plasma and serum, which include CPR, ICAM and hemozoin (Stauga *et al*., 2013). However, apart from being invasive, these biomarkers may not always correlate with the severity of the disease, which undermines the sensitivity and/ or specificity of most of the tests presently used for routine diagnosis of the disease (Suaga *et al*., 2013).

University of Ghana http://ugspace.ug.edu.gh

There is therefore a recognized need to expand and improve biomarker identification and quantification (Kim *et al*., 2010). The discovery and characterization of biomarkers of malaria, particularly, from the urine of infected individuals, is more advantageous because of several reasons. First, urine is the only biofluid that can be obtained in large quantities through non-invasive procedures (Decramer *et al*., 2008). Secondly, urine has been reported to contain several hundreds of biomarkers of diseases which can be explored for diagnostic purposes (Nolen *et al*., 2013). Also, urinary biomarkers are relatively more stable to rough handling and harsh storage conditions than other biomarkers (Nolen *et al*., 2013).

For these reasons therefore, the studies reported in this chapter were conducted to extract and characterize biomarkers of malaria from the urine of infected individuals.

5.1.1 Aim

The aim of the experiments conducted in this chapter was to isolate biomarkers of malaria from the urine of infected individuals and characterize them towards the generation of monoclonal antibodies for diagnosis of malaria.

5.1.1 (b) Specific Objectives

1) To characterize the biomarkers of malaria from infected human urine.
2) To characterize the association between urinary hepcidin, haemoglobin concentration and malaria parasitaemia.

5.2.0 MATERIALS AND METHODS

5.2.1 Characterization of the Sample Population for the Studies to Characterize Biomarkers of Malaria from Infected Urine

A cohort of 290 subjects out of the larger study population participated in the pilot study reported in this chapter. They comprised 103 children and 187 adults of whom 58 were pregnant women.

University of Ghana http://ugspace.ug.edu.gh

5.2.2 Determination of Malaria Infection Status of Study Participants

Thick and thin blood smears prepared from finger prick blood and read by microscopy was used to determine the presence or absence of malaria parasites as described in section 4.2.3.1. Also, malaria parasite density (parasitaemia) among parasite positive individual was calculated as described in section 4.2.3.1.

5.2.3 Determination of Blood Hemoglobin Concentration and the Presence or Absence of Anaemia

The Kubota Model 3100 micro-hematocrit centrifuge (Kubota Corporation, Tokyo, Japan) was used to determine packed cell volume of blood from which the concentration of haemoglobin in the blood of each participant was estimated as described in section 4.2.3.2 above. Also the presence or absence of anaemia was determined for each individual by comparing the haemoglobin concentration.

5.2.4 Extraction of *Plasmodium* Proteins from Malaria Positive Urine

The isolation of *Plasmodium* proteins from the urine of infected individuals has been illustrated by the flow diagram below, and was carried out using the "Farr Technique" protocol described by Bosompem *et al*. (1996*a*) with modification. The Farr Technique describes the process of precipitating antigens with 33-50% saturated ammonium sulphate solution $\{(NH_4)_2SO_4\}$ (Mayer, 2010).

Following aliquotting, the rest of the urine samples from positive malarial subjects, as determined by blood film microscopy, quantitative buffy coat test (QBC) and "CareStart" rapid diagnostic test for malaria, were pooled together in 3 litter conical flasks. While stirring, an equal volume of saturated ammonium sulphate solution $\{(NH_4)_2SO_4\}$ was then added to the content of the flask. The resulting solution in each conical flask was then placed in an ice-

University of Ghana http://ugspace.ug.edu.gh

box at 4ºC. The ice-box and urine solution mixture was then placed on an electronic magnetic stirrer (Stuart Scientific, United Kingdom) and then stirred till the urine-$(NH_4)_2SO_4$ mixture was cloudy, an indication that urinary proteins had been precipitated out. The urinary malaria protein precipitate was then poured into 50 ml Falcon centrifuge tubes and then taken through a series of centrifugation, re-suspension with distilled water, re-addition of 50% (v/v) saturated $(NH_4)_2SO_4$, stirring and re-centrifugation steps as illustrated by the flow-chart below, until the final urine pool 2J (UP2J), also called *Plasmodium* infected human urinary protein (PAgHU), was obtained. The final UP2J or PAgHU was then dialyzed with a cellulose dialysis tubing of membrane cuff-off pore size MWCO 12,400 (Sigma, D9527). Following dialysis, urinary *Plasmodium* antigen isolates were aliquotted into eppendorf micro-centrifuge tubes, examined for protein concentration using the BioRad protein assay technique (as described below) and then stored at -20ºC until used.

University of Ghana http://ugspace.ug.edu.gh

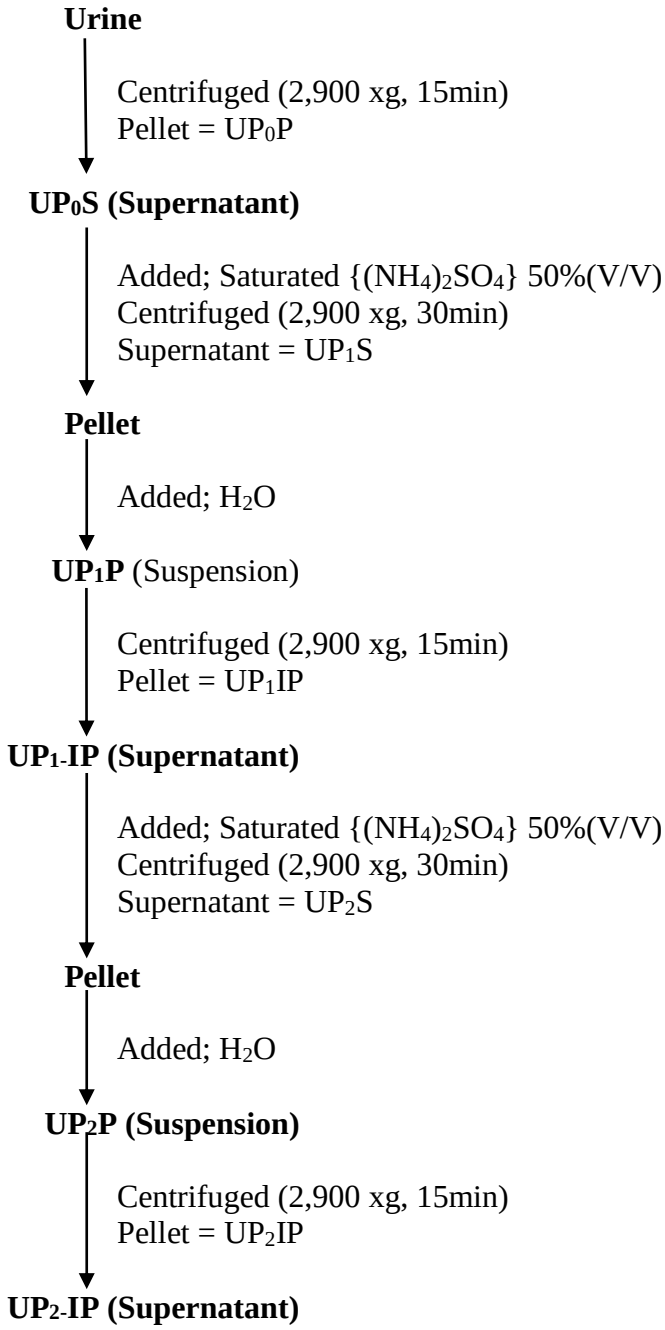


Figure 11: Flow chart for extraction of *Plasmodium* antigens from urine of infected individuals

University of Ghana http://ugspace.ug.edu.gh

5.2.5 Urine analysis by BioRad protein assay

The aliquotted urine samples were retrieved from storage at -20^{o}C and thawed and the pH was adjusted to between 7 and 9 to solubilize precipitates in them. Using the BioRad protein assay (Bio-Rad Laboratories 2000, CA 94547) and BSA standard reagents, the concentration of proteins in each urine sample was determined.

In carrying out the experiment, bovine serum albumen (BSA) standard reagent was titrated at 1:2 dilutions (v/v) with PBS into a flat-bottomed 96-well micro-titre plate. Aliquot of each urine sample was titrated similarly, alongside the BSA reagent. Ten microlitres of BioRad protein assay reagent diluted with de-ionized water according to the ratio, three (3) parts of BioRad protein assay reagent with one (1) part (v/v) of de-ionized water, was added to each well and incubated at room temperature for 5 min with gentle shaking. The optical density of the resulting reaction in each well was measured using the micro-plate reader. A semi logarithmic graph of OD versus BSA standard reagent concentration (C_n) was plotted and then used to estimate the concentration of protein in each urine sample by extrapolation from the curve of log C_n versus OD.

5.2.6 Test Principle for Detection of *Plasmodium* Antigens in Samples by Different Commercial Rapid Diagnostic kits:

The malaria rapid diagnostic test (RDT) cassette contains a membrane strip, which is pre-coated with mouse monoclonal antibodies (MAbs) specific to the histidine-rich protein 2 (HRP2) antigen of *P. falciparum* on the test line labelled *P.f* and/ or mouse MAbs specific to the pan-(genus) specific lactate dehydrogenase antigen (pLDH) of the four human malaria parasite species (*P. falciparum, P. vivax, P. malariae and P. ovale*) on the test line marked by Pan respectively. Included in the test kit is a hemolyzing buffer containing parasite (P.f or pLDH)- specific mouse MAb labelled with colloidal gold conjugate as a detectable marker.

University of Ghana http://ugspace.ug.edu.gh

The mixture of the hemolyzing buffer-mouse MAb colloidal gold conjugate specific to HRP2 or pLDH with the *Plasmodium* antigen in the blood or urine sample results in the binding of the antigen to the specific antibody conjugate (figure 5). They then move along the membrane to the test regions (*P.f and* Pan) and form a visible line as the antibody-antigen-antibody gold particle complex which is observed as a reddish-brown line with high degree of sensitivity and specificity. Both the test line and control line in the results window are not visible before application of the sample.

5.2.7 Detection of *Plasmodium* Antigens in Infected Urine, Blood and Cultured *P. falciparum Parasites* by Rapid Diagnostic Test

A rapid diagnostic test was carried out on aliquots of *Plasmodium* antigens (CPfAg) and (PAgHU) extracted from *in vitro* cultured 3D7 *P. falciparum parasites* and infected human urine respectively, according to the method described by Access Bio Inc (2008) with modification. Briefly, using 20-200µl ranged single channel finnipipette pipette (Thermo Fisher Scientific Incorporated, USA.), 20 µl aliquots of *Plasmodium* antigen extracts, and unprocessed urine samples from malaria negative and positive individuals were dispensed into the sample wells of rapid diagnostic test kit cassettes: : CareStart^{TM} Malaria Pf/Pv combo test kit (AccessBio Inc., New Jersey, USA); SD BIOLINE malaria antigen test kit (Standard Diagnostics Inc., Republic of Korea) or First response malaria antigen Pf (HRP2) detection rapid card test (Transnational Technologies Inc., United Kingdom) for testing. Drops of buffer containing *Plasmodium* parasite (HRP2 or LDH) specific mouse monoclonal antibody conjugated to colloidal gold particles was then added to the buffer well of each cassette according to the manufacturer's specification, and then allowed to develop for 15 min at room temperature (22–25 ^{o}C). Positive dipstick results showed a horizontal reddish brown line in

University of Ghana http://ugspace.ug.edu.gh

addition to a control line on each test strip in a distinct detection zone. Negative results showed only the positive control line.

During the analysis of blood, however, drops of blood were dispensed into the sample wells using the micropipettes included in the test kits according to the manufacturers' specifications. Also, the hemolyzing washing buffer that was added was to remove the hemoglobin and allow visualization of coloured lines as shown in the diagram below. Apart from these minor differences the test on blood samples was similar to that on urine samples.

5.2.8 Detection of Malaria Proteins in Infected Urinary and Cultured Antigen Extracts by SDS-PAGE

Electrophoresis of antigens was conducted using the sodium dodecyl sulfate (SDS) tris-glycine discontinuous buffer system described by Laemmli (1970). The ATTO Cooperation Slab Gel Apparatus (Bunkyo-ku, Tokyo, Japan) was used. The gel casting apparatus comprised a plastic clamp, a pair of glass slabs (one notched), a comb and U-shaped Teflon spacer. The gels were cast in a mold formed by the glass slabs assembled together and sealed with the U-shaped Teflon. The assembled apparatus stabilized by the base of the clamp holder, was mounted vertically on the working bench.

A 10% separating gel (30% acrylamide mix, 1.5M Tris pH8.8, 10% SDS, 10% ammonium persulfate, TEMED, and dH_20) with dimensions of 8 cm (length of separating distance) x 6 cm (width) x 0.75 mm (thickness) was cast by pouring the separating gel solution into the gel casting mold. It was overlaid gently with de-ionized water (dH_2O) to prevent the surface from cracking and allowed to polymerize. The dH_2O was poured off the polymerized gel and rinsed with excess de-ionized dH_2O. The remainder of the gel mold was filled with 5% stacking gel solution (30% acrylamide mix, 1.0M Tris pH 6.8, 10% SDS, 10% ammonium persulfate, and dH_20) after which the comb was inserted to create wells for loading of the

University of Ghana http://ugspace.ug.edu.gh

sample preparations. The gel was then placed upright in an electrophoresis chamber filled with electrode buffer (0.25 M trizma base, 0.192 M glycine, 0.1% SDS, pH 8.3, TEMED) in both upper and lower reservoirs. The samples including extracts from *Plasmodium* infected and non infected urine, cultured *P. falciparum* antigen extracts and *Plasmodium* MSP119 antigen, were diluted with an equal volume of sample buffer [0.5 M trizma base (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 0.02% bromophenol blue, 2.85% 2-mecaptoethanol] and then heated at 100^{o}C for 5 min. The samples were quickly chilled after heating and kept on ice until loading. The samples were loaded (15µg/well) into the wells alongside of a molecular weight marker (mwm) and run at a constant current of 70 mA until the bromophenol blue tracking dye had entered the separating gel. The current was then reduced to 40 mA and allowed to run until the dye had almost run out of the separating gel. After electrophoresis the glass slabs were cautiously separated and the gel was left attached to one slab. The stacking gel was cut off and discarded, and a small portion of the separating gel below the tracking dye was cut off to mark the front side of the gel followed by staining or western immunoblotting. Other gels were prepared without staining using different *Plasmodium* infected and non-infected urine (pos and neg) and used for western immunoblotting.

5.2.9 Coomassie Brilliant Blue R-250 Staining of Gel

The gel after electrophoresis, was gently placed in a container and stained with coomassie brilliant blue R-250 (CBB) solution [1% (w/v) CBB, 50% methanol, 10% Acetic acid] for 10 min with continuous rocking on an electronic shaker (Stuart Scientific-STR6). The stained gel was then de-stained in excess volume of de-staining solution-1 (40% methanol, 7% acetic acid) and then in de-staining solution-2 (5% methanol, 7% acetic acid). The gel was washed several times with excess de-staining solution 2 and then removed when

University of Ghana http://ugspace.ug.edu.gh

the background was clear and the protein bands visible (Appendix 1). The molecular weight (M_{wt}) of each protein band was then determined by comparison of its relative mobility (R_m) with those of several marker proteins of known molecular weight (M_{wt}). A semi logarithmic graph of the logarithms of M_{wt} of the protein standards as functions of the R_m values was plotted and the unknown M_{wt} of the samples was estimated using extrapolation from the curves of log M_{wt} versus R_m.

5.2.10 Identification of Biomarkers of Malaria by Western blotting Analysis

The gels were prepared as described above without staining. The protein bands in each gel were transferred onto PVDF membrane (Immobilon-P transfer membrane, Millipore Cooperation, Cat. No. IPVH00010) by electro-blotting using a transfer apparatus (Milliblot-Graphite Electroblotter 1, Millipore Cooperation), which had been previously immersed in transfer buffers. The PVDF membrane containing the transferred bands was then probed with different anti-*Plasmodium* monoclonal antibodies (MAbs) to identify the *Plasmodium* antigen in each band.

In carrying out the experiment, two pieces of blotting paper (9 x 7 cm) were placed on the anode pole and wetted with anode buffer I (0.3 M trizma base, 10% methanol, pH 10.4). Another blotting paper was wetted with anode buffer II (25 mM trizma base, 10% methanol, pH 10.4) and placed on them. The PVDF membrane pre-wetted in methanol to remove traces of air bubbles and render it hydrophilic, was rinsed in de-ionized water (dH_2O) and laid on the set of the anode buffer-wetted blotting paper. The gel containing the transferred protein bands was immediately placed on the PVDF membrane with face down. Two pieces of blotting paper wetted in cathode buffer (25 mM Trizma base, 192 mM glycine, 20% methanol, pH 9.4) was then placed carefully on the gel. A pipette was gently rolled over the blotting paper-PVDF membrane-gel-blotting paper layer to remove all traces of air

University of Ghana http://ugspace.ug.edu.gh

bubbles. The setup was then supplied with constant current of 200 mA for 55 min after covering with the cathode pole.

The PVDF membrane with the blotted proteins was rinsed in phosphate buffered saline (PBS) washing buffer and then incubated in blocking buffer [5% (w/v) skimmed milk in washing buffer] at 25^{o}C for 30 min. The membrane was transferred into a 1:50 dilution of anti-*Plasmodium falciparum* histidine rich protein 2 (*pf*HRP2) MAb and incubated at 25^{o}C for 2 h. The membrane was removed, rinsed once with washing buffer and then washed two times with washing buffer at 10 min intervals on a shaker at room temperature. The membrane was probed with horseradish peroxidase conjugated to goat anti-mouse IgM (Sigma, A8786) antibodies diluted 1:500 in blocking buffer. The membrane was incubated in substrate solution (100 mM phosphate buffer, pH 6.4) until blue-black bands were observed. The experiment was repeated several times with a different probing anti-*Plasmodium* antibody for each blot.

5.2.11 Characterization of urinary hepcidin from *Plasmodium* infected human urine

Urinary hepcidin was measured using the surface-enhanced laser desorption/ ionization time-of-flight mass spectrometry (SELDI-TOF MS) as described by Kemna *et al*. (2005). Urine samples were thawed, vortexed, and centrifuged at 12,000 rpm for 25 minutes. Seven microlitres (7 uL) of the supernatant of each ample was applied in triplicate (3 spots) on an NP20 Ciphergen ProteinChip® (CPC) and incubated in a moist chamber for 30 min. Each CPC was washed three times with high performance liquid chromatography (HPLC) grade water for 10 min to remove excess/ unbound proteins. The CPC arrays were air-dried, and 1.0 μL of alpha-cyano-4-hydroxy-cinnamic acid (CHCA, Ciphergen) in 50% acetonitrile and 0.25% trifluoroacetic acid was added to the array surface and allowed to dry in air.

University of Ghana http://ugspace.ug.edu.gh

The arrays were then analyzed on a CPC Reader (Model PBS II, Ciphergen). The data were collected using the settings described by WHO (1968) as follows. Two warming shots at laser intensity 175 (not collected); 10 shots at laser intensity 170 at every five positions between 20 and 80; high mass 4500 Da; mass deflector 100 Da, detector sensitivity 7; acquired mass range from a mass-to-charge (m/z) ratio of 500 to 4500. Peak annotation was conducted using CPC Software (version 3.2.0) after baseline subtraction and adjustment had been carried out. The peak for hepcidin was recorded at a characteristic mass-to-volume (m/v) ratio of 2790 when the signal-to-noise ratio (s/n) is > 3:1. Although the molecular weight of hepcidin is 2789, it is ionized as a charged (protonated) molecule on the SELDI-TOF mass spectrometer; thus, the average peak on low-resolution mass spectrometry is one unit higher than the average molecular weight because of the proton gained.

Urinary creatinine was measured using a commercial ELISA (Quidel Corporation, San Diego, CA) according to the manufacturer's protocol. Between run coefficients of variation (CV) for urinary creatinine were 11.9% and 5.9% for high and low controls, respectively. Urinary hepcidin concentrations were expressed as intensity per mmol/L creatinine. Means and standard deviations were calculated for descriptive statistics. Anemia was defined as hemoglobin < 11 g/dL for children and pregnant women, < 12 g/dL for non-pregnant adult women, and < 13 g/dL for men (WHO, 1968). Both malaria parasitemia and urinary hepcidin (mmol/L creatinine) were continuous variables that were highly skewed and were transformed by $\log_e$ to normalize the distribution. Linear regression models were used to examine the relationship between urinary hepcidin and other variables such as parasitemia and hemoglobin.

University of Ghana http://ugspace.ug.edu.gh

5.3.0 RESULTS

5.3.1 Detection of *P. falciparum* HRP2 Antigen in Infected Human Urine by Commercial Diagnostic Kits

Plate 1 below illustrated the results of an experiment to identify *Plasmodium* antigens in infected human urinary antigen extract (PAgHU). According to the results, the anti-*P. falciparum* histidine-rich protein 2 (HRP2) antibody-based rapid diagnostic test (RDT) kit was reactive to PAgHU as shown by the test result line labelled "**T**" in the plate below. The mark labelled "C" was the test control line, which indicated that the test was successful.

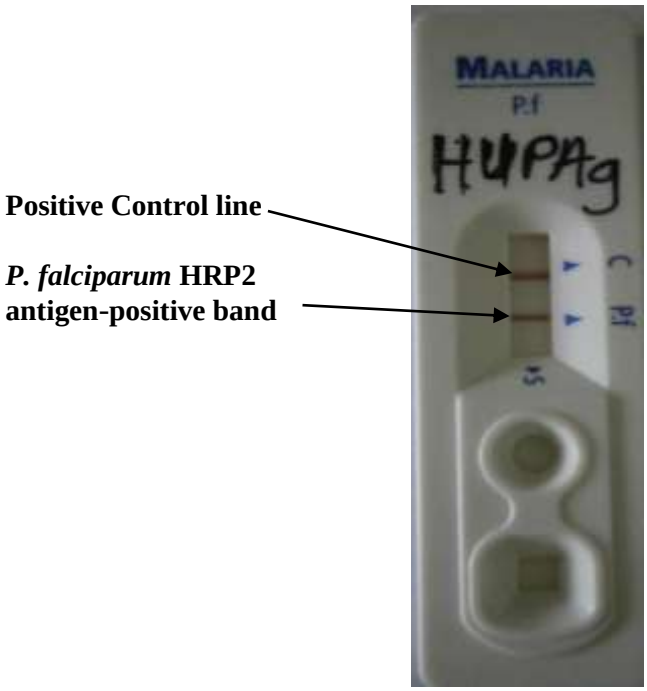


Plate 1: A commercial RDT cassette showing the presence of P. *falciparum* histidine rich protein II (pfHRP2) in crude antigens (PAgHU) extracted from malaria infected human urine. C, is the control line, Pf is the pfHRP2 band.

University of Ghana http://ugspace.ug.edu.gh

5.3.2 Detection of *Plasmodium* LDH Antigens in Infected Human Urine by the CareStart™ Rapid Diagnostic Kit

Plate 2 below illustrated the results of an experiment to identify *Plasmodium* antigens in infected human urinary antigen extract (PAgHU). According to the results, the anti-*Plasmodium* pan (genus)-specific lactate dehydrogenase (LDH) antibody-based RDT kit was reactive to PAgHU, as indicated by the test result line labelled "**Pan**" in the plate below. The mark labelled "C" was the test control line, which indicated that the test was successful.

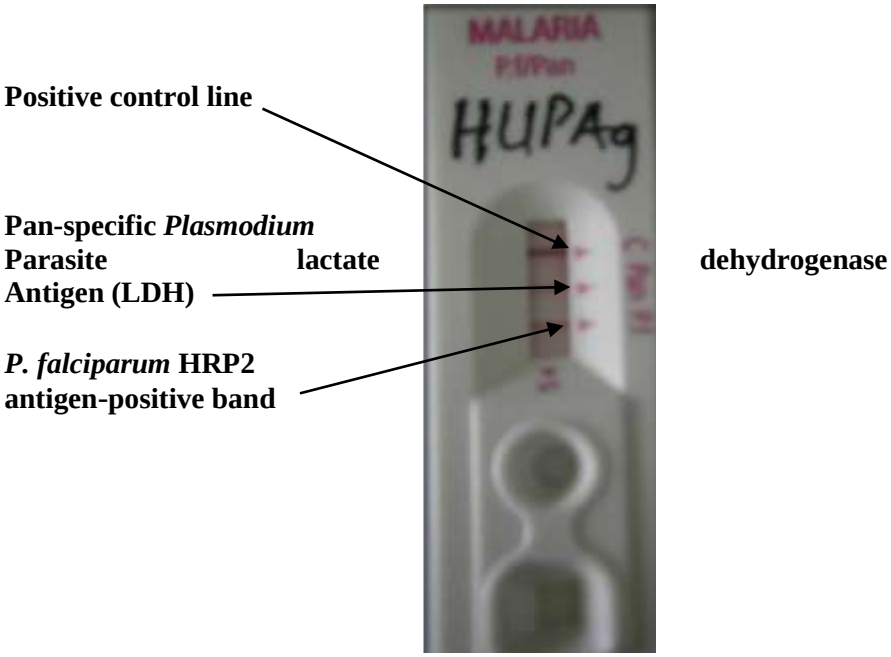


Plate 2: A commercial RDT cassette showing the presence of *P. falciparum* histidine rich protein 2 (pfHRP2) in crude antigens (PAgHU) extracted from malaria infected human urine

5.3.3 Urine analysis by BioRad protein assay

Following the extraction of *Plasmodium* proteins from infected human urine and *in vitro* cultured *Plasmodium* parasites, the BioRad protein assay was used to determine the concentration of proteins in the antigen extracts by extrapolating sample ODs on a standard

University of Ghana http://ugspace.ug.edu.gh

curve of bovine serum albumen (BSA) (Fig 32, below) and the results show that the concentration of proteins in the human urinary antigen extract (PAgHU) was 0.75 mg/ml (0.75g/l) whilst that of the *in vitro* cultured *P.falciparum* pararasite antigen extract (CPfAg) was 0.50 mg/ml.

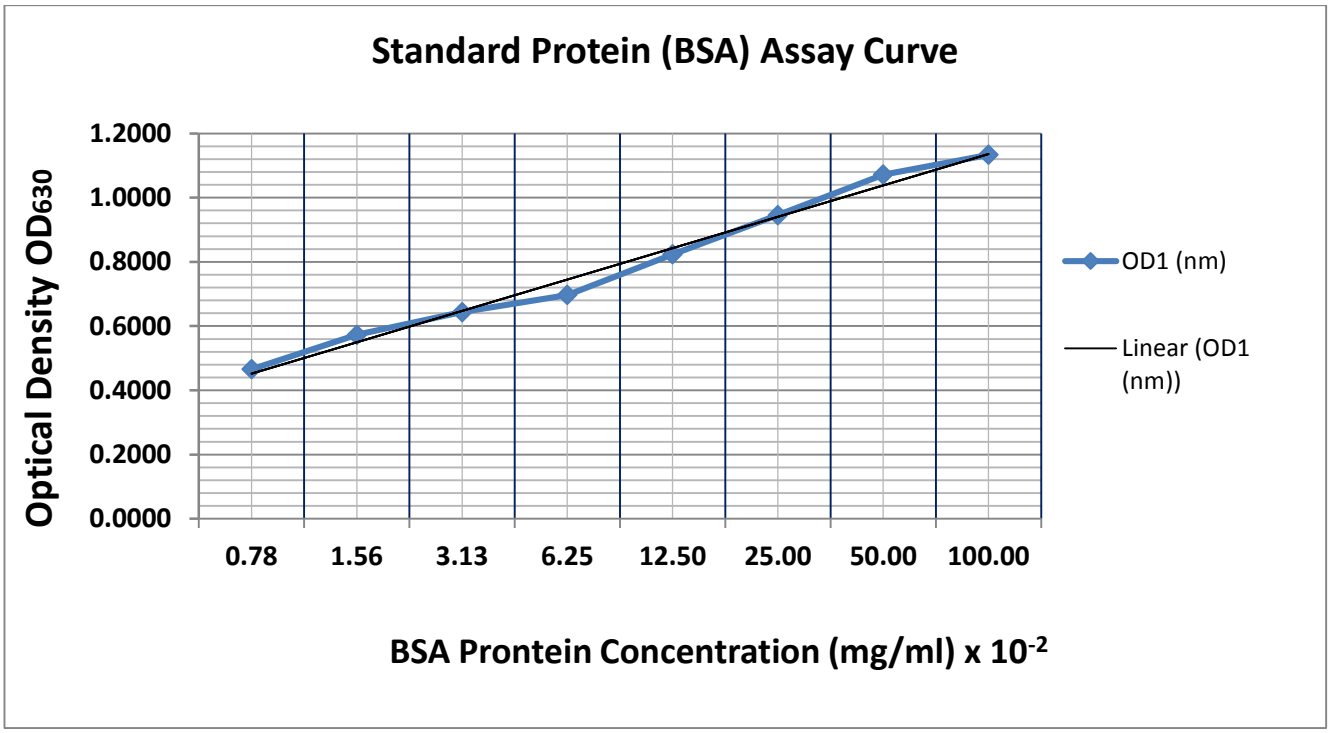


Figure 12: Determination of Concentration of Proteins in *Plasmodium* Infected Urine by BioRad Assay

University of Ghana http://ugspace.ug.edu.gh

5.3.4 Characterization of proteins in Different Urine and *Plasmodium* Antigens by SDS-PAGE

Figure 13 below, summarized the results of the SDS-PAGE experiment to characterize the proteins within different antigen preparations. As shown, PAgHU, lane 2, was one of the 2 proteins with the highest number (10) of bands within the protein profile. Three (3) of these bands were most prominent and had MWs of 10.4, 27.2 and 50 kDa. The urine sample from the 3+ malaria positive individual (DUMI-2, lane 8), was the other antigen preparation with the highest number (10) of proteins within the profile. Two of its most prominent bands had MW of 27.2 and 63 kDa.

The 2+ malaria infected urine sample (DUMI-3, lane 9), had the profile with the next highest number of bands. The profile contained 6 bands, 2 of which had joined together to form a thick prominent band. The third band which was also prominent and located slightly above the merged bands, had a MW of 27.5 kDa. DUMI-3 had another prominent band with MW being 63 kDa like DUMI-2, but which was less prominent than those described earlier.

A urine sample from 1+ malaria positive individual (DUMI-4, lane 10) had the least number (3) of visible bands of MW, 10.4, 27.5 and 32 kDa, of which the 10.4 kDa protein was the most prominent. In addition to the antigen samples described above with bands in their profile, was the *Plasmodium MSPl19* antigen (lane 6) which had a huge prominent band located below the 7 kDa MW level.

Finally, the remaining 3 samples, comprising the malaria negative urine from Ghana (lane 3), malaria negative urine from USA (lane 4), and the cultured *Plasmodium* parasite antigen extract (CPfAg, lane 5) did not have any observable bands within their profiles.

University of Ghana http://ugspace.ug.edu.gh

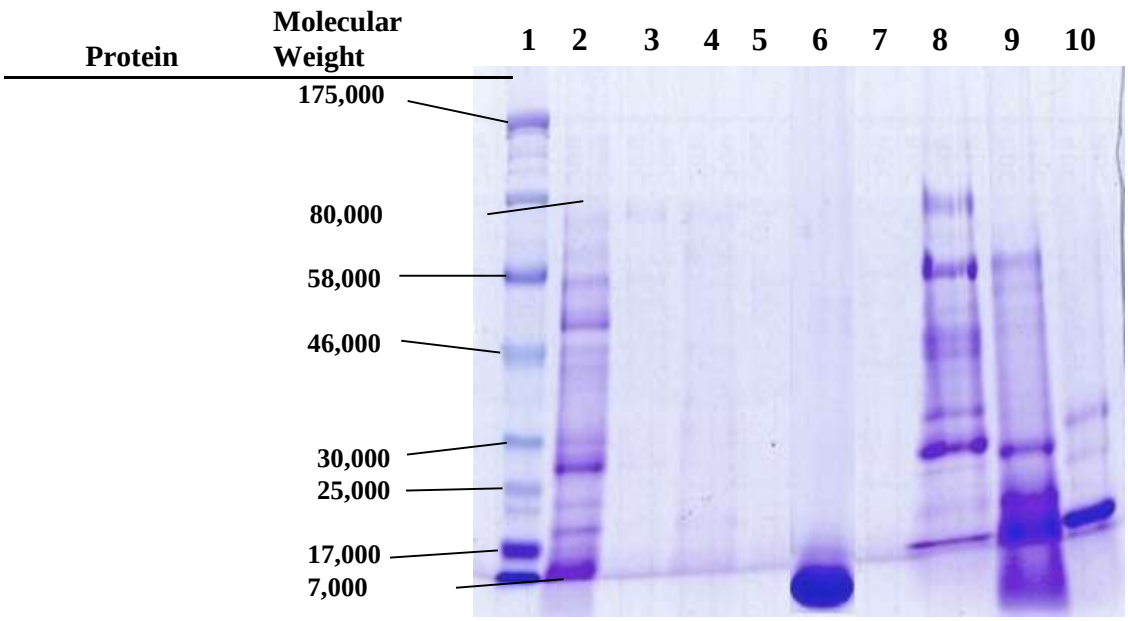


Figure 13: Coommassie blue gel electrophoresis results

Lane 1 Prestained SDS-PAGE broad range molecular weight marker
Lane 2 Crude urinary *Plasmodium* antigen extract (PAgHU)
Lane 3 Urine from *Plasmodium* negative individual from Ghana
Lane 4 Cultured 3D7 strain *P. falciparum* parasite antigen extract
Lane 5 Urine from *Plasmodium* negative individual from USA
Lane 6 *Plasmodium* $MSP1_{19}$ antigen
Lane 7 *Plasmodium* parasite aldolase antigen
Lane.8 Urine from 3+ *Plasmodium* positive individual
Lane 9 Urine from 2+ *Plasmodium* positive individual
Lane 10 Urine from 1+ *Plasmodium* positive individual

University of Ghana http://ugspace.ug.edu.gh

5.3.5 *Plasmodium* Proteins in Different Antigen Preparations by Western Blotting Analysis

Figure 14 showed the results of the western blotting experiment to identify *Plasmodium* proteins in 6 antigen preparations using mouse anti-*Plasmodium* polyclonal antibodies (PAbs). As shown, the mouse anti-*Plasmodium* PAb detected bands in protein profiles of only 2 of the 6 antigen preparations analyzed. These antigen preparations were the PAgHU and the direct urine sample from a 3+ malaria positive individual (DUMI-2). According to the results, the PAb detected fewer protein bands in the profile of PAgHU than that of DUMI-2. The 2 protein bands detected in PAgHU comprised a 50.9 kDa antigen labelled **Ω** and a 54.6 kDa antigen labeled **β.** Out of these 2 proteins, the band size of **Ω** was bigger and darker compared to that of **β**.

On the other hand, the 3 proteins detected by PAb within the profile of DUMI-2 were a 27.2, 46 and a 52.7 kDa proteins labelled **π**, **δ** and **λ** respectively. Also, these results showed that the band size of the protein labelled **λ** was larger and darker than those of **δ** and **π**.

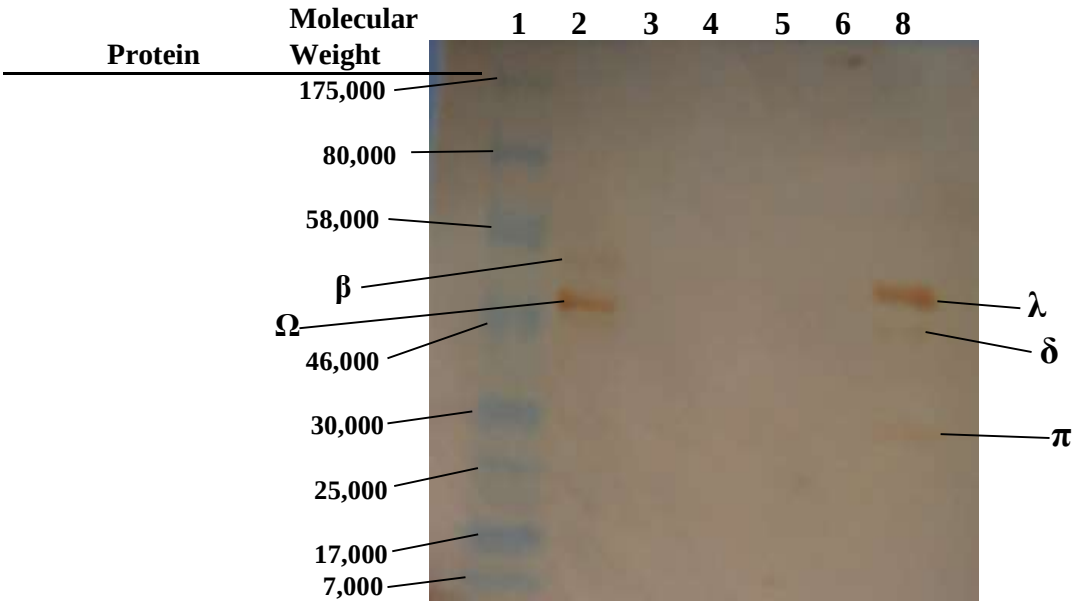


Figure 14: Western blotting results

Lane 1 Prestained SDS-PAGE broad range molecular weight marker
Lane 2 Crude urinary *Plasmodium* antigen extract (PAgHU)

University of Ghana http://ugspace.ug.edu.gh

Lane 3 Urine from *Plasmodium* negative individual from Ghana
Lane 4 Urine from *Plasmodium* negative individual from USA
Lane 5 Cultured *P. falciparum* crude antigen extract (CPfAg)
Lane 6 *Plasmodium* MSP119 antigen
Lane 8 Urine from 3+ *Plasmodium* positive individual

5.3.6 Characterization of Hepcidin from *Plasmodium* Infected Human Urine

Table 8 summarized the results of the experiments to characterize hepcidin as a biomarker for malaria in the urine of 199 malaria infected and uninfected subjects whose infection status had been determined earlier by blood smear microscopy. As shown, out of the 199 study participants whose urine samples were examined, 83 (41.7%) were children, 82 (41.2%) were adults and 31 (15.6%) were pregnant women. Urine samples from 3 (1.5%) of the participants who were children, were not included in the study because of the lack of data on their ages. This therefore made the total number of children involved in this aspect of the study to be 86 (43.2%).

According to the results, the prevalence of malaria among this cohort of study population was 69.1% among children, 44.7% among adults and 41.4% among pregnant women. Also, the prevalence of anaemia among this study participants was 79.1% among children, 82.9% among adults and 90.3% among pregnant women.

In addition, the results showed that the minimum and maximum levels of parasitaemia among these study participants were 40 and 394,480 among children, 40 and 195,040 among adults and 40 and 8,960 among pregnant women. Furthermore, the results showed that the minimum and maximum haemoglobin levels among the study participants were 3.8 and 12.6 g/dL among children, 7.9 and 17.3 g/dL for adults and 6.2 and 12.6 g/dL for pregnant women.

Finally, the mean urinary hepcidin concentration among healthy control individuals, as determined by the experiments, was 0.52 intensity/ mmol creatinine, while that among the

University of Ghana http://ugspace.ug.edu.gh

infected individuals ranged from 3-6 intensity/ mmol creatinine. These results were however not shown in the data summarized below.

Table 8: Characterization of Hepcidin from *Plasmodium* Infected Human Urine and Study Subjects Characteristics

Characteristics	Mean (SD) or %, unless noted		
	Children (n = 83)	Adults (n = 82)	Pregnant women (n = 31)
Age	7.9 (5.1)	31.3 (13.0)	24.4 (5.1)
Female (%)	44.2	56.1	N/A
Haemoglobin concentration (g/dL)	9.5 (1.8)	11.3 (1.6)	9.0 (1.7)
Anaemia (%)	79.1	82.9	90.3
Log parasitaemia	6.84 (4.36)	3.45 (3.98)	3.95 (3.56)
Geometric mean parasitaemia (parasites/ 200 white blood cells)	934	31.5	51.9
Hepcidin concentration (intensity/ mmol creatinine)	4.2 (1.4, 9.5)	0.7 (0.3, 3.6)	0.7 (0.2, 5.9)

5.3.7 Association between urinary hepcidin level and parasitaemia, anaemia and haemoglobin level

Table 9 summarized the relationship between urinary hepcidin levels and parasitaemia, haemoglobin and anaemia in each of the 3 study groups, and in all the 3 groups combined, as determined by uni- and multi-variable linear regression models. According to the results, examination of the data by a univariable linear regression model indicated that the level of hepcidin (intensity/ mmol creatinine) in subjects urine was significantly associated with log parasitaemia among the different study participant groups comprising 86 children (regression coefficient, $\beta = 0.086$, standard error, SE = 0.035, $P < 0.017$); 82 adults ($\beta = 0.184$, SE = 0.043,

University of Ghana http://ugspace.ug.edu.gh

$P < 0.0001$); 31 pregnant women ($\beta = 0.218$, SE = 0.085, $P < 0.016$) and in all groups combined ($\beta = 0.171$, SE = 0.025 and $P < 0.0001$).

On the other hand, urinary hepcidin levels were not significantly associated with haemoglobin levels in the children ($P = 0.58$), adults ($P = 0.79$), pregnant women ($P = 0.54$) and in all the groups combined ($P = 0.10$). Also, urinary hepcidin levels were not significantly associated with anaemia in the children ($P = 0.38$), adults ($P = 0.07$), pregnant women ($P = 0.54$) and all the groups combined ($P = 0.13$).

Furthermore, examination of the data by a multi-variable linear regression model showed that $\log_e$ urinary hepcidin was significantly associated with $\log_e$ parasitaemia among the study participants ($\beta = 0.174$, SE = 0.026, $P < 0.0001$), but not $\log_e$ haemoglobin levels ($\beta = 0.022$, SE = 0.058, $P = 0.71$). In addition, the results showed that there was no significant association between $\log_e$ parasitaemia and haemoglobin levels as determined by the linear regression models.

Finally, examination of the severely anaemic individuals data by an alternative multivariable linear regression model also showed that $\log_e$ urinary hepcidin was significantly associated with $\log_e$ parasitaemia ($\beta = 0.248$, SE = 0.056, $P < 0.0001$), but not haemoglobin levels ($\beta = 0.211$, SE= 0.192, $P = 0.28$).

University of Ghana http://ugspace.ug.edu.gh

Table 9: Association between urinary hepcidin level and parasitaemia, haemoglobin level and anaemia

Characteristics	Regression coefficient Beta	95% Confidence interval (CI)	Standard error (SE)	P-value (*P*)
Children (n = 83)				
Log parasitemia	0.086	0.016-0.156	0.035	0.017
Anemia	0.048	−0.123–0.221	0.086	0.58
Hemoglobin	0.345	−0.426–1.112	0.388	0.38
Adults (n = 82)				
Log parasitemia	0.184	0.099–0.268	0.043	< 0.0001
Hemoglobin	−0.030	−0.262–0.201	0.116	0.79
Anemia	0.900	−0.068–1.869	0.487	0.07
Pregnant women (n = 31)				
Log parasitemia	0.218	0.043–0.392	0.085	0.016
Hemoglobin	−0.110	−0.516–0.276	0.193	0.54
Anemia	0.677	−1.541–2.986	1.083	0.54
All groups combined (n = 196)				
Log parasitemia	0.171	0.122–0.221	0.025	< 0.0001
Hemoglobin	−0.101	−0.222–0.020	0.061	0.10
Anemia	0.481	−0.143–1.106	0.317	0.13

University of Ghana http://ugspace.ug.edu.gh

5.4.0 DISCUSSION

The studies described in this chapter sought to characterize possible biomarkers of malaria from the urine of infected individuals. The strategies adopted were partly to determine the levels of various biomarkers of malaria in the urine of infected and uninfected individuals, using the Multistix 10 SG reagent strips (Miles Diagnostics, Elkhart, Ind.), extract proteins from the urine samples and then characterize them by SDS-PAGE, western blotting assay and mass spectrometry sequencing. Finally, the strategies adopted were also to determine the level of association of the identified biomarkers with malaria among the study participants.

Identification of biomarkers of malaria in urine samples by commercial rapid diagnostic test (RDT) kits

The positive reactivity of the histidine rich protein 2 (HRP2) based test kit with the malaria antigen extract from infected human urine (PAgHU) suggested that probably, PAgHU contained *P. falciparumi* HRP2. On the other hand, the non reactivity of the test kit with the negative control urine samples suggested that the HRP2 antigen was not present in those urine samples from malaria negative individuals.

The detection of HRP2 in the urinary antigen extract from malaria positive individuals, unlike the negative control urine samples, therefore, indicated that probably, an HRP2-based RDT could also be developed for diagnosis of malaria in the urine of infected individuals. Since diagnosis of malaria like other diseases is gradually shifting focus from the use of invasive methods to non-invasive techniques, this test when developed, would have an advantage over existing ones because, it is urine-based and therefore non-invasive.

Also, the detection of the *Plasmodium* pan (genus)-specific lactate dehydrogenase (LDH) antigen in PAgHU, indicated that probably, the *Plasmodium* genus-specific LDH antigen was present in the urine of infected individuals. The non-reactivity with the negative control urine

University of Ghana http://ugspace.ug.edu.gh

samples, on the other hand suggested that the antigen was not present in uninfected human urine, just like the HRP2 antigen. These results therefore implied that a urine-based commercial RDT kit could also be developed for malaria, which when available, would be more attractive to health care givers because of its non-invasive nature.

Characterization of biomarkers in different antigen preparations by SDS-PAGE

The experiments to characterize proteins in different fractions of urine and *Plasmodium* antigens by SDS-PAGE revealed that PAgHU and the direct urine samples from malaria infected individuals (DUMIs) contained higher numbers of proteins in their profiles than the negative control and the rest of the samples. It has been reported that humans infected with *Plasmodium falciparum* frequently have elevated levels of proteins in their urine (Rodriguez-del Valle *et al*., 1991). In view of this, the higher number of proteins as observed in PAgHU and DUMIs compared to the negative samples, confirmed the report that infection by malaria parasites leads to the excretion of proteins in the urine of affected individuals.

The absence of such protein bands from the profiles of the negative control urine samples therefore, gave the indication that the identified proteins in the urine of infected individuals might be malaria-related biomarkers, which could be explored by further studies to determine their functions in the *Plasmodium* parasite, their possible association with malaria and suitability for development of non-invasive urine-based diagnostic tests for malaria.

In order to obtain PAgHU, urine samples from over 400 malaria infected individuals, some of whom harboured >100,000 parasites/ ul blood, were pooled together before extraction of the antigen. Therefore, the higher numbers (>10) of protein bands in PAgHU and the 3+ malaria positive individuals' urine (DUMI-2), which decreased with decreasing parasite density (parasitaemia) to 6 protein bands in the 2+ malaria positive individuals urine (DUMI-3) and lastly, to 3 bands in the 1+ malaria positive individuals urine (DUMI-4), suggested that the

University of Ghana http://ugspace.ug.edu.gh

separation and identification of proteins in urine, using SDS-PAGE, could be explored to quantify parasite density in the urine of malaria infected individuals.

Also, the results suggested that the separation and characterization of proteins in infected urine could be used to predict the severity of the disease in infected individuals. In addition, the fact that some of the protein bands were more prominent than others suggested that probably, the proteins with the darker bands had higher relative abundance (or concentrations) within the respective antigen preparations.

The presence of only one band in the protein profile of the $MSP1_{19}$ antigen as shown by the results, also confirmed the fact that the $MSP1_{19}$ antigen preparation was a pure recombinant antigen comprising only one protein.

Finally, the fact that the negative control urinary antigen preparations did not show any protein bands in their profiles suggested that probably, the donors were not suffering from any disease which could lead to the excretion of proteins in the urine. These results therefore confirms the findings that the presence of proteins in the urine was suggestive of a defective urine filtration system in the Bowman's capsule. This condition is said to arise as a result of an infection that causes inflammation and its consequent alteration of the fine structure of the filtration membrane within the Bowman's capsule.

Characterization of biomarkers in different antigen preparations by western blotting assay

In the western blotting experiment to characterize the proteins in different malaria infected urinary and *Plasmodium* antigen preparations, the mouse anti-*Plasmodium* polyclonal antibody (PAb) was observed to have reacted to five (5) protein bands within PAgHU and DUMI-2. These results suggested that probably, those protein bands were biomarkers of malaria, and should therefore be studied further for possible exploration for development of non-invasive urine-based diagnostic tools for malaria.

University of Ghana http://ugspace.ug.edu.gh

Also, the fact that the 54.6 kDa protein labelled **Ω** in the profile of PAgHU and the 52.7 kDa protein labelled **λ** in the DUMI-2 profile had stronger reactivity to the PAb than the other protein bands in the 2 profiles, suggested that probably, those proteins had higher relative abundance in the respective antigen preparations than the others. On the other hand, it could be because, those proteins were more immunogenic, and therefore, induced the production of higher titres of antibody against those proteins than the others which reacted weakly to the PAb.

Furthermore, the similarity of the intensity of the reactivity of the 2 proteins (**Ω** and **λ**) to PAb coupled with the closeness of their molecular weights also suggested that probably, the 2 proteins were isoforms of the same biomarker of malaria which were being expressed at slightly different molecular weights from each other. On the other hand, it was also possible that the method used in extracting PAgHU, might have modified the protein in PAgHU compared to DUMI-2, which was a direct unprocessed urine sample. This probable modification of the proteins in PAgHU might have led to the slight difference in molecular weight of the same protein in the 2 different antigen preparations.

In addition, the fact that there was no observable reactivity between the PAb and the cultured *Plasmodium* crude antigen extract (CPfAg), suggested that probably, the concentration of proteins in CPfAg was far below the threshold that was necessary for identification by PAb.

Indeed, the BioRad protein assay showed that the cultured parasite antigen (CPfAg) had lower concentration of proteins (0.5 µg/µL) than PAgHU which had 0.75 µg/µL of proteins. This observation therefore was a factor that supports the statement that CPfAg probably had lower than the amount of proteins required to show observable reactivity between it and the PAb.

Lastly, the non reactivity of the negative control urine samples with the PAb suggested that possibly, they did not contain any malaria-related biomarker at all.

University of Ghana http://ugspace.ug.edu.gh

Characterization of urinary hepcidin as a biomarker of malaria in the urine of study subjects:

Two hundred and ninety (290) urine samples were originally obtained for identification and characterization of malaria-related biomarkers in a pilot study to ascertain the suitability of urine samples for development of a non-invasive diagnostic test for malaria. However, only 199 urine samples were finally utilized in the study that led to the discovery of urinary hepcidin as a biomarker for malaria. This was because, following the repetitive use of the urine samples for preliminary experiments, 91 urine samples ran out.

Also, though 199 samples were analysed for urinary hepcidin, data from 196 samples were used in the statistical analysis, because 3 samples did not have data on the ages of the participants and were therefore excluded from the analysis.

According to these preliminary studies, the highest prevalence of malaria (69.1%) recorded for children, compared to the 44.7% for adults and 41.4% for pregnant women confirmed the findings that children, especially, those below age 5, are among the group at highest risk of infection by the disease.

Also, the lowest malaria prevalence rate of 41.4% that was obtained for the pregnant women among the 3 groups, was attributable to the fact that those women were part of the Intermittent Preventive Treatment (IPT) programme that pregnant women in the study community were involved in.

The results showed again that contrary to expected findings, the prevalence of anaemia, unlike malaria infection, was lowest (79.1%) among children than in adults (82.9%) and pregnant women (90.3%). These results therefore suggested that probably, anaemia among the children was not attributable to malaria infection. However, the highest prevalence of anaemia obtained among pregnant women in this study confirmed reports that pregnancy induces anaemia among affected women (Anderson, 2002).

University of Ghana http://ugspace.ug.edu.gh

Finally, the highest range of parasitaemia (40 and 394,480) among children as compared to adults and pregnant women, further supported the highest malaria prevalence rate in suggesting that children were among the most affected group in susceptibility to malaria. In addition, the lowest range of parasitaemia (40 and 8,960) that was obtained for pregnant women as compared to the children and adults supported the fact that probably, IPT contributed to the reduction of malaria infection rates among these women.

Hepcidin is a recently discovered peptide hormone synthesized by liver cells, and is considered as an important modulator of iron metabolism in homeostasis. Hepcidin produced by chronic inflammation is known to block the release of iron from liver cells, macrophages and intestinal epithelial cells, leading to hypoferremia and therefore, is thought to contribute to anaemia in affected individuals.

Malaria causes inflammation, and chronic inflammation leads to the synthesis of hepcidin (Anderson *et al*., 2002). Since both malaria and increased hepcidin concentration in the body are known to lead to anaemia, they were considered to have something in common. In view of this, in the efforts to identify and characterize biomarkers of malaria from the urine of infected individuals, the relationship between urinary hepcidin concentration and malaria parasitaemia, anaemia or blood haemoglobin levels was determined.

The results showed that the mean hepcidin concentration among healthy control individuals was 0.52 intensity/ mmol creatinine while that among infected individuals ranged from 3-6 intensity/ mmol creatinine. Also, the examination of the data by the linear regression models indicated that high levels of parasitaemia was significantly associated with increased urinary hepcidin concentration.

These results together suggested that increased levels of urinary hepcidin among infected individuals might have been caused by inflammation resulting from increasing malaria parasite load among the participants. The results therefore implied that urinary hepcidin concentration

University of Ghana http://ugspace.ug.edu.gh

could be used as a biomarker for malaria parasitaemia levels. Further studies are however required to explore this finding in development of a urine-based quantitative test for malaria using hepcidin levels as a marker for diagnosis.

Furthermore, the results suggested that urinary hepcidin concentration could not be used as a marker for determination of the haemoglobin or anaemia status of infected individuals. The results therefore suggested that haemoglobin levels and anaemia among individuals may be contributed by several factors, including nutrition as indicated by earlier findings (Santana-Morales *et al*., 2013).

This study as a whole, provided the initial information on the relationship between urinary hepcidin concentration and parasitaemia among malaria infected individuals, even though it was limited by the lack of indepth studies to clarify the apparent lack of association between urinary hepcidin levels and haemoglobin and anaemia.

Since these findings would contribute towards identification, characterization and development of new biomarkers for non-invasive diagnosis of malaria and its consequences, further studies are therefore required to examine the longitudinal variation in urinary hepcidin concentration and its association with changes in haemoglobin concentration, anaemic status and iron status indicators.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER SIX

GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST *PLASMODIUM FALCIPARUM* ANTIGENS USING URINARY AND *IN VITRO* CULTURED PARASITES

6.1.0 INTRODUCTION

Global eradication of malaria has not achieved expected success (Shiff, 2002) partly because of the limitations in diagnostic accuracy (Eibach *et al*., 2013; Reyburn *et al*., 2007). Tools presently available on the market for malaria detection are invasive and sometimes involve the use of complex techniques or sophisticated equipment which are difficult to find outside the health facility (Azikiwe *et al*., 2012; Eibach *et al*., 2013). This need for blood makes most of the available tests non user friendly.

To overcome these diagnostic limitations, this study seeks to explore the development of a rapid urine-based dipstick test (RUBDA) for diagnosis of the disease. For the RUBDA to function as an effective diagnostic tool, monoclonal antibodies (MAbs) may be used as essential reagents that can specifically recognize the malaria parasite antigens in diagnosis. Several authors have generated MAbs against *Plasmodium* antigens (Dzakah *et al*., 2013; Seth *et al*., 2013 Kattenberg, *et al*., 2012) some of which have been utilized in simple ICT based diagnostic tests for malaria, such as CareStart Malaria^{TM}, Paracheck, Malaria Rapid Test, ICT Malaria and Parasight F test for detection of *P. falciparum* histidine rich protein 2 (Eibach *et al*., 2013; Reyburn *et al*., 2007). However, MAbs to malaria specific urinary antigens are yet to be reported.

University of Ghana http://ugspace.ug.edu.gh

In view of this, the studies described in this chapter were conducted to generate MAbs against urinary *Plasmodium* parasite antigens suitable for development of the RUBDA for diagnosis of malaria using suspected patient urine.

Hybridoma products from cell fusion require cloning, expansion by cell culture, harvest, purification and formulation into a target antigen -specific MAb reagent. MAbs generated through cell fusion was therefore characterized to determine antibody isotype and binding characteristics in different assay systems including micro-plate ELISA and Western Blotting analysys.

6.1.1.0 Aim

The aim of the studies conducted in this chapter was to generate MAbs by cell fusion against *Plasmodium* antigens present in infected human urine and characterize them by isotyping and reactivity in microplate ELISA.

6.2.0 MATERIALS AND METHODS

6.2.1 Preparation of Immunogens for Immunization of BALB/c Mice

The immunogen (antigen preparation) for immunization of one mouse was prepared by dispensing 15µg of antigen extract and 50µl of Freund's complete adjuvant (FCA) into a clean 5ml glass vial, and equilibrating the volume to 100µl by addition PBS (pH 7.4). The content of the vial was thoroughly emulsified by mixing with a 2ml syringe fitted with 18xG hypodermic needle. To make provision against wastages an additional 30µl of immunogen per mouse was added to the original 100µl computed for each mouse. Also, to minimize the loss of immunogen during preparation, the antigen preparation for immunization of 10 mice was mixed together in

University of Ghana http://ugspace.ug.edu.gh

the same vial. After preparation the immunogen was dispensed into a 1.0ml hypodermic syringe fitted with a 26G x ½ needle before inoculation into the mice.

6.2.2 Immunization of BALB/c Mice

Eight to twelve weeks old BALB/c mice were immunized intraperitoneally (Plate 4) for production of anti-*Plasmodium falciparum* antibodies and were given three boosters at two-week intervals. A final booster a few days prior to cell fusion was also administered. Prior to immunization, 20 µl of tail vein blood was collected (Plate 3) into eppendorf tubes containing 1000 µl of PBS as shown in Plate 2. This was important for monitoring the progress of immune response in the mice. Immunization was performed by administering 15 µg/100 µl of *In vitro* cultured *Plasmodium* parasite antigen extract (CPfAg) emulsified in 50% (v/v) Freund's Complete Adjuvant (Sigma F-5881) using 26G x ½ needles as shown in Plate 3. Booster immunization consisting of similar concentration of the same immunogen in Freund's Incomplete Adjuvant (Sigma F-5506) was given on days 14, 28 and 42 after the first immunization. The final booster consisting of 15µg/100 µl of proteins isolated from *Plasmodium* infected human urine (PAgHU) without adjuvant was administered 4 days prior to cell fusion. PAgHU was used as a final booster inoculum to increase the population of B-lymphocytes bearing the gene coding for biosynthesis and secretion of anti- PAgHU antibody. The mice were bled from the tail veins before each immunization and the sera tested for the presence of anti- PAgHU antibodies using the micro-plate ELISA method.

University of Ghana http://ugspace.ug.edu.gh

Plate 3: Collecting blood from the tail of BALB/c mouse before and after immunization

Plate 4: Inoculating BALB/c mouse with PAgHU or CPfAg Immunogen

University of Ghana http://ugspace.ug.edu.gh

6.2.3 Anti-*Plasmodium* parasite Antibodies in Immunized Mouse Sera by Microplate ELISA

Microplate ELISA was conducted to screen sera of immunized BALB/c mice for anti-urinary and cultured *Plasmodium* parasite antibodies at the end of secondary immunization. Mice showing high antibody titre values in terms of optical density were selected and given a final booster of immunization with PAgHU before cell fusion. The steps for microplate ELISA were followed as described earlier (section 3.2.6), except that pre-immunization (normal mouse) sera were used as negative controls.

6.2.4 Feeder Cell Preparation and *In Vitro* Propagation of Myeloma Cells

Feeder cells were used to condition the culture medium in order to provide an environment that ensured optimum growth and multiplication of myeloma cells retrieved from storage in liquid nitrogen at -196^{o}C. Thymus gland was dissected out using a pair of dissecting scissors from a two-week old BALB/c mouse into a Petri dish. The dish contained 5 ml of Iscove's Modified Dulbecco's (IMDM) culture medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 50 µg/ml phosphate gentamycin prior to dissection. Dissection was done under aseptic conditions in a Clean Bench (Hitachi, Type PCV). The thymus gland was washed 3 times to get rid of blood in IMDM and then transferred into 0.5 ml of the medium. It was minced using the head of a sterile syringe plunger in order to free individual thymocytes. The freed thymus cells were re-suspended in a 50 ml Falcon centrifuge tube containing 10 ml medium and washed two times by centrifugation at 252 x g for 5 min at 37^{o}C. The cells were re-suspended again after washing in fresh medium and then distributed into the wells of a 96-well plate at 100 µl per well. Myeloma cell lines X63/NS1/1-Ag4-1 (supplied by Biological Resources Center, Tokyo, Japan) were added to the same wells (100

University of Ghana http://ugspace.ug.edu.gh

µl/well) after washing and then cultured together in a humidified incubator conditioned with 5% CO_2 in air. The wells with growing myeloma cells at the log phase were selected and expanded in large flasks. The growth of the cells was monitored using an inverted microscope (Olympus, CK30-F200, Japan).

6.2.5 Cell Fusion and Selection of Hybridomas

BALB/c mice with the highest serum antibody titres were given a final booster of immunization as described in section 6.2.2 towards cell fusion. On the fourth day following the final booster immunization of mice, cell fusion was performed according to the methods described by Ansar and Ghosh (2013) and Pandey (2010) with modifications. The splenocytes of the most hyperimmunized mice were fused with myeloma cell lines using polyethylene glycol (PEG) as fusion agent. The spleen cells from hyperimmunized mice were prepared as described above as described for feeder cells in section 6.2.4 above. The cells were then similarly washed and resuspended in 10 ml PBS. An aliquot of the cell suspension was diluted with Trypan Blue (0.1% in PBS). The cells were counted using improved Neubauer Counting Chamber and the density determined using the formula stated below. Myeloma cells also in log growth phase were also washed in serum-free medium and the cell density determined.

$$X = ¼ \times C_n \times 10^4 \times F_d$$

Where:

X =	the number of cells per ml
¼ =	the mean of the 4 quadrants of the Neubauer chamber
C_n =	the total number of cells in the 4 quadrants of the Neubauer chamber
10^4 =	is a constant
F_d =	the dilution factor of stained suspension and is defined as the ratio of the total volume of dilution to the initial volume of cell suspension

The myeloma cells were mixed with spleen cells at a ratio of 3:1 myeloma to spleen cells, in a 50 ml tube. The mixed cell suspension was then centrifuged at 252 g for 5 min at

University of Ghana http://ugspace.ug.edu.gh

37ºC and the supernatant completely aspirated. The 50 ml tube containing mixed cell pellet was placed in a beaker (200 ml) containing water warmed at 37ºC. One milliter (1 ml) of fusion solution [50% polyethylene glycol (PEG) 8000 and 5% dimethyl sulfoxide (DMSO) in PBS] also warmed to 37ºC, was added drop wise to the cells over a period of 1min, while gently mixing the cells by gently shaking the tube in the beaker. The content was gently stirred with the same pipette for another 1min. Soon after, 2 ml of PBS was added over a period of 2 min while mixing as described above to dilute the PEG. A final 7 ml PBS was added over a period of 3 min and the suspension centrifuged as described before. Care was taken to ensure that the total time the cells spent in PEG solution alone did not exceed 8 min. The fusion products were then resuspended in 10 ml IMDM culture medium and washed by centrifugation as before. The final product was re-suspended in 20 ml culture medium and then distributed over two 24-well tissue culture plates at 500 µl/well. The plates were kept in the 37ºC incubator conditioned with 5% CO_2 in air as described above.

On the following day, half of the medium in each well was aspirated out and a similar quantity of HAT-medium (IMDM supplemented with $10x10^{-4}$ M Hypoxanthine, $4x10^{-7}$ M Aminopterin and $1.6x10^{-5}$ M Thymidine) added to each well. The procedure was repeated on days 2, 4 and 6. Between days 8 and 13, wells with single large colonies of hybrid cells, as determined by observation under an Inverted Microscope were marked. The medium was allowed to become acidic (yellowish) and then screened by microplate-ELISA for the presence of antibodies. Hybridomas from selected wells were cloned while some stabilized as soon as possible. When hybridomas were growing well, (Plates 8 and 9 below) the HAT-medium was replaced with normal growth medium. Other cell fusions were performed using some of the remaining mice showing high serum antibody titers.

University of Ghana http://ugspace.ug.edu.gh

6.2.6 Screening, cloning and cryopreservation of hybridoma cells

Following cell fusion, culture supernatants were screened by microplate-ELISA for antibody activity as described in section 3.2.6 above. The microtitre plates were coated with either cultured *P.falciparum* parasite antigen (CPfAg) or *Plasmodium* infected human urinary antigen (PAgHU) at a concentration of 5.0 µg/well. Wells showing detectable antibody activity as revealed by optical densities were selected for cloning by limiting dilution. Some hybridomas, not cloned, were transferred into 6-well culture plates and allowed to grow and then cryopreserved. Cells ($1x10^6$/ml) were cryopreserved (-196^{o}C) with 1ml culture medium supplemented with 5% (v/v) DMSO and 34% (v/v) FBS in vial tubes. Hundred microliters (100 µl) of cell suspension estimated to have 1 cell (ie 1 cell/ 100 µl \suspension), was added to each well and cultured. Supernatants from wells containing single cell colonies were finally screened again by microplate-ELISA for antibody reactivity.

6.2.7 Determination of cut-off point for ELISA negative reactivity results

The absorbance ODs were measured at 414nm.

Cut off point (Cf) for negative reactivity was calculated using the formula:

Cf = Mean of negative controls + 2 x standard deviation (SD) of negative controls.

Cf for plates coated with PAgHU = OD 0.0985nm; and for plates coated with CPfAg = OD 0.1345nm

Table 10: Mode of grading of monoclonal antibody reactivity patterns

Grading of OD Values	Reaction with UPAg (ODs)	Reaction with CPfAg (ODs)
Negative Results	Below 0.099	Below 0.135
Weak positive/ Trace **(+/-)**	0.099 – 0.499	0.135 to 0.499
Moderately strong positive **(+)**	0.500 – 1.499	0.500 – 1.499
Strong positive **(2+)**	1.500 – 2.499	1.500 – 2.499
Hyper positive **(3+)**	2.500 – 3.499	2.500 – 3.499

University of Ghana http://ugspace.ug.edu.gh

6.2.8 Immunoglobulin Classes of Monoclonal Antibodies in Hybridoma Culture Secretions

The monoclonal antibodies (MAbs) produced in hybridoma cell culture supernatant were examined for immunoglobulin classes using the Pierce rapid isotyping kit (PRIK) for mouse sera (Thermo Scientific, Rockford, IL USA). This was done according to the manufacturer's specification with modification. Briefly, the culture supernatant from each of the selected wells was diluted (1:5) by addition of 250 µl of PRIK sample buffer to 50 µl of the hybridoma cell culture supernatant in a 1.5 ml centrifuge (eppendorf) tube. 150 µl of the culture supernatant-buffer complex mixture was dispensed into the sample well of the PRIK cassette and then allowed to incubate for 15-20 minutes at room temperature (25^{o}C). The test outcome indicated by the appearance of reddish-purple lines were read and graded visually by noting the immunoglobulin class label at the end of each line together with the intensity of coloration of the reddish-purple lines. The immunoglobulin class labels were IgG_{1}, IgG_{2a}, IgG_{2b}, IgG_{3}, IgM, and a control line labelled C; while the visual gradings were **+**, **2+**, **3+** and **4+**.

6.3.0 RESULTS

6.3.1 Immune Response in BALB/c Mice to Urinary and Cultured *Plasmodium* Antigens

During immunization of BALB/c mice towards generation of MAbs, 15.0µg of urinary (PAgHU) and cultured (CPfAg) *Plasmodium* parasite antigens were used throughout the primary and secondary immunizations. Upon testing sera from the immunized mice for response to immunization, the results showed that only 20% (6/30) of the mice had a weak

University of Ghana http://ugspace.ug.edu.gh

response (absorbance OD values ranged between 0.36 and 0.45, when measured at 414nm. Negative results had an absorbance of 0.12 and below) to the PAgHU. The majority of them showed no response at all. Also, the results showed that virtually all the mice exhibited no observable immune response to the CPfAg. Except for 10% (3/30) of them which exhibited a weak response (with absorbance ranging from 0.27-0.33; negative absorbance values ranging from 0.15 and below). Following the poor overall output of the initial immunizations, a fresh batch of 15 mice were immunized with 30.0µg (instead of 15.0µg) of the *Plasmodium* antigens and the results were presented in sections 6.3.2 and 6.3.3 below, as follows.

6.3.2 Detection of Anti-Urinary *Plasmodium* Antibodies in Immunized Mouse Sera by Microplate ELISA

BALB/c mice immunized with 30.0µg of PAgHU antigens extracted from the urine of infected individuals and cultured parasites were tested for response to immunization by detection of anti-urinary *Plasmodium* antibodies in microplate ELISA. According the results (Figure 15), almost all the mice showed positive immune response to PAgHU. Mouse numbered 2 exhibited the highest immune response of 2.75 while mouse numbered 14 which apparently did not show any appreciable immune response at all, exhibited the lowest immune response OD of about 0.2. Also, mice numbered 11, 12 and 13 showed weak immune response with ODs below 0.3 from the beginning of immunization until the end of secondary immunization 1. However, after the end of secondary immunization 1, immune response to PAgHU increased in the mice until it had gone beyond OD 1.0 to OD 1.75 in mouse numbered 13. Finally, mouse numbered 4 after reaching immune response OD of 0.49 died at the end of secondary immunization 1 and therefore could not complete the immunization.

University of Ghana http://ugspace.ug.edu.gh

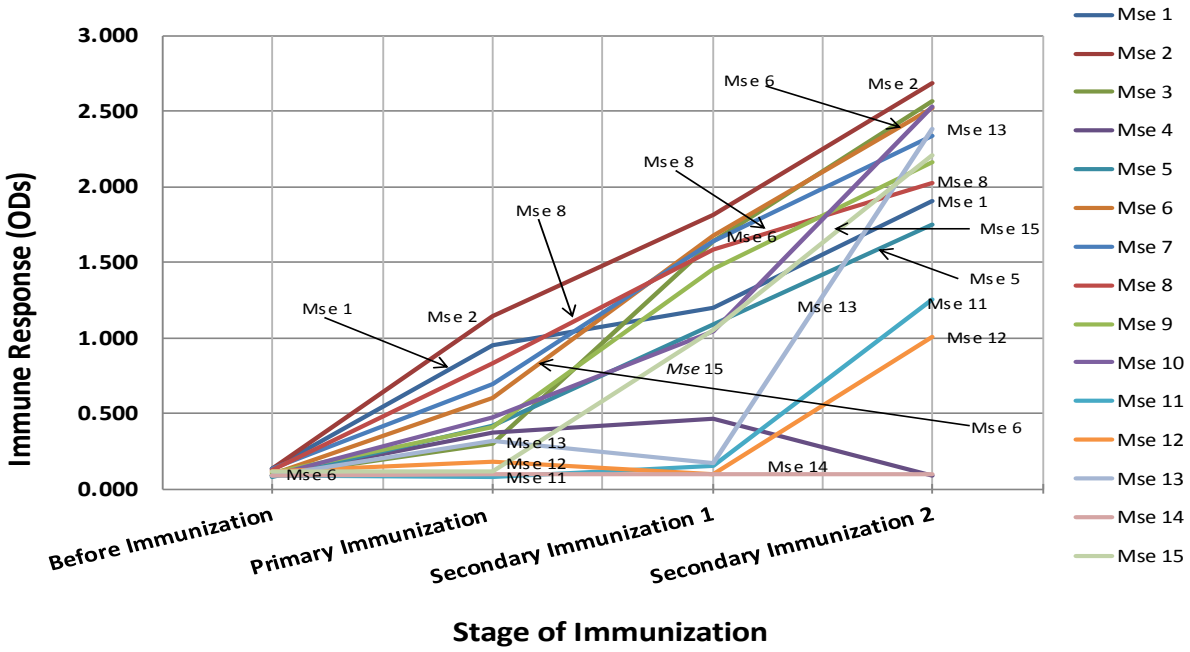


Figure 15: Immune Response of BALB/c Mice to Urinary Malaria Antigens

University of Ghana http://ugspace.ug.edu.gh

6.3.3 Detection of Cultured *Plasmodium* Parasite Antibodies in Immunized Mouse Sera by Indirect ELISA

As summarized in Figure 16 below, the results showed that unlike the response to PAgHU, almost all the mice exhibited weak or no immune response to CPfAg. Mice numbered 6, 11 and 13 however, showed good immune response to the CPfAg, with antibody response ODs being 0.79, 1.6 and 0.55 respectively. Consequently, these 3 mice were selected as the most suitable for cell fusion to generate the hybridoma cells for monoclonal antibody production.

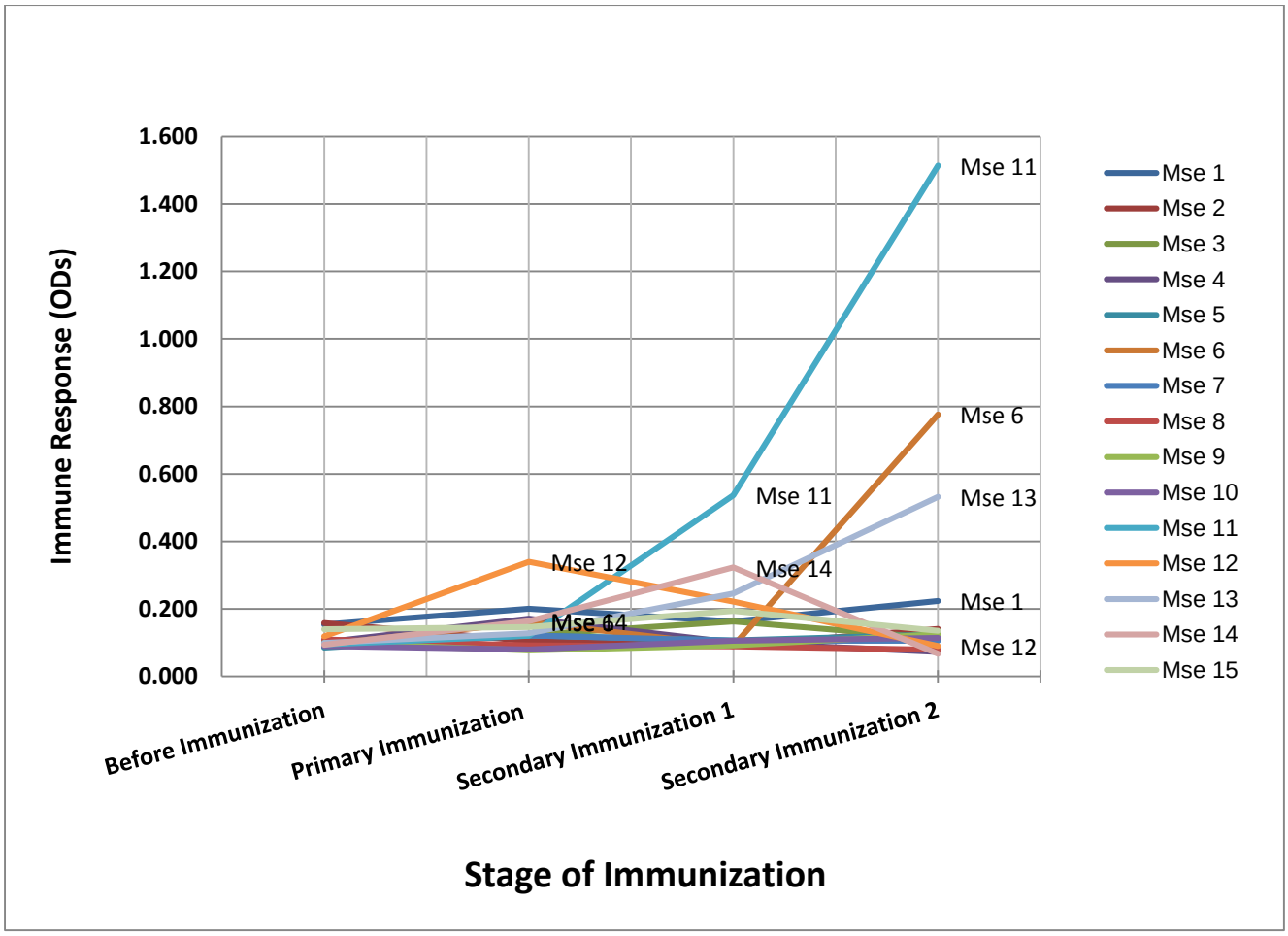


Figure 16: Immune Response in BABLB/c mice to Cultured *Plasmodium* Antigens

University of Ghana http://ugspace.ug.edu.gh

Plates 5 and 6 below are photographs of some of the microtitre plates after ELISA to determine immune response of BALB/c mice to urinary (PAgHU) and cultured (CPfAg) *Plasmodium* parasite antigens. The results corroborated what was shown by Figures 15 and 16 above that generally, immune response in the mice to the immunizing antigens increased with time. Also, the plates showed that before immunizations (columns 1 and 7), the mice were not showing response to any *Plasmodium* antigens, therefore, their sera reacted negatively to PAgHU and CPfAg in microplate ELISA. However, after primary immunizations with PAgHU and CPfAg, immune response to these antigens rose gradually until after secondary immunization 2 when immunizations had ended.

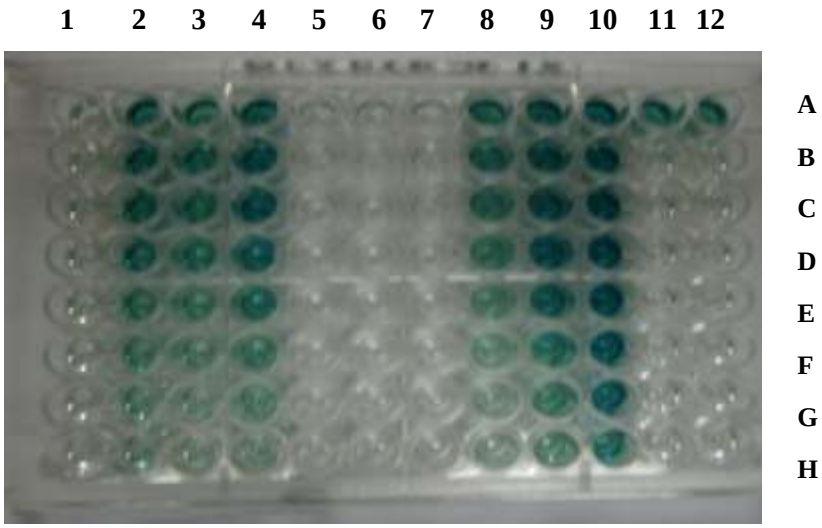


Plate 5: A microtitre plate showing the response of mice 1 and 2 to urinary *Plasmodium* antigens as determined by microplate ELISA: Columns 1 & 7 = Response before immunization; Colum's 2 & 8= Response after primary immunization; Columns 3 & 9 = Response after secondary immunization 1; Columns 4 & 10 = Response after secondary immunization 2; Wells A11 and A12 = positive control sera

University of Ghana http://ugspace.ug.edu.gh

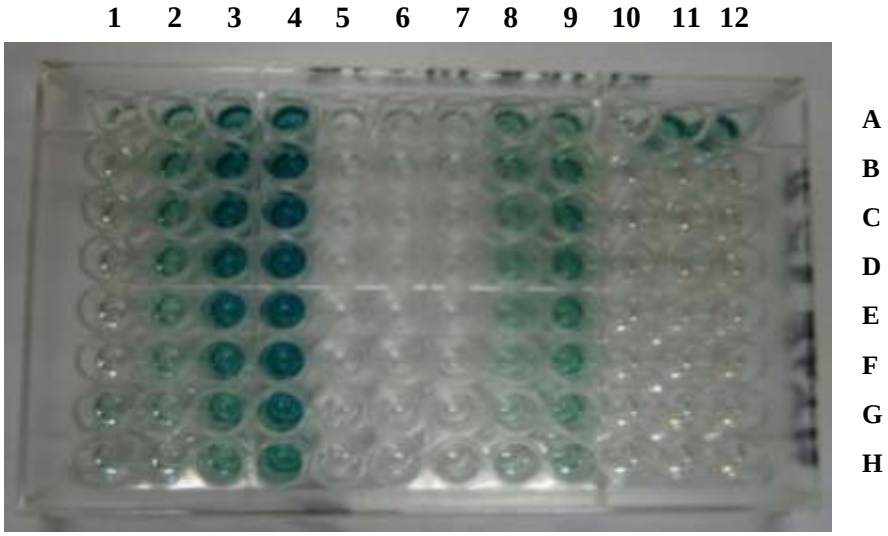


Plate 6: A microtitre plate showing response of mice 3 & 4 to urinary *Plasmodium* antigens as determined by microplate ELISA: Columns 1 & 7 = Response before immunization; Colum's 2 & 8= Response after primary immunization; Columns 3 & 9 = Response after secondary immunization 1; Columns 4 & 10 = Response after secondary immunization 2; Wells A11 and A12 = positive control sera

University of Ghana http://ugspace.ug.edu.gh

Plate 7

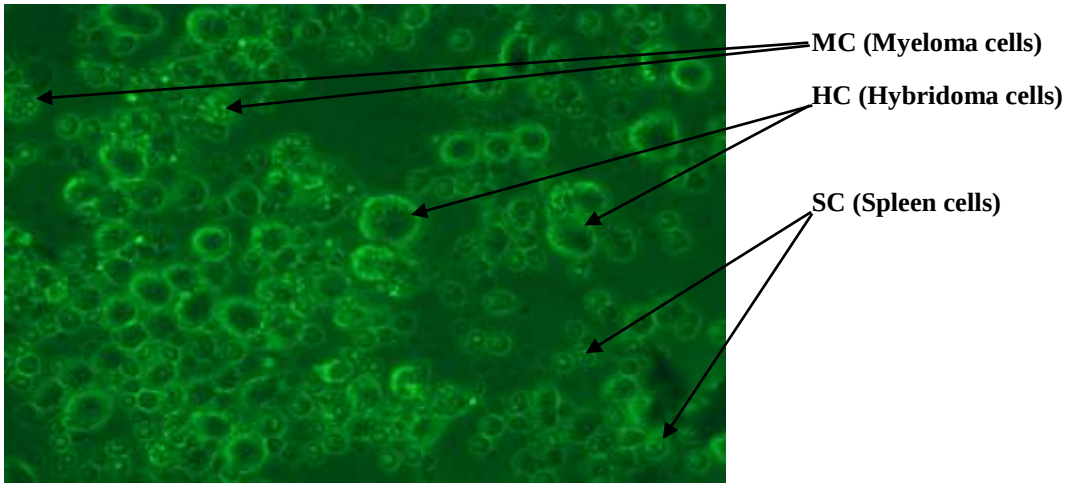


Plate 7: A cell fusion product on day-3 post cell fusion, showing a well on a 24-well tissue culture plate containing a mixture of unfused myeloma cells (MC; large cells that are degenerating), spleen cells (SC; small cells that are degenerating) and hybridoma cells (HC; large cells that are either dividing or are about to divide).

Plate 8

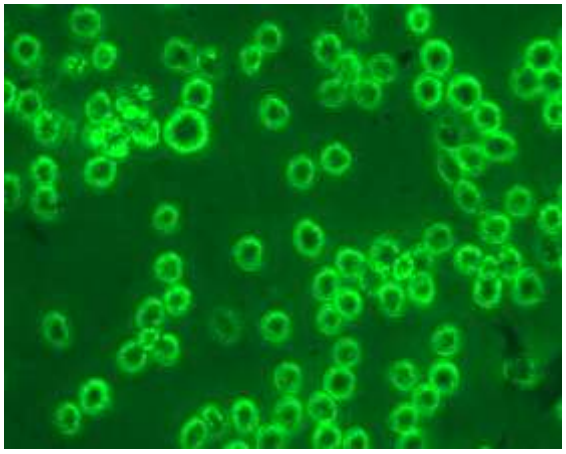


Plate 9

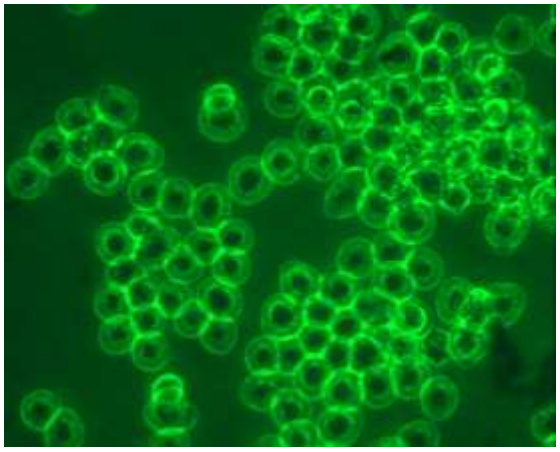


Plate 8: Hybridoma cell colonies in various phases of growth on day 9 post cell fusion.

Plate 9: Hybridoma cell colonies in various phases of growth on day 14 post cell fusion.

University of Ghana http://ugspace.ug.edu.gh

6.3.4 Characterization of Anti-*Plasmodium* Monoclonal Antibodies by Microplate ELISA

In all 96 hybridoma cell clones were obtained after the 5th cell fusion to generate monoclonal antibody (MAb) secreting hybridoma cells. Analysis of these MAbs by microplate ELISA (Figure 17) showed that all the 96 clones secreted MAbs that were reactive to human urinary *Plasmodium* parasite antigen extract (PAgHU); and 66.8% (66/96) of these 96 hybridoma cell clones were also secreting MAbs that were reactive to cultured *P. falciparum* parasite antigens (CPfAg). Also, 31.3% of the MAbs produced were observed to be reactive to PAgHU only, 66.8% were reactive to both PAgHU and CPfAg and none was reactive to CPfAg alone.

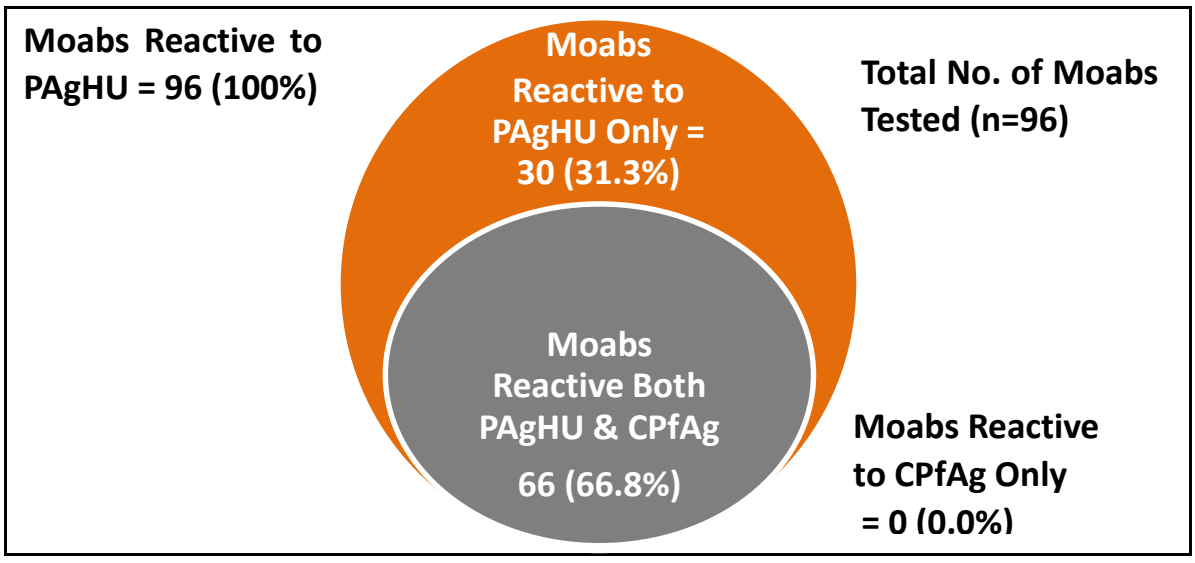


Figure 17: Reactivity of Anti- *Plasmodium* Monoclonal Antibodies by Microplate ELISA

University of Ghana http://ugspace.ug.edu.gh

6.3.5 Immunoglobulin Class and Reactivity of Monoclonal Antibodies by Rapid Isotyping Kit (PRIK)

Table 11 below showed the results of the experiment to determine the immunoglobulin classes of selected hybridoma secretions (monoclonal antibodies), using the PRIK for mouse sera (Thermo Scientific, Rockford, IL USA). The results showed that the majority (8/14; 57.14%) of the hybridoma secretions characterized by isotyping contained monoclonal antibodies (MAbs) of the immunoglobulin IgM class (isotype) with very strong reactivity (**4+** and **3+**). The results also showed that 6/14 (42.86%) of the hybridoma cells were secreting the IgG class of MAbs. Of these number of hybridoma cells secreting the IgG class of MAbs, 3 cells were secreting the IgG_1 subclass of MAbs with reactivity grades **4+**, **3+** and **2+** respectively. Two cells were secreting IgG_{2a} subclass of MAbs with strong (**2+**) reactivity; while one (1) was secreting the IgG_3 subclass of MAbs with a moderately strong (**+**) reactivity. Finally, all the isotyping kit cassettes functioned normally, with the control line labelled "C", appearing on each of the cassettes.

Table 11: Immunoglobulin classes of selected hybridoma culture secretions

Sample Serial Number (No)	Monoclonal Antibody Sample Code	Reactivity to Antigens		Immunoglobulin Class Detected	Isotyping Reactivity Strength
		PAgHU	CPfAg		
8	UCP_1W_8	1.57 **(2+)**	0.78 **(+)**	**IgM**	**3+**
12	UCP_1W_{12}	1.37 **(+)**	0.46 **(+/-)**	**IgM**	**4+**
13	UCP_1W_{13}	0.21 **(+/-)**	0.08 **(-)**	**IgM**	**4+**
30	UCP_2W_6	0.39 **(+/-)**	0.08 **(-)**	**IgM**	**4+**
31	UCP_2W_7	0.87 **(+)**	0.09 **(-)**	**IgG_{2a}**	**2+**
32	UCP_2W_8	0.91 **(+)**	0.12 **(-)**	**IgG_3**	**+**
33	UCP_2W_9	0.36 **(+/-)**	0.08 **(-)**	**IgG_{2a}**	**2+**
56	UCP_3W_8	2.82 **(3+)**	2.13 **(2+)**	**IgM**	**3+**
60	UCP_3W_{12}	2.98 **(3+)**	3.08 **(3+)**	**IgM**	**4+**
61	UCP_3W_{13}	0.60 **(+)**	0.60 **(+)**	**IgM**	**4+**
78	UCP_4W_6	0.62 **(+)**	0.18 **(+/-)**	**IgM**	**3+**
79	UCP_4W_7	1.73 **(2+)**	0.92 **(+)**	**IgG_1**	**3+**
80	UCP_4W_8	2.87 **(3+)**	0.29 **(+/-)**	**IgG_1**	**2+**
81	UCP_4W_9	0.92 **(+)**	0.28 **(+/-)**	**IgG_1**	**4+**

University of Ghana http://ugspace.ug.edu.gh

6.3.6 Cloning of Hybridoma Cells the Limiting Dilution Method

Hybridoma cell colonies secreting monoclonal antibodies (MAbs) with highest antibody titres were cloned by the method of limiting dilution to obtain single clones of cells which were further expanded in order to obtain single clones of MAb producing cells. However, upon testing the culture supernatants for secretion of MAbs, the results were negative suggesting the absence of immunoglobulins. Attempts at further cloning of additional hybridoma cells, were not successful due to failure of the incubators to discharge carbon dioxide at the required concentration of 5% in air. The hybridoma cells were therefore cryopreserved to prevent them from dying, until the incubators are repaired.

University of Ghana http://ugspace.ug.edu.gh

6.4.0 Discussion

Since Kohler and Milstein invented the B-cell hybridoma technology for generating monoclonal antibodies (MAbs) in 1975, MAbs have become valuable molecular tools in both basic biochemical research and in medicine for disease diagnosis and treatment (Casadevall *et al*., 2004; Dozier, 2014). In order to obtain the antibodies required for diagnosis of malaria therefore, the experiments reported in this chapter were carried out with a main objective to develop MAbs against PAgHU. The MAbs were produced with the expectation that some would be useful in specifically detecting *Plasmodium* antigens in urine and therefore, serve as a promising tool for diagnosis of malaria in urine. The strategy was to immunize BALB/c mice with urinary (PAgHU) and cultured (CPfAg) *Plasmodium* antigens, and use immunized mice spleen cells in cell fusion to generate MAb against PAgHU and CPfAg.

The *in vitro* culture hybridoma technology was adopted for production of MAbs in this study other than the *in vivo* ascites production technique. This was because, the ascites are often contaminated by other immunologically active compounds including viruses and bacteria that may be present in the mouse abdominal cavity (Dewar *et al*., 2005; Lipman *et al*., 2005), which compromise the specificity of the MAbs produced.

Laboratory bred BALB/c mice were immunized for generation of MAbs instead of other animals such as rabbits, or hamsters, because the myeloma cell lines (NS-I and X63) available for cell fusion were from mouse origin. The decision to use myeloma cell fusion partners of the same species was based on the report by Grimaldi and French (1995) that "the myeloma cell fusion partner should be genetically compatible with the immunized spleen-cell source because hybridomas generated from cells of the same species are more stable than hybridomas generated from different species".

University of Ghana http://ugspace.ug.edu.gh

To obtain immunized murine spleen cells for cell fusion and generation of monoclonal antibodies (MAbs), laboratory bred BALB/c mice were immunized via the intraperitoneal route (IP) because it has several advantages over other routes which include the following. Firstly, the IP route is relatively more simple and easier and therefore, the most frequently and widely used channel for immunization of rodents (Shimizu, 2004; FSUIRAP, 2013). Secondly, it allows a wide distribution of immunogen to lymphoid tissues which leads to the induction of the heightened immune response (Bhowmick *et al*., 2009; Jerusalem and Eling, 1969), requisite for generation of highly reactive MAbs.

In addition, even though it is comparable to the intravenous (IV) route of inoculation, in terms of the intensity of immune response induced, it is several times safer than the IV route. The IV route demands a great deal of caution and can lead to the death of most or all of the animals before the completion of immunization (Jerusalem and Eling, 1969). In spite of the numerous advantages of the IP route over other immunization roots, it has some disadvantages which include the risk of anaphylactic shock if booster immunogens are absorbed too rapidly in the vascular system. For these reasons therefore, the IP route was adopted for immunization of mice towards the generation of MAbs for development of the dipstick test for malaria.

During the immunization of mice towards MAb development, the higher level of immune response induced by 30.0µg compared to 15.0µg of immunogen per mouse per immunization might be because 15.0µg of the crude antigen was not immunogenic enough. Indeed, it has been documented that the intensity of immune response that is mounted is partly dependent on the molecular size of antigen inoculated, such that a minimum of 5 kDa (8.30265×10^{-15} µg) of antigen is required to induce a minimum level of immunity; and the larger the size of the antigen

University of Ghana http://ugspace.ug.edu.gh

the higher the immunogenicity (Varun, 2012). Therefore, the fact that 30.0µg of the crude antigens induced a more heightened level of immune response implied that it contained enough amount of antigen capable of stimulating humoral immune response in the mice against the malaria antigens.

Also, the observation that mouse number 2 in the experiment showed the strongest immune response while those numbered 12 showed the weakest intensity of immune response even though they were all inoculated with relatively equal amounts of the immunogen (Figure 15) could be due to inherent genetic differences among them, which make some of them to exhibit better immunological response than others.

In addition, the induction of a higher level of immune response in the mice by the urinary malaria antigens (PAgHU) compared to the cultured *Plasmodium* parasite antigens (CPfAg) (Figures 15 and 16) suggested that PAgHU was more immunogenic than CPfAg. In fact, a similar observation was made by Amanor *et al*. (1996), who reported that mice immunized with urine-based *Schistosoma hematobium* antigens exhibited a higher immune response than those immunized with *S. haematobium* soluble egg antigens.

In all, 96 MAb-secreting hybridoma cell colonies were produced after cell fusion. The reactivity of all the 96 (100.0%) MAbs clones to PAgHU compared to only 68 (70.8%) which were reactive to CPfAgs supported the earlier statement that the former antigen was more immunogenic, and could therefore induce a higher immune response leading to the production of a greater number of reactive MAbs than CPfAg. Also, the detection of both PAgHU and CPfAg by 68 (70.8%) of the monoclonal antibodies suggests that these MAbs have a wider range of diagnostic applicability and could therefore be the most promising for development of the urine-

University of Ghana http://ugspace.ug.edu.gh

based diagnostic test for malaria, provided that they are specific to malaria antigens only, and do not cross-react with any other antigens.

In addition, the reactivity of 68 of the MAbs to both PAgHU and CPfAg suggests that the immunogenic factors that induced the production of these MAbs are shared in common by both antigens. Furthermore, that 30 (31.2%) of the MAbs could react with only PAgHU suggests that the immunogenic component of the malaria antigens that induced the production of such MAbs originated from PAgHU alone. Finally, the fact that none of the MAbs could react to only CPfAg implies that probably, none of the immunogenic factors of the antigens that induced the production of the MAbs came from CPfAg only.

Antibodies are grouped into different immunoglobulin (Ig) classes known as isotypes which are characterized by differences in their biological features, including structure, distribution, function as well as affinity and specificity for different antigens. In view of this, the knowledge of the Ig classes of antibodies is useful in determining the probable nature of immune reaction that can occur between a particular MAb and the target antigen, and therefore helps in the selection of a given MAb for detecting particular disease antigen. Also, knowledge of the Ig class may be very useful in immunochemical engineering to improve upon the specificity and sensitivity of a selected MAb for diagnosis of a given disease.

For these reasons, 7 out of the 96 MAb clones generated were examined for their Ig isotypes and strength of reactivity to the isotyping antisera, PAgHU and CPfAg. Only 7 MAbs were examined for Ig classes because, logistical challenges prevented the analysis of more than 7. The fact that almost all the 7 clones of MAbs examined had the IgM isotype compared to only 4 of them that had the IgG itotype suggested that the IgM producing spleen cells were more successful in fusing to myeloma cells than their counterpart IgG producing spleen cells. Also, the

University of Ghana http://ugspace.ug.edu.gh

stronger reactivity (4+, 4+, 4+ 4+, 3+, 3+ and 3+) of the IgM isotype of MAbs compared to that (4+, 2+, 3+and 2+) of the IgG isotype, indicated that probably, there were more molecules of the IgM isotype than the IgG in each clone of MAb examined. Alternatively, it could be due to the fact that IgM being pentameric, had a higher specificity and affinity for the isotyping antisera than their IgG counterpart.

Interestingly, however, in terms of the reactivity to PAgHU, the culture supernatant that contained more than one class/ subclass of the IgG isotype (i.e. IgG_1 in addition to IgG_{2a}, IgG_{2b} and/ or IgG_3) together with IgM, also had the highest reactivity for PAgHU, thereby suggesting that the presence of the IgG subclass istotypes, especially, IgG_1 in the culture supernatant, heightens the reactivity of the MAb to PAgHU. Finally, in terms of the reactivity of the various classes of MAb to CPfAg, no clear reactivity pattern was observed between the Ig classes and CPfAg, thereby suggesting that the reactivity of MAbs to CPfAg is not influenced by Ig class characteristics.

The single or monospecificity of monoclonal antibodies (MAbs) for their target antigens is necessary for differentiating between the target antigen and others, and forms a true basis upon which accurate diagnosis relies to control malaria by chemotherapy. The cloning of hybridoma cells to obtain a cell line originating from a single parental hybridoma cell is required to produce MAbs with monospecificity for target antigens. In order to generate MAbs with monospecificity for the PAgHU, therefore, the hybridoma cell colonies secreting multiple clones of MAbs with the highest antibody titre (ODs), were cloned by limiting dilution (Pandey, 2010) into single cells which were further multiplied in culture to obtain colonies of hybridoma cells capable of producing large amounts of MAbs.

University of Ghana http://ugspace.ug.edu.gh

Upon testing the culture supernatants for reactivity to PAgHU and CPfAg, however, the results were negative, suggesting the absence of MAbs that were reactive to the target antigens. The observed absence of MAbs that were reactive to PAgHU and CPfAg in the culture supernatants, could however be due to abortion of antibody secretion by the monoclonal hybridoma cell lines. Indeed, these results confirms reports by several authors that monoclonal hybridoma cell lines sometimes abort MAb secretion, making them to function like myeloma cell lines.

Attempts at further cloning by limiting dilution, however, were not successful due to failure of the incubators to discharge carbon dioxide at the 5% concentration in air needed for successful culture of the cells. The cultures were therefore suspended, and hybridoma cells were cryopreserved in liquid nitrogen to prevent them from dying until the incubators are repaired.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER SEVEN

REACTIVITY OF MOUSE ANTI-*PLASMODIUM* MONOCLONAL AND POLYCLONAL ANTIBODIES IN ELISA AND WESTERN BLOTTING ASSAY

7.1.0 INTRODUCTION

Polyclonal antibodies (PAbs) have several important uses as stated earlier in chapter two of this thesis report. In addition to these remarkable qualities, PAbs are the first kind of antibodies to be produced during infection and/ or immunization, and can give indication on whether a particular immunization exercise towards MAb generation has been successful or not. Therefore, in order to determine whether the immunization of mouse towards the generation of monoclonal antibodies as reported in this work, has been successful or not, PAbs produced against urinary and cultured *Plasmodium* parasite antigens were tested for reactivity against these antigens.

Knowledge of the reactivity of the PAbs was necessary to select the best immune responder mice for cell fusion towards MAb Production. It was also necessary for characterization of the malaria diagnostic antigens present in infected human urine. In addition, the reactivity of the PAbs was required for optimization experiments to determine the best conditions for ELISAs and western blotting assays needed to characterize the MAbs being generated.

In order to select the best hybridoma cells for cloning to generate MAbs specific to the urine-based malaria proteins, it was necessary to test the multiple clones of MAbs produced in hybridoma culture solutions for reactivity to the target urinary *Plasmodium* antigens. For these reasons, therefore, the experiments below were conducted to determine the reactivity of the monoclonal and polyclonal antibodies in ELISA and western blotting assays.

University of Ghana http://ugspace.ug.edu.gh

Accurate diagnosis of malaria plays important roles in the managegent and control of the disease (Eibach *et al*., 2013). Also, it is necessary for the success of intervention programmes towards eradication of the disease (Eibach *et al*., 2013). The accuracy of diagnosis in turn, is largely dependent on the sensitivity and specificity of the tests used. This is because, a non-sensitive test would give rise to false negatives which could cause curative drugs not to be given to truly infected individuals who really need them. This factor could lead to complications and death in high risk groups (Reyburn *et al*., 2007).

Also, a nonspecific test would result in false positives which could lead to dispensing of curative drugs to individuals who do not have the disease. Indeed, this factor is known to be the driving force behind parasite development of drug resistance (Reyburn *et al*., 2007).

To ascertain whether the anti-mouse MAb and PAb produced were reacting accurately to the urinary and cultured *Plasmodium* parasite antigens, therefore, the sensitivity and specificity of these antibodies to the parasite antigens were determined using microplate ELISA and the result were compared to those of microscopy and other biomarkers of malaria.

7.1.1 Overall Aim

To determine the reactivity of mouse anti-*plasmodium* monoclonal and polyclonal antibodies to different antigen preparations by microplate ELISA and western blotting assay

Specific objectives

- To determine the optimum conditions for conducting microplate ELISA experiments using MAbs and PAbs

University of Ghana http://ugspace.ug.edu.gh

- To determine the reactivity of mouse anti-P*lasmodium* monoclonal antibodies by indirect microplate ELISA
- To determine the prevalence of *Plasmodium* infections in exposed individuals by mouse anti-*Plasmodium* UCP4W7 MAb microplate ELISA
- To determine the prevalence of *Plasmodium* infections in exposed individuals by mouse anti-*Plasmodium* PAb microplate ELISA
- To determine the reactivity of the UCP4W7 MAb to different antigen fractions by western blotting assay

7.2.0 MATERIALS AND METHODS

7.2.1 Optimum Conditions for ELISA

Microplate ELISA experiments were carried out as described in section 3.2.5, to determine the best conditions, such as incubation temperature, time and type of microtitre plates for conducting microplate ELISA in this study. The findings were reported in the results section below.

7.2.2 Reactivity of Anti-*Plasmodium* Monoclonal Antibodies by Microplate ELISA

The MAbs produced in hybridoma culture were tested for reactivity against the urinary *Plasmodium* antigen extract (PAgHU) and the cultured parasite antigen extract (CPfAg) using indirect microplate ELISA. Briefly the microtitre plates were coated with PAgHU (5 µg/ well) or CPfAg (5 µg/ well), and probed further with 100 µl of the supernatant from each well of the

University of Ghana http://ugspace.ug.edu.gh

hybridoma cells in culture following cell fusion. The microplate ELISA experiment was then carried out as described in section 3.2.5 to determine the reactivity of the various MAbs to PAgHU and CPfAg.

7.2.3 Prevalence of Malaria in the Sample Population by MAb Microplate ELISA

Microplate ELISA was carried out using one of the most reactive monoclonal antibodies (UCP4W7) as determined above, against direct unconcentrated urine samples from 420 individuals. This was done in order to compare the sensitivity and specificity of UCP4W7 with those of microscopy, quantitative buffy coat (QBC) test and polyclonal antibody ELISA. The 420 individuals were study participants whose blood samples had previously been examined by microscopy and QBC. The microtitre plates were coated (50 µl/ well) with urine sample preparation comprising 25 µl of urine from each participant and 25 µl of phosphate buffered saline (PBS, pH 7.2). The wells of the plate were coated overnight at 4^{o}C, rinsed 2 times with excess washing buffer and then blocked (200 µl/ well) with blocking buffer comprising 5% w/v casein (Wako Pure Chemical Industries Ltd., Japan, Cat. No. 030-01505) in Dulbecco's PBS (DPBS, pH 7.2) for 1hr.

The wells were then incubated with the UCP4W7 MAb (100 µl/ well) for 2hrs at room temperature (25^{o}C), flipped empty, banged on tissue paper to dislodge excess unbound MAb, and then washed 2 times with washing buffer. The wells were then incubated (50 µl/ well) with secondary antibody (goat anti-mouse polyclonal IgGAM conjugated with horse raddish peroxidase) (Abcam, Cambridge, UK; Cat No. ab6006), diluted (1:2000) in DPBS blocking buffer. The plates were flipped empty as before, banged and washed 5 times. ABTS [2,2 bis-azino-di-(3-ethylbenzthiazoline-6-sulphonate)] substrate solutions A and B were added (100 µl/

University of Ghana http://ugspace.ug.edu.gh

well), which gave a green colour reaction for positive results. Optical densities (ODs) of ELISA results were measured within 30 min using spectrophotometer (Multiskan Ascent Microplate Reader, Model 354; ThermoLab systems, Finland) at 414 nm. Cut off point (Cf) for negative reactivity was determined as described in section 6.2.7 above.

After determination of the positive and negative results, the sensitivity and specificity of the UCP4W7 ELISA were determined using microscopy as a gold standard test.

7.2.4 Prevalence of Malaria in the Sample Population by anti-*Plasmodium* PAb Microplate ELISA

Microplate ELISA to determine the reactivity of anti-*Plasmodium* PAbs to the 420 direct urine samples examined above was carried out as described for the UCP4W7 MAb above. Except for the PAb which was incubated (50 µl/ well) at 1:1250 dilution, all other aspects of the ELISA as well as the estimation of the relative sensitivity and specificity, were carried out as described in section 7.2.3 above.

7.2.5 Reactivity of Anti-*Plasmodium* PAbs to Different Antigen Preparations by Western Blotting Analysis

Following the characterization of the proteins in the different antigen preparations by SDS-PAGE as described in sections 5.2.1 and 5.2.2 above, western blotting assay was used to determine the reactivity of the anti-mouse PAb to the different protein bands obtained as described in section 5.2.3 above.

University of Ghana http://ugspace.ug.edu.gh

7.2.6 Reactivity of UCP4W7 MAb to different antigen preparations by SDS-PAGE and western blotting assay-1

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to characterize the proteins in different fractions of urine and cultured *Plasmodium* crude antigen extract as described in sections 5.2.1 and 5.2.2 above.

Following the separation of proteins in different sample preparations by SDS-PAGE, western blotting assay was carried out to determine the reactivity of the UCP4W7 MAb to the various protein bands using the chemilluminescent detection method described by Mathews *et al.* *(*2009) with modification.

After SDS-PAGE, the gel was washed in transfer buffer for 10 min, with gentle shaking. PVDF membrane about the same size as the gel (75 cm^2) was pre-soaked in 100% methanol for 15 sec with gentle rocking and then transferred into the Invitrogen NuPAGE® Transfer Buffer (Life Technologies Corporation, California, USA) to soak for 5min with gentle rocking. The gel, the membrane and the pieces of blotting paper were assembled as illustrated in Figure 18 below, and then run for 1hr at 90mA.

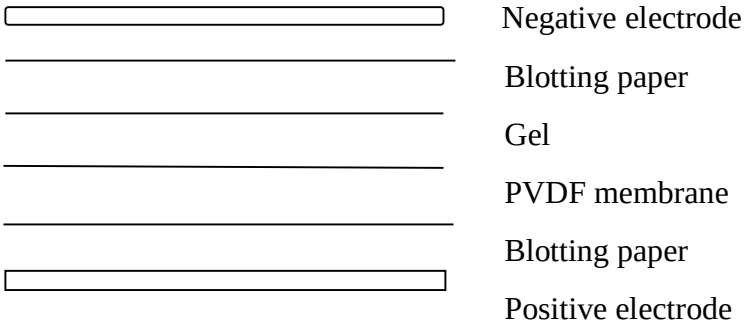


Figure 18: Mode of arrangement of the western blotting apparatus and gel system for western blotting analysis

University of Ghana http://ugspace.ug.edu.gh

The membrane was washed with excess PBS for 5min after protein transfer and then washed again with PBST (0.05% Tween 20 in PBS) for 15min. The membrane was transferred into blocking buffer, made up of 3% bovine serum albumin (BSA) in PBST to block overnight at 4^{o}C. Following blocking, the membrane was incubated with the primary antibody solution at 1:500 dilution of UCP4W7 MAb in blocking buffer for 1hr at 37^{o}C with gentle rocking. The membrane was washed in excess PBS for 5min, and then in PBST for 15min with gentle rocking. The membrane was incubated with 1:2000 dilutions of the secondary antibody solution (horse raddish peroxidase conjugated goat anti-mouse polyclonal IgGAM antibody) in blocking buffer for 1hr at 37^{o}C. The membrane after incubation was washed once more with excess PBS for 5min and in PBST for 15min.

The membrane was then soaked in the immobilon western chemilluminescence reagent A and B (Millipore) with rocking for 5min to develop and then photographed using the Ez-Capture II ATTO Cooled CCD Camera System (ATTO Corporation, Japan) at 1min exposure.

7.2.7 Reactivity of UCP4W7 MAb to different antigen preparations by SDS-PAGE and western blotting assay-2

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to characterize the proteins in different fractions of urine, cultured *Plasmodium* crude antigen extract as well as antigens and vaccines of non-malarial parasites. The experiment was carried using the method described in sections 5.2.1 and 5.2.2 above.

Following the separation of proteins in different sample preparations by SDS-PAGE, western blotting assay was carried out to determine the reactivity of the UCP4W7 MAb to the various protein bands obtained as described in section 7.2.7 above.

University of Ghana http://ugspace.ug.edu.gh

7.2.8 Estimation of molecular weights of unknown proteins

Following SDS-PAGE, the mobility (retardation factor, R_f) of the various protein bands in the gel, was calculated for the molecular weight (MW) protein standard as well as for the different antigen preparations, using the formula:

$$\mathbf{R_f} = \frac{\mathbf{d2}}{\mathbf{d1}}$$

Where $\mathbf{R_f}$ is the mobility (retardation factor) of the various proteins in the gel,

di, measured in centimetres (cm), is the distance migrated by the tracking dye front, and is equivalent to the full length of the gel,

d2 (cm) is also the distance travelled by the protein in the gel, measured from the top of the gel to the position of the band in the gel.

A graph of the logarithm of molecular weight (log MW) against R_f was plotted and the line of best fit was drawn. Following this, the R_f of each protein band whose MW was to be determined was located on the x-axis and then followed vertically to the line of best fit from which the log MW of each protein was extrapolated. The antilog of the log MW value of each protein band was then taken to determine the approximate molecular weight (MW_1) of the protein.

Also, to improve the accuracy of the MW estimates, the equation of the line of best fit was determined as $y = -1.1868x + 5.1538$, where "y" represented the log MW and "x" was the R_f value for the protein. Using this equation, the R_f of each protein was substituted to determine the corresponding log MW of each protein and the antilog was read to obtain the second approximate

University of Ghana http://ugspace.ug.edu.gh

molecular weight (MW_2) for each protein. The final approximate molecular weight (MW) for each protein was then determined by taking the mean ($MW_1 + MW_2/2$) value of the two molecular weight readings estimated.

7.3.0 RESULTS

7.3.1 Optimum Conditions for ELISA to Ascertain the Reactivity of Anti-*Plasmodium* PAbs and MAbs

Coating plates overnight at 4^{o}C and 30 min at room temperature (22-25^{o}C) gave similar ELISA outcomes (OD values). Coating of plates overnight at 4^{o}C, however, had certain advantages. Firstly, it saves time. Enables trouble shooting to be carried out promptly for solution to unexpected results. Therefore, coating of plates overnight at 4^{o}C was selected for carrying out all ELISA experiments.

Also, coating urinary *Plasmodium* antigens on microtitre plates using carbonate-bicarbonate coating buffer (CCB) and PBS gave similar ELISA outcomes (ODs), however, PBS was selected as the routine buffer for coating of plates to run ELISA because of additional advantages over the CCB. These additional advantages are 1) PBS is the most abundant buffer in our laboratory, and 2) PBS is more readily available (obtainable) in our laboratory than CCB.

Finally, upon comparing the immulon 2 and Nunc microtitre plates for conduct of the ELISA experiments, the immulon 2 plates gave a better output in binding *Plasmodium* antigens in terms of distinguishing between negative and positive control results.

7.3.2 Reactivity of mouse anti-*Plasmodium* MAbs to urinary and cultured parasite antigens by microplate ELISA-1

University of Ghana http://ugspace.ug.edu.gh

Table 12 showed the reactivity of different clones of mouse anti-*Plasmodium* MAbs to parasite antigens, PAgHU and CPfAg, extracted from the urine of infected individuals and cultured parasites respectively. Generally, the results showed that all the MAb clones were more reactive to the urinary *Plasmodium* antigen extract (PAgHU) than the cultured parasite antigen extract (CPfAg).

According to the results, all the 48 (100%) MAb clones showed positive reactivity to PAgHU, but only 20 (41.7%) of them reacted positively to CPfAg. Of the 48 clones that reacted positively to PAgHU, 8 (16.7%) clones numbered 4 to 8, 26, 27 and 37; with code names UCP1W4 to UCP1W8, UCP2W2, UCP2W3 and UCP2W13 showed strong positive (2+) reactivity. 23 (47.9%) were moderate positivity (+), while the remaining 17 (35.4%) showed weak positive (+/-) reactivity.

On the other hand, of the 20 clones that reacted positively to CPfAg, only 1 (2.1%) numbered 8, and coded UCP1W8 showed moderate positive reactivity. The remaining 19 (39.6%), clones showed weak positive reactivity to CPfAg.

Finally, whereas all the MAb clones were reactive to PAgHU, 28 (58.3%) showed no reactivity to CPfAg.

University of Ghana http://ugspace.ug.edu.gh

Table 12: Reactivity of anti-*Plasmodium* MAbs to urinary and cultured parasite antigen extracts-1

Sample		Monoclonal Antibody Reactivity Absorbance OD (Grading)		Sample		Monoclonal Antibody Reactivity Absorbance OD (Grading)	
No	**Code**	**PAgHU**	**CPfAg**	**No**	**Code**	**PAgHU**	**CPfAg**
1	UCP_1W_1	0.54 **(+)**	0.24 **(+/-)**	25	UCP_2W_1	1.43 **(+)**	0.09 **(-)**
2	UCP_1W_2	1.18 **(+)**	0.21 **(+/-)**	26	UCP_2W_2	1.95 **(2+)**	0.11 **(-)**
3	UCP_1W_3	1.01 **(+)**	0.38 **(+/-)**	27	UCP_2W_3	1.84 **(2+)**	0.10 **(-)**
4	UCP_1W_4	1.59 **(2+)**	0.18 **(+/-)**	28	UCP_2W_4	1.13 **(+)**	0.16 **(+/-)**
5	UCP_1W_5	1.51 **(2+)**	0.24 **(+/-)**	29	UCP_2W_5	0.47 **(+/-)**	0.08 **(-)**
6	UCP_1W_6	2.40 **(2+)**	0.43 **(+/-)**	30	UCP_2W_6	0.39 **(+/-)**	0.08 **(-)**
7	UCP_1W_7	1.92 **(2+)**	0.21 **(+/-)**	31	UCP_2W_7	0.87 **(+)**	0.09 **(-)**
8	UCP_1W_8	1.57 **(2+)**	0.78 **(+)**	32	UCP_2W_8	0.91 **(+)**	0.12 **(-)**
9	UCP_1W_9	1.20 **(+)**	0.15 **(+/-)**	33	UCP_2W_9	0.36 **(+/-)**	0.08 **(-)**
10	UCP_1W_{10}	1.39 **(+)**	0.18 **(+/-)**	34	UCP_2W_{10}	1.06 **(+)**	0.18 **(+/-)**
11	UCP_1W_{11}	1.23 **(+)**	0.11 **(-)**	35	UCP_2W_{11}	0.28 **(+/-)**	0.08 **(-)**
12	UCP_1W_{12}	1.37 **(+)**	0.46 **(+/-)**	36	UCP_2W_{12}	0.68 **(+)**	0.09 **(-)**
13	UCP_1W_{13}	0.21 **(+/-)**	0.08 **(-)**	37	UCP_2W_{13}	2.23 **(2+)**	0.14 **(+/-)**
14	UCP_1W_{14}	0.47 **(+/-)**	0.08 **(-)**	38	UCP_2W_{14}	0.29 **(+/-)**	0.08 **(-)**
15	UCP_1W_{15}	0.23 **(+/-)**	0.07 **(-)**	39	UCP_2W_{15}	0.53 **(+)**	0.09 **(-)**
16	UCP_1W_{16}	0.38 **(+/-)**	0.08 **(+/-)**	40	UCP_2W_{16}	0.54 **(+)**	0.08 **(-)**
17	UCP_1W_{17}	0.34 **(+/-)**	0.08 **(-)**	41	UCP_2W_{17}	0.31 **(+/-)**	0.24 **(+/-)**
18	UCP_1W_{18}	0.34 **(+/-)**	0.08 **(-)**	42	UCP_2W_{18}	0.29 **(+/-)**	0.08 **(-)**
19	UCP_1W_{19}	0.58 **(+)**	0.08 **(-)**	43	UCP_2W_{19}	0.75 **(+)**	0.08 **(-)**
20	UCP_1W_{20}	0.38 **(+/-)**	0.09 **(-)**	44	UCP_2W_{20}	1.00 **(+)**	0.21 **(+/-)**
21	UCP_1W_{21}	0.70 **(+)**	0.08 **(-)**	45	UCP_2W_{21}	0.77 **(+)**	0.08 **(-)**
22	UCP_1W_{22}	0.74 **(+)**	0.14 **(+/-)**	46	UCP_2W_{22}	0.55 **(+)**	0.13 **(-)**
23	UCP_1W_{23}	0.37 **(+/-)**	0.08 **(-)**	47	UCP_2W_{23}	0.57 **(+)**	0.13 **(+/-)**
24	UCP_1W_{24}	0.46 **(+/-)**	0.09 **(-)**	48	UCP_2W_{24}	0.30 **(+/-)**	0.19 **(+/-)**

University of Ghana http://ugspace.ug.edu.gh

7.3.3 Reactivity of mouse anti-*Plasmodium* MAbs to urinary and cultured parasite antigens by microplate ELISA-2

Table 13 showed the continuation of the reactivity of different clones of mouse anti-*Plasmodium* monoclonal antibodies to parasite antigens, PAgHU and CPfAg, extracted from the urine of infected individuals and cultured parasites, respectively. Generally, these results also showed that all the MAb clones were more reactive to the PAgHU than to CPfAg.

According to these results, all the 48 (100%) clones were reactive to both PAgHU and CPfAg. Of the clones that were reactive to PAgHU, 10 (20.8%) showed hyperpositive (3+) reactivity, 12 (25.0%) were strong positive (2+), 25 (52.1%) were moderate positive (+) and only 1 (2.1%) showed a weak positive (+/-) reactivity. Of the clones that were reactive to CPfAg, however, only 1 (2.1%) showed a hyperpositive reactivity. Two (4.2%) showed a strong positive reactivity, 16 (33.3%) were moderate positive, while the remaining majority of 29 (60.4%) clones were weak positive.

University of Ghana http://ugspace.ug.edu.gh

Table 13: Reactivity of mouse anti-*Plasmodium* MAbs to urinary and cultured *Plasmodium* antigen extracts

Sample		Monoclonal Antibody Reactivity Absorbance OD (Grading)		Sample		Monoclonal Antibody Reactivity Absorbance OD (Grading)	
No	**Code**	**PAgHU**	**CPfAg**	**No**	**Code**	**PAgHU**	**CPfAg**
49	UCP_3W_1	0.42 **(+/-)**	0.31 **(+/-)**	73	UCP_4W_1	2.74 **(3+)**	0.25 **(+/-)**
50	UCP_3W_2	1.77 **(2+)**	0.55 **(+)**	74	UCP_4W_2	2.86 **(3+)**	0.41 **(+/-)**
51	UCP_3W_3	1.67 **(2+)**	1.85 **(2+)**	75	UCP_4W_3	2.88 **(3+)**	0.21 **(+/-)**
52	UCP_3W_4	2.38 **(2+)**	1.29 **(+)**	76	UCP_4W_4	1.91 **(2+)**	0.54 **(+)**
53	UCP_3W_5	2.19 **(2+)**	0.51 **(+)**	77	UCP_4W_5	0.91 **(+)**	0.38 **(+/-)**
54	UCP_3W_6	2.80 **(3+)**	1.32 **(+)**	78	UCP_4W_6	0.62 **(+)**	0.18 **(+/-)**
55	UCP_3W_7	2.57 **(3+)**	0.53 **(+)**	79	UCP_4W_7	1.73 **(2+)**	0.92 **(+)**
56	UCP_3W_8	2.82 **(3+)**	2.13 **(2+)**	80	UCP_4W_8	2.87 **(3+)**	0.29 **(+/-)**
57	UCP_3W_9	1.98 **(2+)**	0.86 **(+)**	81	UCP_4W_9	0.92 **(+)**	0.28 **(+/-)**
58	UCP_3W_{10}	2.14 **(2+)**	0.69 **(+)**	82	UCP_4W_{10}	2.02 **(2+)**	0.39 **(+/-)**
59	UCP_3W_{11}	2.51 **(3+)**	0.46 **(+/-)**	83	UCP_4W_{11}	0.76 **(+)**	0.28 **(+/-)**
60	UCP_3W_{12}	2.98 **(3+)**	3.08 **(3+)**	84	UCP_4W_{12}	1.34 **(+)**	0.20 **(+/-)**
61	UCP_3W_{13}	0.60 **(+)**	0.60 **(+)**	85	UCP_4W_{13}	3.10 **(3+)**	0.56 **(+)**
62	UCP_3W_{14}	1.40 **(+)**	0.72 **(+)**	86	UCP_4W_{14}	0.67 **(+)**	0.26 **(+/-)**
63	UCP_3W_{15}	0.64 **(+)**	0.77 **(+)**	87	UCP_4W_{15}	1.43 **(+)**	0.25 **(+/-)**
64	UCP_3W_{16}	0.88 **(+)**	0.19 **(+/-)**	88	UCP_4W_{16}	1.11 **(+)**	0.18 **(+/-)**
65	UCP_3W_{17}	0.90 **(+)**	0.17 **(+/-)**	89	UCP_4W_{17}	0.50 **(+)**	0.74 **(+)**
66	UCP_3W_{18}	0.99 **(+)**	0.17 **(+/-)**	90	UCP_4W_{18}	0.78 **(+)**	0.27 **(+/-)**
67	UCP_3W_{19}	1.43 **(+)**	0.16 **(+/-)**	91	UCP_4W_{19}	1.30 **(+)**	0.16 **(+/-)**
68	UCP_3W_{20}	1.04 **(+)**	0.35 **(+/-)**	92	UCP_4W_{20}	1.70 **(2+)**	0.26 **(+/-)**
69	UCP_3W_{21}	1.67 **(2+)**	0.29 **(+/-)**	93	UCP_4W_{21}	1.31 **(+)**	0.52 **(+)**
70	UCP_3W_{22}	1.86 **(2+)**	0.45 **(+/-)**	94	UCP_4W_{22}	1.47 **(+)**	0.46 **(+/-)**
71	UCP_3W_{23}	0.84 **(+)**	0.31 **(+/-)**	95	UCP_4W_{23}	1.25 **(+)**	0.58 **(+)**
72	UCP_3W_{24}	1.16 **(+)**	0.25 **(+/-)**	96	UCP_4W_{24}	0.82 **(+)**	0.30 **(+/-)**

University of Ghana http://ugspace.ug.edu.gh

7.3.4 Summary of the reactivity of mouse anti-*Plasmodium* MAbs to urinary and cultured parasite antigens

Table 14 below summarized the reactivity of 96 mouse anti-*Plasmodium* MAb clones to PAgHU and CPfAg. As shown, the 96 MAb clones were more reactive to PAgHU than to CPfAg. Also, in terms of high reactivity strength, whereas 10/96 (10.4%) MAbs clones were hyperpositive (**3+**), 20 (20.8%) were strong positive (**2+**) and 48 (50.0%) were moderate positive (+) in reactivity to PAgHU. In contrast, only 1 (1.0%), 3 (3.1%) and 16 (16.7%) clones respectively, showed reactivity to CPfAg (Table 14).

In relation to weak positive and negative reactivity, although 18/96 (18.8%) and no (0.0%) MAb clones were reactive to PAgHU more, 48 (50.0%) and 28 (29.2%) MAbs respectively, showed reactivity to CPfAg.

Table 14: Summary of monoclonal antibody reactivity results

Reactivity strength		**No of Monoclonal antibody clones reactive to**	
Name	**Grade**	**PAgHU No (%)**	**CPfAg No (%)**
Hyperpositive	3+	10 (10.4)	1 (1.0)
Strong positive	2+	20 (20.8)	3 (3.1)
Moderate positive	+	48 (50.0)	16 (16.7)
Weak positive	+/-	18 (18.8)	48 (50.0)
Negative	-	0 (0.0%)	28 (29.2)
Total		**96 (100.0)**	**96 (100.0)**

University of Ghana http://ugspace.ug.edu.gh

7.3.5 Reactivity of UCP4W7 MAb to different antigen preparations by SDS-PAGE and western blotting assay-1

Figure 19a showed the results of the separation of proteins in different antigen preparations by SDS-PAGE. As shown, PAgHU (lane 2) and the blood sample from a malaria negative individual from USA (lane 3) had more (>10) clearly visible protein bands each, than the rest of the various antigens separated by SDS-PAGE. These protein bands included those with estimated molecular weights (MW) of about 27.2, 32, 52 and 63.2 kDa.

CPfAg (lane 4), had the next highest number (4) of clearly visible protein bands, which included proteins of MW 10.2, 13.5, 32 and 63.2 kDa. It was followed by the urine sample from a true malaria negative individual from Ghana (GH, lane 6), which also had 2 clearly visible bands of MW between 63.2 and 75.4 kDa. The malaria negative urine sample from JHU (lane 5), showed the least number of visible bands with an MW of about 13.5 kDa.

The remaining antigens showed some bands which however, were not clear enough. Among this category of antigens was the Polio vaccine which showed bands that have joined together to form a smear. Lane 1 was occupied by the prestained SDS-PAGE broad range molecular weight marker

Figure 19b, adjacent the coommassie blue gel electrophoregram, showed the results of the reactivity of the UCP4W7 MAb to the proteins separated by SDS-PAGE. As shown, the UCP4W7 MAb reacted with 10 protein bands in PAgHU, 4 of which were very sharp while the remaining 6 were faint (weak). The 4 sharp protein bands had MWs of about 27.2, 32, 63.2 and 75. 4 kDa.

Also, UCP4W7 showed a high reactivity with 2 protein bands of MW 27.2 and 75.4 kDa in the cultured *Plasmodium* parasite antigen (CPfAg). The remaining antigens however, did not show any observable reactivity with the UCP4W7 MAb.

University of Ghana http://ugspace.ug.edu.gh

1 = Molecular weight marker

2 = Human urinary *Plasmodium* antigen extract (PAgHU)

3 = Malaria negative blood sample

4 = Cultured *Plasmodium falciparum* crude antigen (CPfAg)

5 = Malaria negative urine from USA (JHU –Ve)

6 = Malaria negative urine from Ghana (GH –Ve)

7= *Schistosoma mansoni* soluble egg antigen (*Sm*. SEA)

8 = *Schistosoma haematobium* adult worm antigen (*Sh.* AWA)

9= Polio Vaccine

10= Measles vaccine

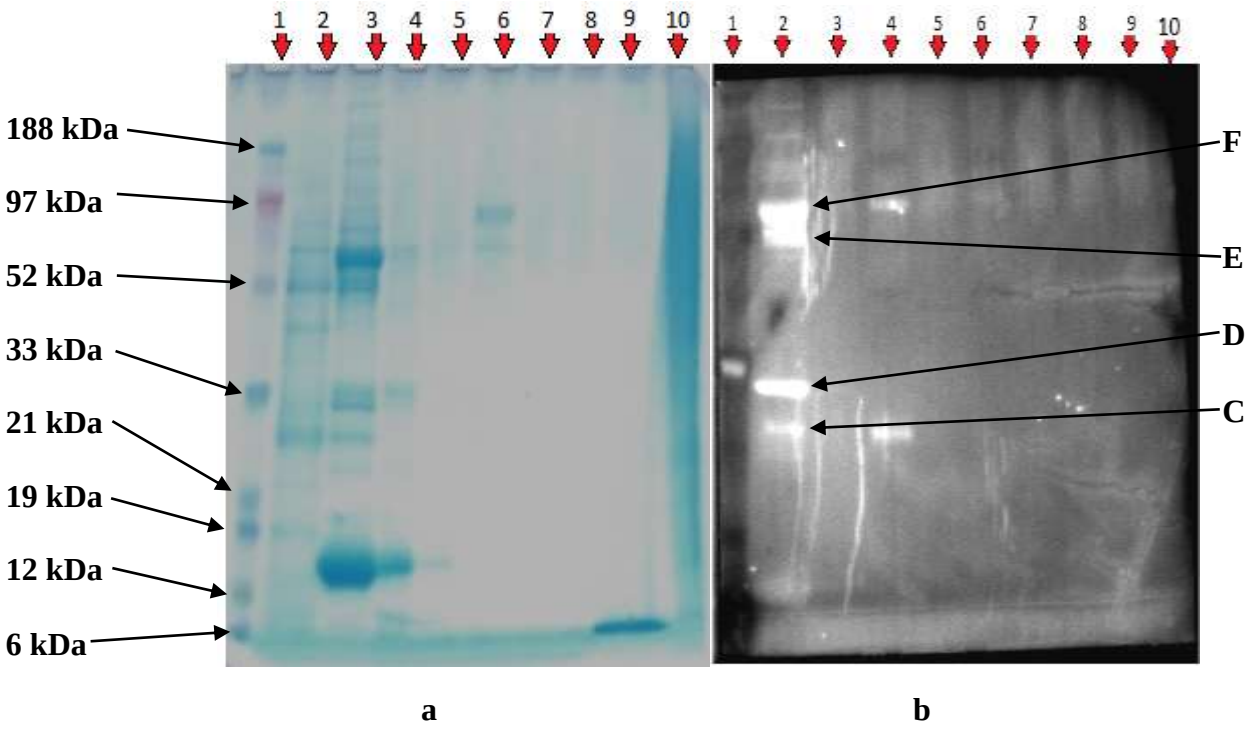


Figure 19: a) Analysis of proteins in different antigen preparations by SDS-PAGE-1 and b) UCP4W7 probed western blotting assay-1.

University of Ghana http://ugspace.ug.edu.gh

7.3.6 Separation of proteins in different antigen preparations by SDS-PAGE and reactivity of UCP4W7 by western blotting assay-2

Figure 20a summarized the results of the separation of proteins in different antigen preparations by SDS-PAGE. As shown, with the exception of the JHU negative urine sample which had bands that were not distinguishable, all the proteins exhibited multiple bands after staining. Among the protein profiles with the multiple bands, the malaria negative urine sample from Ghana (GH-ve, lane 8), had the profile with the least number (2) of bands with estimated molecular weight (MW) being 63.2 and 75.4 kDa.

CPfAg (lane 6), had 4 bands, 2 of which were clearly visible and the other 2 being faint. The 2 sharp bands were proteins of MWs being 13.5 and 63.2 kDa, while the weak bands had MWs of 10.2 and 32 kDa. The 2 direct urine samples (DUMIs) from the 3+ (DUMI-2, lane 2) and 4+ (DUMI-1, lane 3) malaria positive individuals had the next highest number of bands. Each of these 2 samples with similar protein profiles had eight (8) visible bands, 4 of which were most prominent. These prominent protein bands had MWs being 27.2, 32, 45.6 and 63.2 kDa.

The remaining samples comprising the PAgHU (lane 4), the malaria negative blood sample (MNBS, lane 5) and the yellow fever vaccine (lane 9), had protein profiles with the highest number of bands (>10) each. Of these, the yellow fever vaccine which apparently had the highest number of bands, had most of the bands within the upper part of the profile joining together to form a smear.

Furthermore, the protein profile of the MNBS had a 13.5 kDa protein band (**B**) which was also present in the CPfAg and the yellow fever vaccine, but not in the profile of any other antigen preparation. In addition, MNBS had a 63.2 kDa band (**E**) which was also observed in all the other protein profiles. Finally, the results showed that the band sizes of proteins **B** and **E** were relatively bigger and darker than all the other bands observed in the entirety of the gel.

Figure 20b showed the results of the reactivity of the UCP4W7 MAb with the bands in the protein profiles of the different antigen preparations as determined by western blotting assay. The

University of Ghana http://ugspace.ug.edu.gh

results showed that generally, the UCP4W7 MAb was reactive to PAgHU, CPfAg, and DUMIs, but did not react with MNBS or the GH-ve and US-ve urine samples.

The UCP4W7 MAb reacted specifically with more than 3 similar bands within the profiles of the DUMIs (lanes 2 and 3). The most prominent of these bands, labelled **Z**, with MW <6 kDa, was present only in the DUMIs, but not in PAgHU, CPfAg, MNBS, GH-ve or US-ve urine samples. The other prominent bands included a 27.2 kDa protein labelled **C** which was also present in CPfAg with strong reactivity, but in PAgHU with a weak reactivity (even though **C** showed a very strong reactivity in Figure 19b). This band was however not present in MNBS, GH-ve or US-ve. The last prominent band in the DUMIs that UCP4W7 reacted with was a 63.2 kDa protein labelled **E**, which was also present in PAgHU but not in either CPfAg, MNBS, GH-ve or US-ve.

Also, the UCP4W7 MAb reacted with multiple bands within the profile of PAgHU. Three of these bands had the strongest reactivity and were protein **D** (32 kDa), a 63.2 kDa protein labelled **E** as well as a 75.4 kDa protein labelled **F**. Whereas protein **D** was detected in only PAgHU, **F** was also detected in only CPfAg. CPfAg also had 2 bands reacting strongly with the UCP4W7 MAb. These bands were the 27 and 63.9 kDa proteins, labelled **C** and **F** respectively and mentioned above.

Furthermore, there were several bands within the yellow fever vaccine protein profile and one band in the molecular weight marker (lane 1), which reacted non-specifically with the UCP4W7 MAb. Lastly, there were no bands within the MNBS as well as GH-ve or US-ve that showed observable reactivity with the UCP4W7 MAb.

University of Ghana http://ugspace.ug.edu.gh

1 = Molecular weight marker

2 = 3+ Malaria positive urine

3= 4+ Malaria positive urine

4 = Human urinary *Plasmodium* antigen extract (PAgHU)

5 = Malaria negative blood sample

6 = Cultured *Plasmodium falciparum* crude antigen (CPfAg)

7 = Malaria negative urine from USA (JHU –Ve)

8 = Malaria negative urine from Ghana (GH –ve)

9 = Yellow fever vaccine

3+ = 50-100,000 parasites/µL of blood

4+ = >100,000 parasites/ µL of

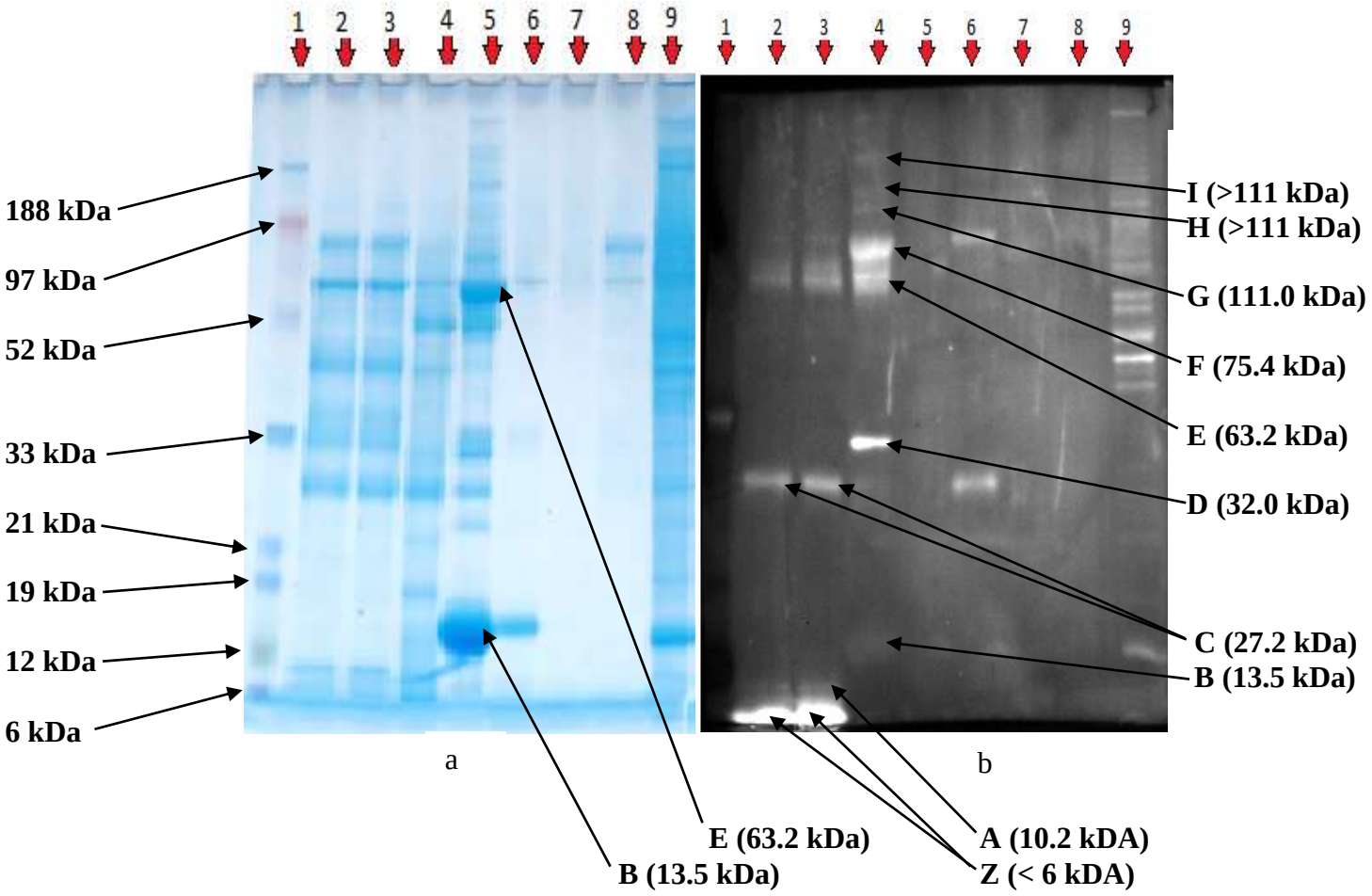


Figure 20: a) Analysis of proteins in different antigen preparations by SDS-PAGE-2. and b): UCP4W7 probed western blotting assay-2.

University of Ghana http://ugspace.ug.edu.gh

7.3.7 Detection of *Plasmodium* Proteins in Different Fractions of Urine and *Plasmodium* Antigens by Western Blotting Analysis

Figure 20 showed the results of the reactivity of mouse anti-*Plasmodium* polyclonal antibodies (PAbs) with 6 of the antigen preparations resolved by SDS-PAGE in sections 5.2.1 and 5.2.2 above, using western blotting assay. As shown, the mouse anti-*Plasmodium* PAb reacted with bands in protein profiles of only 2 of the 6 antigen preparations analyzed. These antigen preparations were the PAgHU and the direct urine sample from a 3+ malaria positive individual (DUMI-2). The PAb reacted with the 50.9 kDa antigen (**Ω**) and the 54.6 kDa antigen (**β**) within PAgHU of which **Ω** was bigger and darker than **β**.

Also, the PAb reacted with 3 bands within DUMI-1, which were the 27.2, 46 and 51 kDa proteins labelled **π**, **δ** and **λ** respectively. Furthermore, these results showed that the protein band **λ** was larger and darker than **δ** and **π**.

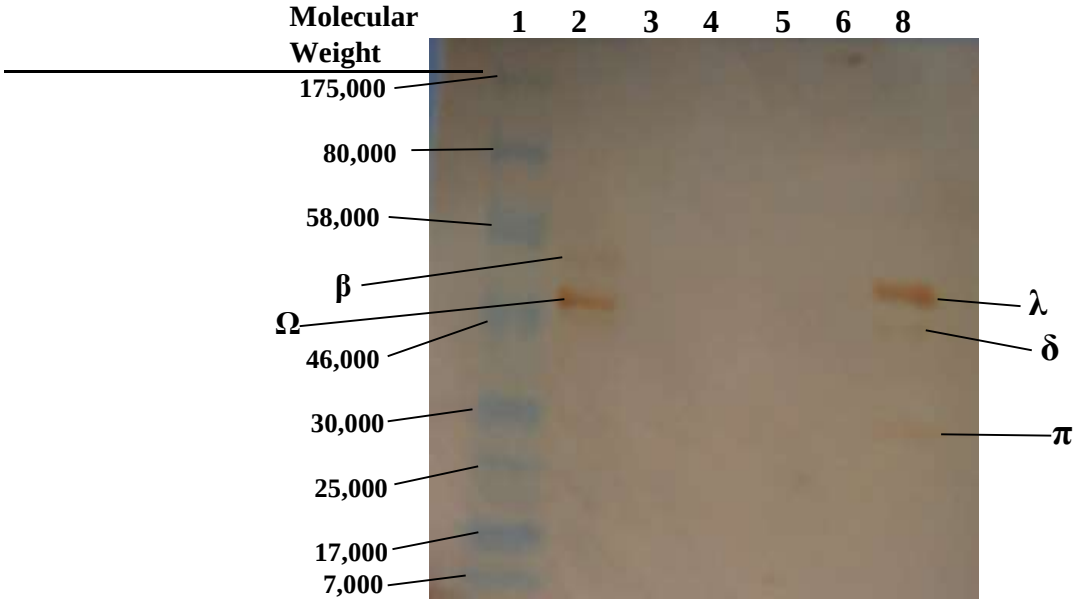


Figure 21: Western blotting results

Lane 1 Prestained broad range protein molecular weight marker
Lane 2 Crude urinary *Plasmodium* antigen extract (PAgHU)
Lane 3 Urine from *Plasmodium* negative individual from Ghana
Lane 4 Urine from *Plasmodium* negative individual from USA

University of Ghana http://ugspace.ug.edu.gh

Lane 5 Cultured *P. falciparum* crude antigen extract (CPfAg)
Lane 6 *Plasmodium* MSP119 antigen
Lane 8 Urine from 3+ *Plasmodium* positive individual

7.3.8 Relationship between Molecular Weights of Standard Proteins and Their Mobility through the NuPAGE-MOPS Gel

Table 15 below summarized the relationship of standard molecular weight proteins and their mobility (retardation factor, R_f) through the NuPAGE-MOPS gel as determined SDS-PAGE. As shown, the molecular weight (MW) of the standard proteins increased from a minimum of 6000 Da (6 kDa) to a maximum value of 188000 Da (188 kDa). The corresponding mobility (R_f) of the proteins on the other hand, reduced from a maximum of 0.94 to a minimum of 0.12. These results showed that the MW of the standard proteins were inversely related to their mobility through the gel. The results also showed that the MW of the various proteins were inversely related to the distance travelled through the gel such that the larger the MW of the protein, the shorter the distance travelled through the gel.

Table 15: Relationship between the molecular weights of standard proteins and their mobility through NuPAGE MOPS gel

Known	Molecular weight (MW) of Standard Protein Bands (kDa)	Molecular weight (MW) of Standard Protein Bands (Da)	Log of MW of Standard Protein Bands	Distance travelled in gel (D1) (cm)	Retardation factor, R_f (D1/4.9)
1	6	6000	3.78	4.6	0.94
2	12	12000	4.08	4.4	0.90
3	19	19000	4.28	3.8	0.78
4	21	21000	4.32	3.5	0.71
5	33	33000	4.52	2.6	0.53
6	52	52000	4.72	1.8	0.37
7	97	97000	4.99	1	0.20
8	188	188000	5.27	0.6	0.12

University of Ghana http://ugspace.ug.edu.gh

7.3.9 Molecular Weights of Standard Proteins and Their Mobility through the NuPAGE-MOPS Gel

Figure 21 below summarized the relationship between the molecular weight of standard proteins (Log MW) and the mobility of standard proteins in the gel. The results showed that the relationship between Log MW of standard proteins and the mobility R_f was linear, and the linear relationship could be determined by the equation (Y = -1.1868x + 5.1538).

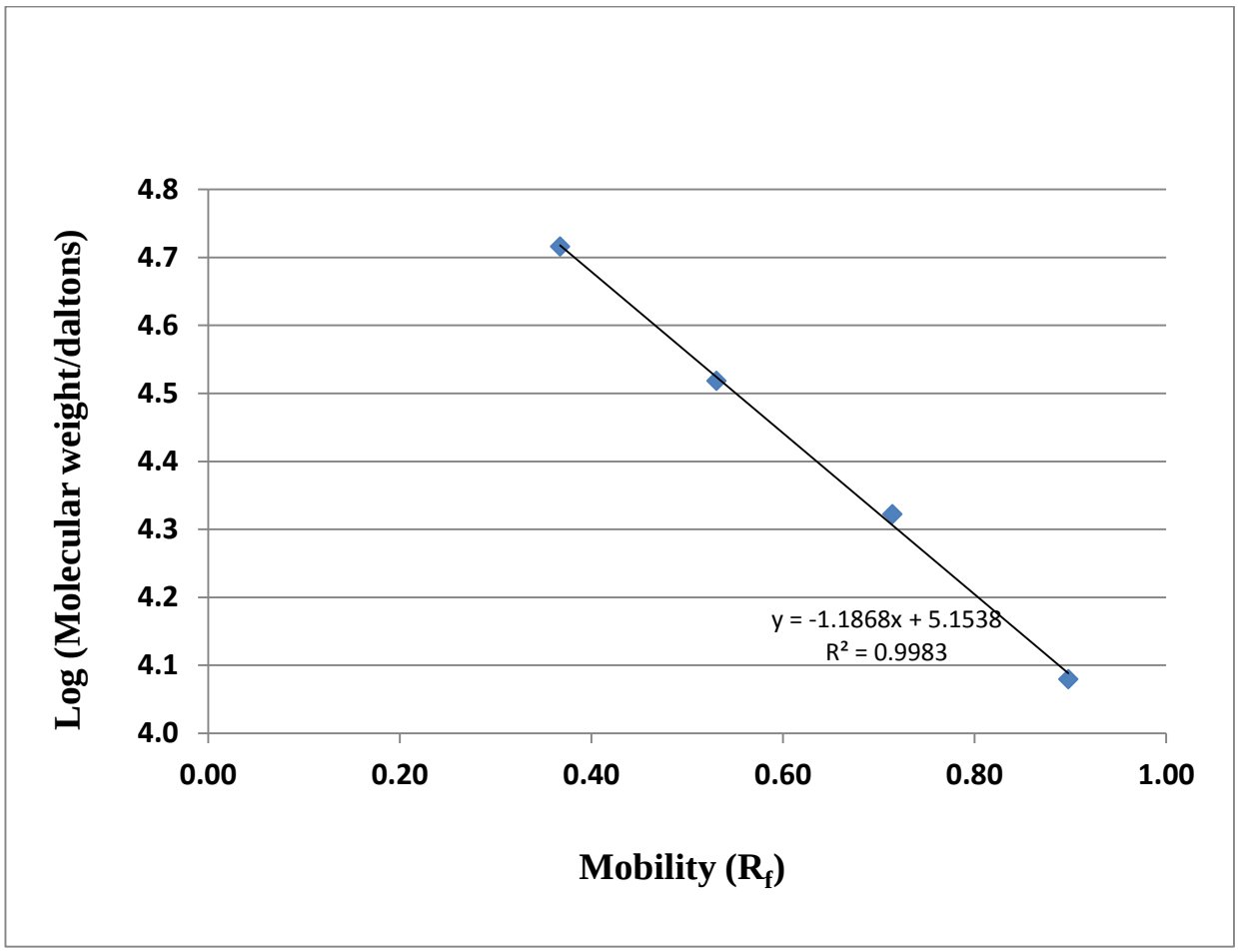


Figure 22: Molecular weights of standard proteins and their mobility through the NuPAGE-MOPS Gel

University of Ghana http://ugspace.ug.edu.gh

7.3.10 Estimation of Molecular Weights (MW1) of Unknown Proteins

Tables 16, 17 and 18 below, showed the estimation of the molecular weights of the different proteins in the various protein profiles that were obtained by SDS-PAGE. As shown, the molecular size (MW) of each protein was inversely related to the distance travelled in the gel such that the larger the MW, the shorter the distance moved within the gel. Also, the MW of the proteins ranged from a minimum of 10.2 kDa for the protein labelled **A**, which moved the longest distance within the gel than all the other proteins. Protein **G** which moved the shortest distance among the proteins in the table had the largest molecular size.

The tables also showed that apart from proteins **A** and **B** which had the same MW values as estimated by both extrapolation from the graph and calculation from the equation of the best line of fit, all the other proteins had different MW values when determined by the two different approaches.

Table 16: Estimation of molecular weights (MW1) of unknown proteins

Unknown protein bands	Distance covered on gel (d2)	Rf (d2/4.9)	y-value (log MW)	Antilog	Estimated MW_1 kDa
A	4.7	0.96	4.01	10232.92992	10.2
B	4.2	0.86	4.13	13489.62882	13.5
C	3.0	0.61	4.44	27542.28703	27.5
D	2.7	0.55	4.51	32359.36569	32.4
E	1.4	0.29	4.79	61659.50019	61.7
F	1.1	0.22	4.86	72443.59601	73.2
G	0.4	0.08	5.03	107151.93052	107.1

University of Ghana http://ugspace.ug.edu.gh

Table 17: Estimation of molecular weights (MW2) of unknown proteins

Unknown protein bands	R_f (d2/4.9)	y-value (log MW)	antilog	Estimated MW_2 (kDa)
A	0.96	4.01	10232.93	10.2
B	0.86	4.13	13489.6	13.5
C	0.61	4.43	26915.34	26.9
D	0.55	4.50	31622.78	31.6
E	0.29	4.81	64565.42	64.6
F	0.22	4.89	77624.71	77.6
G	0.08	5.06	114815.4	114.8

Table 18: Mean molecular weights of unknown proteins

Unknown proteins	Estimated MW_1 (kDa)	Estimated MW_2 (kDa)	Mean Estimated MW (kDa)
A	10.2	10.2	10.2
B	13.4	13.7	13.5
C	27.3	26.7	27.2
D	32.1	31.6	32.0
E	62.1	65.3	63.2
F	73.2	77.2	75.4
G	107.4	114.0	111.0

7.3.11 Relative sensitivity and specificity of tests for different biomarkers of malaria

Table 19 summarized the relative sensitivity (RSe) and specificity (RSp) of tests for different biomarkers of malaria using microscopy as the gold standard. As shown, generally, most of the tests had high specificities which ranged from 82.1% for fever to 99.9% for urobilinogen. Two of the tests, including those for proteinuria and CareStart™ malaria, however, had lower specificities which were 55.6 and 64.6% respectively. The test for anaemia had the lowest specificity which was below 50%.. Unlike the specificities, the sensitivities were rather very low, and mostly below 50%. On individual basis, the commercial

University of Ghana http://ugspace.ug.edu.gh

rapid diagnostic test for malaria was very sensitive (RSe=96.6%) but not specific enough (RSp=64.6%) for diagnosis of malaria. Test for fever was fairly sensitive (RSe=61.0%) and specific (RSp=82.1%) in associating with malaria. The test for urinary proteins was also fairly sensitive (RSe=59.6%) and specific (RSp=55.6%) even though the specificity was lower than that of the test for fever. The tests for microhematuria (blood), leukocytes, bilirubin, urobilinogen, nitrite, ketones and glucose were not sensitive but very specific in detecting biomarkers of malaria compared to microscopy. Microscopy being the gold standard test, was assigned a relative sensitivity and specificity of (100%). The test for urobilinogen had the highest specificity (RSp=99.9%) while that for nitrite showed the lowest sensitivity (RSe=1.9%) in detecting biomarkers of malaria.

Table 19: Sensitivity and specificity of tests for different biomarkers of malaria

Test	Number Tested	Number Positive	Number Negative	Relative Sensitivity (%)	Relative Specificity (%)
Blood smear microscopy[1]	1262	587	675	100	100
CareStart™ malaria[2]	1262	697 (130) [a]	565 (129) [b]	96.6	64.6
Fever	1262	479 (121) [a]	783 (229) [b]	61.0	82.1
Anaemia	1262	655 (364) [a]	607 (296) [b]	49.6	46.1
Micro-hematuria	1262	121 (70) [a]	1141 (521) [b]	8.7	91.9
Proteinuria	1262	648 (298) [a]	614 (239) [b]	59.6	55.6
Leukocytes	1262	101 (66) [a]	1161 (523) [b]	6.0	94.5
Bilirubinuria	1262	27 (7) [a]	1202 (547) [b]	3.4	97.0
Urobilinogen	1262	88 (0) [a]	1174 (500) [b]	15.0	99.9
Nitrite	1262	21 (10) [a]	1203 (557) [b]	1.9	95.7
Ketones	1262	155 (57) [a]	1077 (472) [b]	16.7	89.6
Glucose	1262	19 (7) [a]	1227 (570) [b]	20	97.3

1 Gold Standard test
2 A commercial immunochromatographic (ICT)-based rapid diagnostic test for malaria
a Number of samples that tested negative by Microscopy
b Number of samples that tested positive by Microscopy

University of Ghana http://ugspace.ug.edu.gh

7.3.12 Relative sensitivity and specificity of UCP4W7 ELISA and quantitative buffy coat test for malaria

Table 20 illustrated the relative sensitivity (RS) and specificity (RSp) of UCP4W7 MAb and PAb ELISAs and QBC test for diagnosis of malaria using microscopy as the gold standard. The data showed that the UCP4W7 MAb-based ELISA was very sensitive (RS=96.9%) and highly specific (75.6%) in diagnosing malaria among study participants. The mouse anti-*Plasmodium* polyclonal antibody (PAb) ELISA, on the other hand, was more sensitive (RS=97.4%) than the gold standard microscopy, even though its specificity was poor (RSp=21.3%). The quantitative buffy coat test (QBC) was also sensitive (RS=88.7%) and fairly specific (RSp=66.2%), but relatively less efficient than UCP4W7 ELISA in detecting the disease among the participants. Blood smear microscopy being the gold standard test against which the other tests were evaluated, was assigned a relative sensitivity and specificity of 100%.

Table 20: Sensitivity and specificity of UCP4W7 ELISA and QBC tests

Test	Number Tested	Number Positive	Number Negative	Relative Sensitivity (%)	Relative Specificity (%)
Blood smear microscopy[1]	420	195	225	100	100
QBC[2]	420	245 (72) [x]	175 (26) [y]	88.7	66.2
UCP4W7 MAb ELISA[3]	420	240 (51) [x]	180 (10) [y]	96.9	75.6
PAb ELISA	420	366 (176) [x]	54 (6) [y]	97.4	21.3

1 Gold standard test
2 Quantitative buffy coat test for malaria
3 UCP4W7 monoclonal antibody enzyme linked immunosorbent assay
x Number of samples that tested negative by Microscopy
y Number of samples that tested positive by Microscopy

University of Ghana http://ugspace.ug.edu.gh

7.4.0 Discussion

The aim of the work described in this chapter was to determine the diagnostic applicability of the UCP4W7 MAbs and PAbs produced and described earlier in chapters 3 and 4 of this work, using microplate ELISA and western blotting assay. The rationale was to develop a promising MAb for specific detection of *Plasmodium* antigens in infected human urine to pave the way for the development of a urine-based diagnostic test for malaria.

Determination of optimum conditions for conduct of microplate ELISA

During the experiments to determine the optimum conditions for conducting microplate ELISA to ascertain the reactivity of PAbs and MAbs to urinary and cultured *Plasmodium* parasite antigens, coating of plates overnight at 4^{o}C was selected and used for conducting all ELISA experiments rather than 30 mins at room temperature.

Coating of plates for 30 mins required that antigens for coating were prepared in the morning, followed by dispensing into the plates before allowing them to incubate for 30 mins at room temperature so that the antigens would adsorb onto the wells. This mode of coating is time-consuming, and eventually, caused the time for ELISA, analysis of results, as well as trouble shooting for unexpected results, to extend late into the evening.

During coating of plates overnight at 4^{o}C, however, the preparation of antigens and incubation of plates were all done the previous day. Following the coating of plates, the ELISA experiment itself was carried out the following day without any interference. This option conferred several advantages over the first option, because, it allowed enough time to conduct the ELISA, and analyze the results. Also, in situations where the assay did not perform as expected, there was enough time to do trouble shooting. It was for these reasons therefore that coating of plates overnight at 4^{o}C was used for all ELISA experiments rather than at room temperature for 30 mins.

University of Ghana http://ugspace.ug.edu.gh

Coating of urinary *Plasmodium* antigens onto microtitre plates using carbonate-bicarbonate buffer (CBB, pH 9.6) and phosphate buffered saline (PBS, pH 7.2) gave similar ELISA outcomes (ODs). However, PBS was chosen and used for coating of plates for microplate ELISA because, PBS was the most abundant reagent obtainable in our laboratory, in that it was prepared in large quantities, was obtainable at all times and used at all times.

Finally, upon comparing the immulon 2 and Nunc microtitre plates for conduct of ELISA, the immulon 2 plates gave a better output in binding *Plasmodium* antigens, in terms of their ability to distinguish between blank, negative and positive results. Consequently, the immulon 2 plates were selected and used for all ELISA experiments reported in this thesis.

Reactivity of mouse anti-Plasmodium PAgHU and CPfAg by microplate ELISA

The results showed that both the MAbs and PAbs were reactive to PAgHU and CPfAg (Tables 12, 13 and 14), as well as direct/ unconcentrated urine samples (DUMIs) from malaria infected individuals (Figures 18, 19 and 20), but with different reactivity strengths. The differential reactivity of the MAbs and PAbs to the antigen preparations mentioned could be due to the fact that the antigens that induced the expression and production of the antibodies had different immunogenicities. Also, it could be due to the fact that the *Plasmodium* antigens being reacted to in the different crude protein preparations, occurred in these protein extracts at different concentrations that affects the reactivity of the antibodies detecting them (Murray *et al*., 2008; Yzerman *et al*., 2002). Lastly, it could be because the different antibodies had different degrees of affinity to the antigens being reacted to in the different antigen extracts (Khor *et al*., 2013). Any of the factors stated above could have altered the biochemical activity of either the antigens, the antibodies or both, which therefore led to the patterns of differential reactivity observed in the ELISA experiments.

University of Ghana http://ugspace.ug.edu.gh

The UCP4W7 MAb reacted in microplate ELISA with direct urine samples from microscopy positive and negative individuals (DUMIs). However, they did not react with urine from true malaria negative individuals from Ghana (GH-ve) and USA (US-ve).

The inability of the UCP4W7 MAb to react with some of the microscopy positive urine samples could be due to the fact that they were false positives, as microscopy is capable of giving false positive results, as reported by (Ohrt *et al*., 2002; Rodulfo *et al*., 2007). On the other hand, it is possible that the antigens detected in the majority of the microscopy positive individuals were not expressed in strains of the parasites that infected the discordant microscopy negative individuals (Rosenthal, 2012; Gamboa *et al*., 2010).

Also, the reactivity of UCP4W7 to some of the urine samples from microscopy negative individuals in ELISA (Table 16) could be due to the fact that those individuals were infected but failed to be detected by microscopy. This observation also confirms the findings by other workers that microscopy is not sensitive enough for accurate diagnosis of malaria (Steenkeste *et al*., 2010; Okell *et al*., 2009).

In addition, the ability of the UCP4W7 MAb to react with PAgHU and CPfAg, but not with the true negative urine samples (GH-ve) and (US-ve) suggested that UCP4W7 would be capable of accurately distinguishing between malaria negative and positive individuals and therefore, promising for further exploration and development of a urine-based diagnostic test for malaria.

Resolution of proteins in different antigen preparations by SDS-PAGE

The observation of higher numbers of protein bands, by SDS-PAGE, in the profiles of PAgHU, DUMIs and MNBS, than the rest of the antigen preparations suggested that probably, PAgHU, DUMIs, and MNBS contained higher numbers of different proteins than the rest of the antigen preparations with fewer protein bands in their profiles.

University of Ghana http://ugspace.ug.edu.gh

Also, the fact that the band sizes of proteins **B** and **E** in MNBS were larger and darker than those of the other proteins in SDS-PAGE suggested that probably, the relative abundance of **B** and **E** in MNBS was higher than those of the other proteins observed in the gel.

Finally, the presence of several protein bands in PAgHU and the DUMIs but not in GH-ve and US-ve suggested that probably malaria infection led to the excretion of such proteins into the urine. This is particularly because, it is well established that malaria could induce changes in the urine of infected individuals (Sengupta *et al*., 2011; Basant *et al.*, 2010).

Reactivity of the mouse anti-Plasmodium polyclonal antibody (PAb) to different antigen preparations in western blotting assay

The reactivity of the mouse anti-*Plasmodium* polyclonal antibody (PAb) to protein bands in PAgHU and DUMI-1, but not to any band within GH-ve and US-ve suggested that probably, those proteins were truly malaria antigens, hence their absence from the malaria negative urine samples. In addition, the lack of reactivity between the PAb and the *Plasmodium* MSP119 antigen suggested that that antigen was not among the *Plasmodium* proteins that induced the production of the PAb in the mouse.

Furthermore, the reactivity of the PAb to one protein band each in both PAgHU and DUMI-2, with higher intensity and larger band size suggested that these proteins had higher relative abundance in the respective antigens preparations than the others as reported by Goins and Cutle (2000).

Reactivity of the UCP4W7 monoclonal antibody to different antigen preparations in western blotting assay

According to the results (Figure 19), the UCP4W7 MAb reacted in western blotting assay against PAgHU, CPfAg and DUMIs but not with MNBS, GH-ve and US-ve. These

University of Ghana http://ugspace.ug.edu.gh

results confirm the suggestion made earlier that UCP4W7 was capable of accurately distinguishing between malaria positive and negative individuals.

According to the results, a < 6 kDa protein (**Z**) and a 10.3 kDa protein (**A**) were detected by UCP4W7 in the DUMIs but not in PAgHU, CPfAg and the negative samples. This observation could be due to the fact that proteins **Z** and **A** did not originate from the *Plasmodium* parasite, but was a human metabolite that was synthesized in response to infection by the parasite, hence the inability to detect them in CPfAg. Conversely, it could be that probably, it was a *Plasmodium* parasite antigen but got lost in the course of extraction from the cultured parasites, hence its absence from CPfAg.

Also, it has been reported elsewhere (Midgett and Madden, 2007) that the methods used in extraction of proteins from urine could lead to loss or modification of some antigens in the proteins. By this report, it could be inferred that probably, **Z** and **A** were urinary *Plasmodium* proteins, which were lost during extraction, leaving concentrations which were below the sensitivity that could be detected by UCP4W7.

In fact, during extraction from urine, the antigens were dialyzed with a 10, 000 MW cut-off cellulose membrane as reported in the literature. Since protein **Z** for example, was smaller than the 10,000 MW dialysis membrane pore size, it was possible for some to be lost through dialysis, leaving a concentration that was too low to be detected in PAgHU but high enough in the DUMIs. This concentration, even though might be too low in PAgHU, probably might be highly immunogenic and therefore, was able to elicit the immune response that resulted in the production of antibodies against **Z**. These events could therefore, be the probable reasons for the ability of the UCP4W7 to detect this antigen in the DUMIs but not in PAgHU or CPfAg

University of Ghana http://ugspace.ug.edu.gh

Furthermore, the detection of the 13.2 kDa antigen (**B)** and 32 kDa antigen (**D**) (Figures 19 and 20) by UCP4W7 in PAgHU, but not in either CPfAg or DUMIs or the negative samples suggested that probably, the strains of the *Plasmodium* parasites that produced antigens B and D in PAgHU, were different from the strains that constituted CPfAg and the DUMIs. This explanation was arrived at because urine samples from over 400 infected participants were pooled together in order to prepare PAgHU. Therefore, it was possible that these number of subjects were harbouring different strains of the parasites, some of which had certain antigens which are not shared in common by all the strains of the parasite.

A critical observation of the western blotting results also revealed that UCP4W7 was reactive to a 27.2 kDa protein band labelled **C**, in CPfAg, PAgHU and the DUMIs, though the reactivity to PAgHU was weak. Protein **C** however, was not detected in GH-ve, US-ve and the true malaria negative blood sample. This reactivity pattern suggested that protein **C** was probably a *Plasmodium* protein that was excreted into the urine of infected humans, hence its presence in the cultured parasite antigen extract as well as the urine of infected individuals. Also, the fact that protein **C** was not detected in the *Plasmodium* negative human urine and blood samples suggested that probably **C** was not a human antigen. If these deductions were right, then UCP4W7 and antigen **C** together form a promising antibody-antigen pair for diagnosis of malaria in the urine of infected individuals.

In addition, the reactivity of UCP4W7 with the 63.2 kDa protein (**E**) in both PAgHU and DUMIs, but not in CPfAg or the negative samples, suggested that probably, antigen **E** was an RBC membrane bound *Plasmodium* protein that was lost through the removal of RBC membranes during preparation from the cultured parasites. On the other hand, it could be that **E** was an anti-malaria human metabolite that was produced by the host only after infection.

University of Ghana http://ugspace.ug.edu.gh

These could therefore be the possible reasons for the observed reactivity to **E** in PAgHU and DUMIs but not in CPfAg and the negative samples.

The detection of the 75.4 kDa protein band labelled **F** with reducing reactivity strength from PAgHU to CPfAg to DUMI-2 (>100,000 parasites/ µl of blood) and then to DUMI-1 (50,000 - 100,000 parasites/ µl of blood), suggested that antigen **F** was a true *Plasmodium* parasite protein whose concentration in the urine was probably associated with parasite density. This suggestion was confirmed by the fact that the reactivity of UCP4W7 to **F** in PAgHU was stronger than that observed in the other antigens. PAgHU was concentrated from several *Plasmodium* positive urine samples and probably had a higher amount of the antigen than the other samples. The reduction in reactivity to **F** with parasite density therefore suggested that antigen **F** could probably be very promising for estimation of parasite density in infected individuals.

Furthermore, the fact that UCP4W7 detected 3 protein bands (**G**, **H** and **I**) of molecular weight from 111.0 kDa and above as well as proteins **B** and **D** in PAgHU but not in CPfAg, the DUMIs, GH-ve, US-ve or MNBS, suggested that probably, those antigens were *Plasmodium* proteins, which however, were not common to all the parasites detected in the infected individuals. In respect of this, it was reported that some of the *P. falciparum* parasites in Africa and Peru for example, do not express histidine-rich-protein 2 (HRP2) because they lack the gene for expression of the antigen (Gamboa *et al*., 2010). Rosenthal (2012), also reported that a certain fraction of *P. falciparum* parasites in Africa express a modified form of the HRP2 antigen, whiles, other malaria parasites like *P. malariae*, *P. vivax*, *P. ovale* and now *P. knowlesi*, do not express the HRP2 antigen at all, and therefore, any test directed at this antigen would be negative, and would not necessarily mean that the person did not have malaria. On the other hand, owing to the fact that the DUMIs were direct unconcentrated urine

University of Ghana http://ugspace.ug.edu.gh

samples, it was also possible that they contained the said antigens but at concentrations lower than the sensitivity threshold of UCP4W7, hence the inability to detect them in the DUMIs.

The reactivity of UCP4W7 to the proteins **E** (63.2 kDa) and **F** (75.4 kDa) in PAgHU in western blotting assay with a very high intensity and larger band sizes than the other proteins also confirmed the suggestion that probably those proteins had higher relative abundance in PAgHU than all the other antigen preparations.

Relative sensitivity and specificity of tests for different biomarkers of malaria against blood smear microscopy as the gold standard test

To evaluate the sensitivities and specificities of the various tests for diagnosis of malaria, microscopy was used as a gold standard test against which the various tests were measured. This was because, microscopy is presently the standard diagnostic tool of measure for determining the accuracy of performance of other diagnostic tests (Eibach *et al*., 2013; Rosenthal, 2012; Reyburn *et al*., 2007).

The determination of the relative sensitivities and specificities of the tests for various biomarkers of malaria compared to microscopy showed that tests for ketones, microhematuria, leukocytes, nitrites, glucose, bilirubinuria urobilinogen as well as PAb ELISA, were highly specific but had very low sensitivities.

The reactivity patterns of these tests suggest that they would not be very good for accurate diagnosis of malaria, since they could result in high false negative rates which could cause curative drugs not to be given to truly infected individuals who really need them (Murray *et al*., 2008).

Also, the tests for fever and proteinuria, even though their relative sensitivity and specificity were above 50% they would not be very accurate or useful in diagnosis of the disease because, for a test to be clinically useful, the combined relative sensitivity and specificity should not be less than 170% (Wians, 2009).

University of Ghana http://ugspace.ug.edu.gh

The estimation of the relative sensitivity and specificity of the UCP4W7 MAb (Table 16) showed that its combined relative sensitivity and specificity (96.9% + 75.6%) was 172.5%. The fact that its combined reactivity was greater than 170% showed that this test was accurate enough for distinguishing between malaria infected and uninfected individuals (Wians, 2009) and therefore, promising for further development of a urine-based test for diagnosis of malaria. Also, even though this combined reactivity was more than 170% it was lower than the expected ideal of 198%. This shortfall however, could have been because microscopy is not accurate enough to be used as a gold standard test (Alemu *et al*., 2014).

A correction of these microscopy results by the polymerase chain reaction (PCR) technique as performed by (Eibach *et al*., 2013; Hopkins *et al*., 2008), could have reduced the rates of false negatives and positives, and consequently raised the combined reactivity of the UCP4W7 ELISA far above the observed 172.5%. Confirmatory tests by PCR to correct the microscopy results, however, were not carried out because of lack of logistics.

The quantitative buffy coat (QBC) test unlike the UCP4W7 ELISA, had fairly high relative sensitivity and specificity but a lower combined reactivity (154.9%) than the 170% threshold required for accurate diagnosis. Therefore, for it to be used as a reliable test for diagnosis of malaria, especially, in situations like drug sensitivity studies, its sensitivity and specificity need to be corrected by PCR as done presently for blood smear microscopy (Eibach *et al*., 2013; Hopkins *et al*., 2008).

Finally, the higher sensitivity (97.4%) and extremely lower specificity of the PAb ELISA compared to microscopy suggested that probably, it would not be accurate for diagnosis of malaria in the urine of infected individuals, even when microscopy results are corrected.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

Since the identification of malaria as an important global health problem, several efforts have been made to control the disease. These include attempts to eliminate vector *Anopheles* mosquitoes by insecticides, destruction of breeding places of mosquitoes in order to break disease transmission cycle as well as the elimination of causative *Plasmodium* parasites by anti-malarial drugs. Nevertheless, these efforts have not achieved the expected goal of disease eradication (Jain *et al*., 2014). As a result, the prevalence of the disease is still high, especially, in sub-Saharan Africa, where disease burden and death toll are greatest (Jain *et al*., 2014).

Malaria eradication by chemotherapy requires accurate identification of infected individuals in endemic populations, which therefore demands the utilization of fast, simple, sensitive and specific diagnostic tools in order to achieve desired results.

Currently, blood smear microscopy is the gold standard test for routine and confirmatory diagnosis of malaria (Eibach *et al*., 2013, Azikiwe *et al*., 2012). Also, immunochromatographic tests (ICTs), popularly known as rapid diagnostic tests (RDTs), as well as tests based on the use of clinical signs and symptoms, are some of the alternative tests for routine diagnosis of the disease (Eibach *et al*., 2013, Azikiwe *et al*., 2012). However, all of these tests are not accurate enough, they are labour-intensive and/ or invasive which therefore limit their effectiveness for control of the disease (Alemu *et al*., 2014; Steenkeste *et al*., 2010; Okell *et al*., 2009). For these reasons, it has become necessary to develop new tests

University of Ghana http://ugspace.ug.edu.gh

which are more sensitive and specific, faster and non-invasive for improved diagnosis of malaria.

The identification of parasite antigens and anti-parasite metabolites or antibodies in tears, saliva and urine of infected humans (Nolen *et al*., 2013; Ayong *et al*., 2005), highlighted the importance and the possibility of using such metabolic fluids for development of non-invasive tools for malaria diagnosis. Urine, in particular, was considered in this study as the most reliable excretory/ secretory metabolic by-product for development of non-invasive diagnostic tools for malaria because of the following reasons. Firstly, it is the only body fluid which can be obtained readily in large quantities non-invasively (Decramer *et al*., 2008). Secondly, urinary proteins are more stable to harsh environmental conditions than other biofluids such as the widely used serum and plasma (Nolen *et al*., 2013; Decramer *et al*., 2008). Also, it is ethically easier to sample urine than blood, which is equally, a rich source of biomarkers (O'Riordan *et al*., 2007). Lastly, various researchers have reported the detection of *Plasmodium* antigens and anti-malarial human metabolites in the urine of infected individuals (Thomas *et al*., 2010; Howard *et al*., 2007; Katzin *et al*., 1988; Rodriguez-del Valle *et al*., 1991).

For these reasons, this study directed efforts at producing a mouse anti-malarial MAbs that could be used to develop a rapid tool for diagnosis of the disease through a MAb-based detection of parasite antigens and/ or anti-malarial metabolites in the urine of infected individuals.

In the studies described in chapter four, malaria infected and uninfected subjects were identified from the endemic population using blood smear microscopy, quantitative buffy coat (QBC) test and commercial rapid diagnostic test (RDT) kits. These studies were carried out partly with a view to obtain infected urinary antigens for immunizations towards MAb

University of Ghana http://ugspace.ug.edu.gh

generation. Also, the studies were conducted in order to select true negative control urine samples for ascertaining the specificity of the MAb-based tests to be developed.

The successful discrimination of infected from uninfected subjects among the study population enabled the successful extraction of malaria related antigens for generation of the anti-malarial MAbs.

A high malaria prevalence of 46.5% was found among the study population, which was not significantly different from the 52.0% prevalence obtained in a pilot study conducted 9 years earlier in the same community. These findings confirmed that reported by Jain *et al*. (2014), who stated that in areas with stable transmission in sub-Saharan Africa, malaria prevalence could be as high as 52.0%. It also confirmed the statement by some writers that even though several efforts have been put in place to control malaria, their overall impact has been lower than expected (Eibach *et al*., 2013; Reyburn *et al*., 2007; Shiff, 2002). These results therefore justified the need for new intervention tools to intensify efforts towards the eradication of the disease.

According to the studies, malaria was significantly associated with sex, age, and fever, but not with anaemia. The occurrence of higher prevalence of malaria among females in these studies, even though it confirmed what Ayele *et al*. (2012) have reported, was suspected to be linked to behaviour more than to sex, as reported by other authors such as Steketee *et al*. (2001). The studies reported in chapter four, however, lacked the immunological and biochemical analyses needed to ascertain the link between malaria and sex. Therefore, it was not concluded that the higher prevalence of malaria obtained in females than males was sex-linked. However, the fact that no conclusive agreement have been reached by researchers on this issue, showed clearly that further studies are required to clarify the relationship between malaria and sex.

University of Ghana http://ugspace.ug.edu.gh

Authors such as Griffin *et al*. (2014) and Schellenberg *et al*. (2004) have reported that understanding the relationship between malaria burden and age is important in selecting high risk groups in which priority intervention measures could be focused in order to prevent malaria catastrophe. Therefore, the observation of significantly higher prevalence of malaria and higher parasite density among children than adults in these studies were important findings that confirmed reports by other writers that children are more susceptible to the disease because they lack the immunity against the disease.

These studies have therefore consolidated the findings that children are among the topmost priority group on which targeted intervention measures should be focused in order to minimize the disease burden in endemic populations. The observation of the highest parasite density among children as revealed by these studies has also shown that children are the group to concentrate on in order to obtain malaria antigen-rich urine samples for further studies.

The ability of these studies to determine the association between malaria and fever as reported by other writers has also corroborated the findings made.

Anaemia is known to be triggered by a variety of factors which include parasitic infections and malnutrition (Santana-Morales *et al*., 2013). Therefore, the fact that anaemia could not be linked to malaria in the studies described in this work, confirmed that probably, other factors could be involved in the anaemia observed among the study population. The involvement of other factors in the anaemia observed, however, was not investigated because that was not part of the study objectives.

In studies described in chapter five of this write-up, *P. falciparum* species-specific HRP2 and *Plasmodium* pan-specific antigens were detected in the urine of infected individuals by the commercial blood-based RDT kits. These findings provided useful information on the potential of urine as a reliable source of biomarkers of malaria. Also, they

University of Ghana http://ugspace.ug.edu.gh

indicated that urine is a promising candidate for development of non-invasive rapid tests for diagnosis of malaria.

In the SDS-PAGE experiment to characterize biomarkers of malaria from infected human urine (chapter five), the number of protein bands observed in the profiles of infected urine samples decreased with parasite density in infected individuals. This observation suggested that probably the urine of infected individuals would serve as a good material for determination of efficacy of antimalarial drugs. This finding has promising implications particularly, for antimalarial drug developers. It demonstrated that urine of infected subjects could constitute a better alternative for studies on anti-malarial drug efficacy than blood, because, it is ethically easier to sample urine than blood (O'Riordan *et al*., 2007).

The detection of some proteins in the profiles of infected urine samples at high concentrations by SDS-PAGE and western blotting assay (chapter five), also supported reports that urine samples from infected subjects contained adequate amount of proteins for studies on such malaria-related biomarkers (Thomas *et al*., 2010).

In those experiments, the mouse anti-*Plasmodium* PAb detected 2 proteins of MW 50.9 and 54.6 kDa in PAgHU and 3 proteins with MW being 27.2, 46.0 and 52.7 kDa in DUMI-2. This observation indicated that those proteins could be potential urine-based diagnostic markers of malaria.

Also, the strong reactivity of the 50.9 kDa protein (**Ω**) in PAgHU and 52.7 kDa protein (**λ**) in DUMI-2 to the PAb, showed that probably, those proteins would induce the production of MAbs with very high sensitivity. The fact that the 2 proteins had thicker bands as detected by the PAb, showed that those proteins had higher relative abundance or concentrations in their respective antigen preparations. In their quantitative analysis of the complex protein mixtures, Gygi *et al*. (1999) reported that measured differences in protein expression in yeast correlated with its metabolic function under glucose-repressed conditions. Since the level of

University of Ghana http://ugspace.ug.edu.gh

expressed proteins is directly related to the concentration or the relative abundance of that protein in a biological sample, it implied that the proteins **Ω** and **λ** which showed thicker bands compared to the others, probably, play more important biological roles within the *Plasmodium* parasite (McAfee *et al*., 2006).

Indeed, it is also possible that the higher immunogenicity of the 2 proteins as observed in the studies was caused by their higher concentrations in the urine samples.

On the other hand, the concentration of proteins in a urine sample at a given time is known to reflect the resistance of such proteins to the degradation caused by factors such as handling and storage conditions as well as the proteolytic enzyme activity that is ongoing in the sample (Pisitkun *et al*., 2006). In relation to this report, therefore, it is possible that the 2 proteins which exhibited thicker bands, as observed above, were more stable to the degradative effect of the factors mentioned above.

The concentration of proteins in urine has also been reported to reflect the ability of such proteins to pass through the filtration membranes within the glomerulus (Nolen *et al*., 2013). In relation to this therefore, the higher concentrations of the 2 proteins in the urine samples as revealed by their thicker band sizes, indicated that possibly, such proteins had greater ease of crossing the glomerular barrier into the urinary bladder than the others. This finding, therefore, has important implications for further studies on such proteins in urine.

Finally, the proteins **Ω** and **λ** appeared to have important biological functions in the *Plasmodium* parasites as revealed by their strong reactivity to the PAb, and thicker band sizes that were suggestive of high relative abundance in the urine samples. The veracity of these observations however were not ascertained by the studies reported in this write-up. It is therefore suggested that further studies should be conducted to investigate the importance of these proteins to the *Plasmodium* parasite and the implication for diagnosis and control of the infection due to these parasites.

University of Ghana http://ugspace.ug.edu.gh

In the study to determine the association of malaria, haemoglobin levels and anaemia with urinary hepcidin levels in malaria infected subjects (chapter five), the highest prevalence of malaria and parasite density were observed among children, while the lowest prevalence of the disease and parasitaemia were observed among pregnant women. Those observations confirm the findings that children were among the group of individuals at the highest risk of infection with malaria (Genton *et al*., 2008). The rather low prevalence of the disease and parasitaemia in the pregnant women who are also among the highest risk group, however, might be attributable to the fact that they were undergoing intermittent preventive treatment (IPT), as part of the Ghana Ministry of Health/ Ghana Health Service and the National Malaria Control Programme policy on management of malaria among pregnant women. Also, the lowest haemoglobin level and anaemic status observed among the children, even though they had the highest disease burden among the cohort of participants examined confirmed the findings that anaemia among affected individuals might be attributable to several factors, including malnutrition and hookworm infection rather than to malaria alone (Santana-Morales *et al*., 2013).

Increased levels of hepcidin was observed in the urine of malaria infected individuals which was significantly associated with parasitaemia but not haemoglobin levels or anaemia. This observation demonstrated that urinary hepcidin would be a promising biomarker for diagnosis of malaria. It also indicated that perhaps, changes in urinary hepcidin levels with parasitaemia could be explored for development of a quantitative diagnostic test for malaria. In addition, the decreasing levels of urinary hepcidin with parasitaemia, would be very useful in the evaluation of anti-malaria drug efficacy.

The apparent lack of association between urinary hepcidin and haemoglobin levels or anaemia, however, suggested that urinary hepcidin levels would not be a useful biomarker for determination of haemoglobin levels or anaemia. Probably, this apparent lack of association

University of Ghana http://ugspace.ug.edu.gh

might have been caused by the fact that several confounding factors combined together to give rise to the haemoglobin level or anaemic status of an average individual. Further studies are therefore, required to characterize the longitudinal variation in urinary hepcidin levels with changes in haemoglobin levels and anaemia in the presence or absence of malaria, with or without other concomitant infections which are also implicated in the blood haemoglobin levels or anaemic status of an individual.

In chapter six of this thesis, MAbs were generated by cell fusion, using hybridoma technology. The ascites production method were not used because of the possibility of contamination by unwanted immunologically active compounds in the peritoneum of the mice, which might interfere with the specificity of the MAbs generated as reported (Dewar *et al.*, 2005; Lipman *et al.*, 2005).

It has been reported that MAb-secreting hybridoma cells generated using spleen cells from the same species of animal as the myeloma cell line oringin, are more stable than those generated from different species of fusion partners (Grimaldi and French, 1995). In the immunization experiments towards MAb generation, therefore, BALB/c mice were used because, the myeloma cell lines that were available for cell fusion were of mouse origin.

Also, the route of immunization of laboratory animals towards antibody production has been reported to be very instrumental in the quality of immune response and the corresponding sensitivity of the antibodies generated (Mohanan *et al.*, 2010). In this study therefore, BALB/c were immunized via the peritoneal route because it was relatively easier, safer and could lead to the induction of the high immune response needed for generation of MAbs with high sensitivity. It was therefore not surprising that at the end of the immunization experiments, only one (6.67%) out of the 15 mice finally immunized, died, before obtainment of the last immune sera for determination of immune response in the mice. In addition, the

University of Ghana http://ugspace.ug.edu.gh

high reactivity of the immune sera and the MAbs produced to PAgHU could probably have been contributed by the route of immunization used.

Furthermore, in those experiments, the fact that one mouse did not show any immune response at all, suggested that it might have been immunologically suppressed. Indeed, this observation confirmed the reports that there are immunologically suppressed individuals in each population who may not respond positively to invasion by foreign antigens (Kopel *et al*., 2012).

The potential usefulness of the MAbs generated as a promising diagnostic reagent for malaria was demonstrated through their reactivity with both urinary (PAgHU) and cultured *Plasmodium* (CPfAg) antigen extracts. In those experiments, all the 96 clones (100%) of the MAbs reacted with PAgHU, 70.8% reacted with both PAgHU and CPfAg, (31.3%) reacted with PAgHU only, while none reacted with CPfAg alone. This observation was very interesting because, the reactivity of some of these MAbs to either PAgHU only, or to both PAgHU and CPfAg, indicated that perhaps, the MAbs were either species-specific, pan-specific or both. The reactivity also indicated that probably, the MAbs would also have a wide range of applicability. These species-specific and pan-specific qualities might have been induced by PAgHU as demonstrated in chapter 5 of this report that PAgHU contained both the *P. falciparum* species-specific HRP2 and pan-specific LDH antigens. In view of this, the reactivity of all the 96 clones of the MAbs to PAgHU confirmed the report that the urine of infected individuals would be potentially useful as a promising source of biomarker for diagnosis of malaria. Finally, these results demonstrated that MAbs generated against malaria antigens in urine of infected individuals would be very useful in development of a tool for diagnosis of the disease globally.

University of Ghana http://ugspace.ug.edu.gh

In chapter seven, the reactivity of mouse MAb and PAb produced against PAgHU and CPfAg as well as direct urine samples from study participants were determined. Both MAbs and PAbs reacted positively to microscopy negative urine samples in ELISA, however, there was a higher number of false positive reactivity by the PAbs than the MAbs. This observation may be explained as follows.

MAbs are capable of recognizing and binding to only one specific antigenic determinant or epitope (Ansar and Ghosh, 2013) PAbs on the other hand, are capable of recognizing and binding different epitopes on one or different antigens with varying degree of affinity (Nakazawa *et al*., 2010). This higher antigen binding capacity of PAbs gives them and exaggerated reactivity to both specific and non-specific antigens, which results in the higher false positive rates that are characteristic of most PAb-based antigen detection assays (Nakazawa *et al*., 2010). In view of this, the higher reactivity and the corresponding higher false positive rates of the PAbs in microplate ELISA as compared to the MAbs, might be because they detected more *Plasmodium* negative urine samples non-specifically.

Indeed, false positive results in malaria lead to non-judicious administration of anti-malarial drugs to people who do not really need them (Eibach *et al*., 2013; Azikiwe *et al*., 2012; Reyburn *et al*., 2007) and speeds up parasite development of resistant to such drugs (Eibach *et al*., 2013; Reyburn *et al*., 2007). Also, it causes delay in diagnosis and treatment of the actual infections responsible for the febrile signs and symptoms, which may lead to complications and death of the affected individuals (Reyburn *et al*., 2007). In a bid to arrest this diagnostic inaccuracy and its destructive outcomes, therefore, this study was designed to develop a highly sensitive and specific MAb urine-based dipstick test for diagnosis of malaria.

The PAbs also reacted with some of the negative urine samples in ELISA but not in western blotting assay. This observation might be due to the fact that western blotting assay

University of Ghana http://ugspace.ug.edu.gh

is more specific than microplate ELISA, which has a higher propensity to give false positive results (Bresnahan, 2013).

Like the PAbs, the UCP4W7 MAbs also reacted with urine samples from both microscopy positive and negative individuals in ELISA, but not in western blotting assay. However, the lower false positive results in the UCP4W7 ELISA compared to the PAb ELISA might be because, the former was MAb-based, and could mostly detect only specific antigens with minimal cross-reactivity to non-target antigens.

Also, the successful reactivity of the UCP4W7 MAb to protein bands in PAgHU, CPfAg and DUMIs but not with proteins in the negative control samples, measles and polio vaccines as well as the schistosome antigens, demonstrated the superior specificity of the MAb for malaria-related antigens. The differential detection of some protein bands in the profile of PAgHU by UCP4W7, which were not in CPfAg or DUMIs; and some protein bands in the DUMIs which were not present in PAgHU and CPfAg also suggested that perhaps, those proteins were not equally expressed in all the strains of parasites that infected the study participants.

Indeed, this observation confirmed the reports made by Rosenthal (2012) and Gamboa *et al*. (2010). They reported that firstly, some *P. falciparum* parasites do not produce the parasite specific HRP2 because they lack the gene for expression of the antigen. Secondly, some expressed an altered HRP2 antigen. Lastly, some antigens like the HRP2 are not commonly expressed in the different species of the parasite.

These characteristics are diagnostic limitations which lead to false negative results in some of the RDTs in some localities. False negative results, in turn, could lead to failure in treatment of individuals who have the disease. However, failure to treat children below 5 years who have acute *P. falciparum* infections for example, could lead to complicated malaria

University of Ghana http://ugspace.ug.edu.gh

(Rosenthal, 2012). It is for these reasons that there is an urgent need to avoid false negative results in malaria diagnosis (Rosenthal, 2012).

The limitations associated with the pre-existing RDTs provide interesting lessons to apply in the development of new diagnostic tools for malaria. These lessons suggested that perhaps, in the efforts to develop superior RDT-based tests for malaria, MAbs that have the capacity to detect pan-specific antigens in the urine of all infected individuals should be considered most. Also, they suggested that those proteins that were not common to all the samples could be explored for development of differential diagnostic tests required to make decisions on the type of antimalarial drugs to use.

Out of the 3 vaccines and 2 antigens tested for cross-reactivity to the UCP4W7 MAb, yellow fever vaccine was observed to cross-react with the MAb. This observation suggested that probably, the urine of *Plasmodium* infected individuals used in generating the UCP4W7 MAb also had yellow fever vaccine or active viral antigens.

Kim *et al*. (2010) and Goepp *et al*. (1992) have reported that vaccinated individuals do excrete vaccine antigens in their urine which cause false positive results in urine-based antigen detection assays. Also, Martinez *et al.* (2011) reported that yellow fever vaccine RNA or antigens could persist in vaccinated or infected individuals for more than 6 months as detected in their urine samples.

These findings, therefore suggested that perhaps, the cross-reactivity observed between the UCP4W7 MAb and the yellow fever vaccine, was due to the induction of yellow fever binding affinity in the MAb by vaccine and/ or viral antigens which were probably present in the urine samples.

This cross reactivity, however, was a limitation on the specificity of the UCP4W7 MAb for urinary *Plasmodium* antigens, which could lead to false positive results when used

University of Ghana http://ugspace.ug.edu.gh

for diagnosis of malaria in suspected individuals. For these false positive results to be avoided therefore, yellow fever infections or vaccine antigens must be ruled out of such individuals.

Furthermore, it is likely that further cloning of these hybridoma cells by limiting dilution to obtain a colony of cells arising from single parental cell lines, would yield second or third generation MAbs that are specific to only urinary *Plasmodium* antigens.

Finally, the usefulness of the UCP4W7 MAb as a reagent for development of a urine-based diagnostic test for malaria was demonstrated by evaluating its sensitivity and specificity in microplate ELISA using microscopy as a gold standard test. The results showed that the relative sensitivity of the UCP4W7 ELISA was high (96.9%) although the relative specificity (75.6%) was not high enough. The rather low specificity of the UCP4W7 ELISA could be explained by the fact that microscopy was not specific enough which led to high false positive rates and a corresponding low specificity of the MAb ELISA.

Several studies have shown that microscopy is not accurate enough to be used as a yardstick against which other tests should be evaluated (Steenkeste *et al*., 2010; Okell *et al*., 2009). Therefore, the findings of the study above, further emphasized the need for the use of more accurate tools as standards against which the performance of other tests would be assessed. Furthermore, the findings indicated that while the current prevalence of malaria and its destructive outcomes are still being battled with, perhaps, the development of additional tests to improve the diagnosis of the disease, would enhance current efforts to eradicate the disease as reported.

In conclusion, this study provides useful information on the suitability of MAbs as diagnostic reagents for distinguishing between malaria infected and uninfected individuals. Also, it provides the base-line information that SDS-PAGE and/ or MAb-based western blotting assay could be explored in reliable semi quantitative techniques for differentiating between various levels of parasitaemia in infected individuals. Lastly, the study provides the

University of Ghana http://ugspace.ug.edu.gh

evidence that urine is a reliable source of biomarker for development of non-invasive tests for diagnosis of malaria.

Additions to Knowledge

- MAb-secreting hybridoma cells have been produced against urinary *Plasmodium* antigens for the first time
- A paper on the potential of urinary hepcidin as a promising diagnostic marker for malaria has been published
- The research work has shown that SDS PAGE and WBA could be used to develop a urine-based semi-quantitative test for malaria
- SDS-PAGE on infected human urine could be used to evaluate the efficacy of antimalarial drugs
- Infected human urine may be a better alternative for assessment of antimalarial drug efficacy than blood

Recommendations

Further work, using PCR technology is required to confirm the true infection status of microscopy negative subjects. This is because, determination of the number of individuals who are truly negative is essential for ascertaining the specificity of the MAb-based test being developed. It is recommended that in further studies towards this end, more urine samples from individuals living outside malaria endemic regions would be used as true negative controls, compared with the microscopy determined negative control urine samples from the study community, to optimize the accuracy of the of the MAbs for detection of true positive infections.

Also, additional work is required to produce single clones of the promising UCP4W7 MAb-secreting hybridoma cells for the purpose of obtaining MAbs for development and commercialization of the dipstick assay being developed.

In addition, there is a need to investigate into the possibility of using membrane-based SDS-PAGE to ascertain malaria parasite density in infected and uninfected individuals. This

University of Ghana http://ugspace.ug.edu.gh

is because, the success of this study could lead to the development of a quantitative or semi-quantitative urine-based test for malaria which is requisite for studies on anti-malaria drug efficacy.

Furthermore, studies are required to investigate into the cross-reaction of the UCP4W7 with the yellow fever vaccine antigen. It is also highly desirable that further studies are conducted to determine the cross-reactivity of the UCP4W7 MAb with additional antigens from parasitic infections that share common clinical signs and symptoms with malaria which, however, were not examined in the studies reported in this thesis write-up.

University of Ghana http://ugspace.ug.edu.gh

REFERENCES

Ahmed, R. and D. Gray (1996). Immunological memory and protective immunity: Understanding their relation. Science 272, 54–60.

Alemu, A., H.-P. Fuehrer, G. Getnet, A. Kassu, S. Getie and H. Noed (2014). Comparison of Giemsa microscopy with nested PCR for the diagnosis of malaria in North Gondar, north-west Ethiopia. **13**: 174-178.

Amanor, J. D., K. M. Bosompem, T. Arishima, R. K. G. Assoku and S. Kojima (1996). Characterization of monoclonal antibodies reactive with *Schistosoma haematobium* soluble egg and infected human urinary antigens. *Hybridoma*, **15**(3): 219-224.

Amino, R., S. Thiberge, B. Martin, S. Celli, S. Shorte, F. Frischknecht, and R. Menard (2006). Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nature Medicine*. **12**:220–224.

Anderson, G. J., D. M. Frazer, S. J. Wilkins, E. M. Becker, K. N. Millard, T. L. Murphy, A. T. McKie and C.D. Vulpe (2002). Relationship between intestinal iron-transporter expression, hepatic hepcidin levels and the control of iron absorption. *Biochemical Society Transactions* **30***:* 724–726.

Ansar W. and S. Ghosh (2013). Monoclonal antibodies: a tool in clinical research. Indian *Journal of Clinical Medicine* **4**: 9-21.

Anstey, N. M., B. Russell, T. W. Yeo, R. and N. Price (2009). The pathophysiology of vivax malaria. *Trends in Parasitology* **25** (5): 220-227.

Antranik (2012). Inflammation and the Pathophysiology of Fever**:** Physiology, Science. Downloaded from "http://antranik.org/inflammation-and-the-pathophysiology-of-fever/"

University of Ghana http://ugspace.ug.edu.gh

Asante, F. A. and K. Asenso-Okyere (2003). Economic Burden of Malaria in Ghana. A Technical Report Submitted to the World Health Organization (WHO), African Regional Office (AFRO).

Ayele D. G. T. T. Zewotir and H. G. Mwambi (2012). Prevalence and risk factors of malaria in Ethiopia. *Malaria Journal* **11**: 195-203.

Ayong, L. S., C. B. Tume, F. E. Wembe, G. Simo, T. Asonganyi, G. Lando and J. L. Ngu (2005). Development and evaluation of an antigen detection dipstick assay for the diagnosis of human onchocerciasis. *Tropical Medicine and International Health*, **10** (3): 228–233.

Azikiwe, C. C. A., C. C. Ifezulike, I. M. Siminialayi, L. U. Amazu, J. C. Enye and O. E. Nwakwunite (2012). A comparative laboratory diagnosis of malaria: microscopy versus rapid diagnostic test kits. *Asian Pacific Journal of Tropical Biomedicine*, **2**(4): 307-310.

Baird, J. K. (1995). Host age as a determinant of naturally acquired immunity to *Plasmodium falciparum*. *Parasitology Today* **11**: 105-111.

Baird, J. K., *et al*., (1991). Age-dependent acquired protection against *Plasmodium falciparum* in people having two years exposure to hyperendemic malaria. *American Journal of Tropical Medicine and Hygiene* **45** (1): 65–76.

Baird, J. K., Purnomo, and T. R. Jones **(**1992). Diagnosis of malaria in the field by fluorescence microscopy of QRC capillary tubes. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **86:** 3–5.

Bannister, L. H. and I. W. Sherman (2009). *Plasmodium*. *In: Encyclopaedia of Life Sciences (ELS)*. John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a 0001970.pub2.

University of Ghana http://ugspace.ug.edu.gh

Bannister, L. H., J. M. Hopkins, A. R. Dluzewski, G. Margos, I. T. Williams, M. J. Blackman, C. H. Kocken, A. W. Thomas and G. H. Mitchell (2003). Plasmodium falciparum apical membrane antigen 1 (PfAMA-1) is translocated within micronemes along subpellicular microtubules during merozoites development. *Journal of Cell Science* 116: 3825-3834.

Bartoloni A. and L. Zammarchi (2012). "Clinical aspects of uncomplicated and severe malaria". *Mediterranean Journal of Hematology and Infectious Diseases* **4** (1): 2035-3006. e2012026. doi:10.4084/MJHID.2012.026. PMC 3375727. PMID 22708041

Basant, A., M. Rege, S. Sharma and H. M. Sonawat (2010). Alterations in urine, serum and brain metabolomics profiles exhibit sexual dimorphism during malaria disease progression. *Malaria Journal*, **9**: 110-122.

Bhowmick, S., T. Mazumdar and Nahid Ali (2009). Vaccination Route That Induces Transforming Growth Factor Production Fails To Elicit Protective Immunity against *Leishmania donovani* Infection. *Infection and Immunity*, **77** (4): 1514–1523.

Boa-Amponsem, K., S. E. H. Price, E. A. Dunnington and P. B. Siegel (2001). Effect of route of inoculation on humoral immune response of white leghorn chickens selected for high or low antibody response to sheep red blood cells. *Poultry Science* **80**: 1073–1078.

Bosompem, K. M., I. Ayi, W. K. Anyan, F. K. Nkrumah and S. Kojima (1997). A monoclonal antibody-based dipstick assay for diagnosis of urinary schistosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **91**: 554-556.

Bosompem, K. M., T. Arishima, T. Yamashita, I. Ayi, W. K. Anyan, and S. Kojima (1996*a*). Extraction of *Schistosoma haematobium* antigens from infected human urine and generation of potential diagnostic monoclonal antibodies to urinary antigens. *Acta Tropica*, **62**(2): 91-103.

University of Ghana http://ugspace.ug.edu.gh

Bradley, T. (1996). Malaria and drug resistance. MBChB special study module project report. Department of Microbiology and Immunology, University of Leicester.

Breman, J. G., and I. Arita (1980). The confirmation and maintenance of smallpox eradication. *New England Journal of Medicine*, **303**: 1263-73.

Bresnahan, P. (2013). Immunoassays: Protein Arrays vs. ELISA and Westerns. Downloaded from "http://www.gracebio.com/blog/protein-array-elisa-western/" on 24th June, 2014.

Brewster, D., D. Kwiatkowski and N. J. White (1990). Neurological sequelae of cerebral malaria in childhood. *Lancet*, **336**: 1039-1043.

Brown M. R., K. D. Clark, M. Gulia, Z. Zhao, S. F. Garczynski, J. W. Crim, R. J. Suderman and M. R. Strand (2008). An insulin-like peptide regulates egg maturation and metabolism in the mosquito *Aedes aegypti*. *Proceedings of the National Academy of Science of USA*, **105**(15): 5716-5721.

Carey, M. F., C. L. Peterson and S. T. Smale (2012). Experimental Strategies for the Identification of DNA-Binding Proteins. *Cold Spring Harbour Protocols*, (Cold Spring Harbor Laboratory Press); 17-33.

Casadevall A, E. Dadachova, and L. Pirofski (2004). Passive antibody therapy for infectious diseases. *Nature Reviews Microbiology*, 2004, 2:695-703.

CCAC (Canadian Council on Animal Care) (2002). CCAC Guidelines on: Antibody Production, Ottawa ON: CCAC. (Available online: http://www.ccac.ca/english/ gdlines/antibody/ antibody.pdf).

CDC and WHO (1998). Centers for Disease Control and Prevention and World Health Organization. Infection Control for Viral Haemorrhagic Fevers in the African Health Care Setting. Atlanta, Centers for Disease Control and Prevention, 1998: 1-198.

University of Ghana http://ugspace.ug.edu.gh

Chattopadhyay, R., A. Sharma, V. K. Srivastava, S. S. Pati, S. K. Sharma, B. S. Das, C. E. Chitnis, (2003). Plasmodium falciparum infection elicits both variant-specific and cross-reactive antibodies against variant surface antigens, *Infections and Immunity* **71** (2): 597–607.

Chavatte J. M., F. Chiron, A. Chabaud and I. Landau (2007). "Probable speciations by "host-vector 'fidelity'": 14 species of Plasmodium from magpies" (in French). *Parasite* **14** (1): 21–37. PMID 17432055.

Cinquetti, G., F. Banal, C. Rondel, D. Plancade, C. de Saint Roman, D. Adriamanantena, C. Ragot, S. Védy and B. Graffin (2010). Splenic infarction during Plasmodium ovale acute malaria: first case reported *Malaria Journal* **9**: 288-290.

Clark, I. A., A. C. Budd, L. M. Alleva, W. B. Cowden (2006). Human malarial disease: a consequence of inflammatory cytokine release. *Malaria Journal*, **5**: 85-116.

Conway, D. J., R. I. Machado, B. Singh, P. Dessert, Z. S. Mikes, M. M. Povoa, A. M. Oduola and C. Roper (2001). Extreme geographical fixation of variation in the *Plasmodium falciparum* gamete surface protein gene pfs48/45 compared with microsatellite loci. *Molecular Biochemistry and Parasitology* **115**: 145-156.

Cox-Singh, J. and B. Singh (2008). *Knowlesi* malaria: newly emergent and of public health importance? *Trends in Parasitology*, **24** (9): 406-410.

Deb, R., S. Chakraborty, B. Veeregowda, A. K. Verma, R. Tiwari and K. Dhama (2013). Monoclonal antibody and its use in the diagnosis of livestock diseases. *Advances in Bioscience and Biotechnology*, **4** 50-62. doi:10.4236/abb.2013.44A008; Retrieved from "http://www.scirp.org/journal/abb/" on August 7 2013.

Decramer, S., A. Gonzalez de Peredo, B. Breuil, H. Mischak, B. Monsarrat, J.-L. Bascands and J. P. Schanstra (2008). Urine in Clinical Proteomics. *Molecular and Cellular Proteomics*, **7** (10): 1850-1862.

University of Ghana http://ugspace.ug.edu.gh

Dewar, V. P. Voet, F. Denamur, and J. Smal (2005). Industrial implementation of *in vitro* production of monoclonal antibodies. *ILAR Journal* **46** (3): 307-313.

Doolan, D. L. C. Doban˜o, and J. K. Baird (2009). Acquired Immunity To Malaria. *Clinical Microbiology Reviews*, **22**: 13–36.

Douglas, N. M., D. A. Lampah, E. Kenangalem, J. A. Simpson, J. R. Poespoprodjo, P. Sugiarto, N. M. Anstey, R. N. Price (2013). Major Burden of Severe Anemia from Non-Falciparum Malaria Species in Southern Papua: A Hospital-Based Surveillance Study. *PLoS Medicine*. **10**(12): 1371-1387.e1001575. doi:10.1371/journal.pmed.1001575

Dozier, S. (2014). Special section: monoclonal antibodies. Retrieved from "http://altweb.jhsph.edu/

Draper, Simon (2012). Progress in Malaria Vaccine Research. Retrieved from "http://www.ndm. ox.ac.uk/simon-draper-progress-in-malaria-vaccine-research" on 4-1-2014.

Dzakah, E. E., K. Kang, C. Ni, H. Wang, P. Wu, S. Tang, J. Wang, J. Wang and X. Wang (2013). Plasmodium vivax aldolase-specific monoclonal antibodies and its application in clinical diagnosis of malaria infections in China. *Malaria Journal*, **12**:199-206.

ECDPC Report (2013). Unusual increase in reported cases of paratyphoid A fever among travellers returning from Cambodia. *European Centre for Disease Prevention and Control*, Stockholm, 2013.

Eibach, D., B. Traore, M. Bouchrik, B. Coulibaly, N.Coulibaly, F. Siby, G. Bonnot, A.-L. Bienvenu and S. Picot (2013). Evaluation of the malaria rapid diagnostic test VIKIA malaria Ag Pf/Pan™ in endemic and non-endemic settings *Malaria Journal*, **12**: 188-194.

University of Ghana http://ugspace.ug.edu.gh

Escalante A. A. Ayala F. J. (1995). Evolutionary origin of Plasmodium and other Apicomplexa based on rRNA genes. *Proceedings of the National Academy of Science (USA)*, **92**: 5793-5797.

Flahault, A., M. Cadilhac and G. Thomas (2005). Sample size calculation should be performed for design accuracy in diagnostic test studies. *Journal of Clinical Epidemiology* **58**: 859–862.

FM (Filtration Membrane) (2013). Glomerular filtration. Retrieved from "http://courses. washington.edu/conj/bess/filtration/ filtration.htm" on 25-12-2013.

FSUIRAP (2013). Florida State University Immunization Routes for Antibody Production. Downloaded from "http://research.fsu.edu/media/1848/immunizat" on 15-9-2013.

Gamboa, D., M. F. Ho, J. Bendezu, K. Torres, P. L. Chiodini, J. W. Barnwell, S. Incardona, M. Perkins, D. Bell, J. McCarthy and Q. Cheng (2010). A large proportion of *P. falciparum* isolates in the Amazon region of Peru lack pfhrp2 and pfhrp3: Implication for malaria rapid diagnostic tests. *PLoSOne* **5**: e8091.

Genton, B., V. D'Acremont, L. Rare, K. Baea, J. C. Reeder, M. P. Alpers and I. Muller (2008). Plasmodium vivax and Mixed Infections Are Associated with Severe Malaria in Children: A Prospective Cohort Study from Papua New Guinea. *PLoS Medicine* **5** (6): 127-135.

Global Parasite Control Initiative (GPCI) in West Africa Report (2002). Baseline Survey of Parasitic Diseases in the GPCI Model Project District, Dangme-East District, Ghana: Phase1:Ada-Foah sub-district.

Goepp, J. G., M. Hohenboken, J. Almeido-Hill, M. Santosham. (1992). Persistent urinary antigen excretion in infants vaccinated with *Haemophilus influenzae* type b capsular polysaccharide conjugated with outer membrane protein from *Neisseria meningitides*. *The Pediatric Infectious Disease Journal*, **11**(1):2-5.

University of Ghana http://ugspace.ug.edu.gh

Goins, T. L. and J. E. Cutle (2000). Relative Abundance of Oligosaccharides in *Candida* Species as Determined by Fluorophore-Assisted Carbohydrate Electrophoresis. *Journal of Clinical Microbiology*, **38**(8): 2862–2869.

Graham, K., H. Rehman, M. Ahmad, M. Kamal, I. Khan and M. Rowland (2004). Tents pre-treated with insecticide for malaria control in refugee camps: an entomological evaluation *Malaria Journal* **3**: 25-31.

Greenwood, B. M. (1997). Malaria transmission and control. *Parasitology Today* **13**: 90–92.

Greenwood, B. M., K. Bojang, C. J. Whitty and G. A. Targett (2005). "Malaria". *Lancet* **365** (9469): 1487–498. doi:10.1016/S0140-6736(05)66420-3. PMID 15850634.

Griffin, J. T., N. M. Ferguson & A. C. Ghani (2014). Estimates of the changing age-burden of Plasmodium falciparum malaria disease in Sub-saharan Africa. *Nature* Communications, **5**: 3136 | | doi: 10.1038/ncomms4136 |www.nature.com/nature communications

Grimaldi, C. M. and D. L. French (1995). Monoclonal Antibodies by Somatic Cell Fusion. *ILAR Journal*, **37** (3): 125-132.

Gygi, S. P., B. Rist, S. A. Gerber, F. Turecek, M. H. Gelb and R. Aebersold (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nature Biotechnology*, **17**: 994-999. doi:10.1038/13690

Halbert, J, L. Ayong, L. Equinet, K. Le Roch, M. Hardy, D. Goldring, L. Reininger, N. Waters, D. Chakrabarti, C. Doerig (2010). A Plasmodium falciparum transcriptional cyclin-dependent kinase-related kinase with a crucial role in parasite proliferation associates with histone deacetylase activity. *Eukaryotic Cell*, **9** (6): 952-959.

Hanly, W. C., J. E. Artwohl and B. T. Bennett (1995). Review of polyclonal antibody production procedures in mammals and poultry. *ILAR Journal* **37**: 93-118.

University of Ghana http://ugspace.ug.edu.gh

Hendriksen, C. and J. Hau (2003). Production of polyclonal and monoclonal antibodies. In: Handbook of Laboratory Animal Science. 2nd ed. Boca Raton: CRC Press LLC. p 391-411.

Hill, A. V. S. (2011). Vaccines against malaria. *Philosophical Transactions of the Royal Society B* **366**: 2806–2814.

Hopkins, H., L. Bebell, W. Kambale, C. Dokomajilar, P. J. Rosenthal, and G. Dorsey (2008). Rapid Diagnostic Tests for Malaria at Sites of Varying Transmission Intensity in Uganda. *The Journal of Infectious Diseases*, **197**: 510-518.

Hoppenrath, M. and J. F. Saldarriaga (2012). Dinoflagellates. Version 15 December 2012 (under construction). *http://tolweb.org/Dinoflagellates/2445/2012.12.15 in* The Tree of Life Web Project, *http://tolweb.org/*

Horata, N., T. Kalambaheti, A. Craig, and S. Khusmith (2009). Sequence variation of PfEMP1-DBLα in association with rosette formation in *Plasmodium falciparum* isolates causing severe and uncomplicated malaria. *Malaria Journal*; **8**:184.

Howard, C. T. U. S. McKakpo, I. A. Quakyi, K. M. Bosompem, E. A. Addison, K. Sun, D. Sullivan, and R. D. Semba (2007). Relationship of Hepcidin with Parasitemia and Anemia among Patients with Uncomplicated *Plasmodium falciparum* Malaria in Ghana. *American. Journal of Tropical Medicine and Hygiene*, **77** (4): 623–626.

Humphreys, M. (2001). *Malaria: Poverty, Race, and Public Health in the United States*. Johns Hopkins University Press. p. 256. ISBN 0-8018-6637-5.

Huse, K., H.-J. Bo¨hme, and G. H. Scholz (2002). Purification of antibodies by affinity chromatography. *Journal of Biochemical and Biophysical Methods* **51**: 217–231.

Hviid, L. (2007). Adhesion Specificities of *Plasmodium falciparum*-Infected Erythrocytes Involved in the Pathogenesis of Pregnancy-Associated Malaria. *The American Journal of Pathology*. 170(6): 1817–1819.

University of Ghana http://ugspace.ug.edu.gh

Idro, R., K. Marsh, C. C. John, and C. R. J. Newton (2010). Cerebral malaria; mechanisms of brain injury and strategies for improved neuro-cognitive outcome. *Pediatric Research*, 68 (4): 267–274.

Imbert, P, C. Rapp and P. A. Buffet (2009). Pathological rupture of the spleen in malaria: analysis of 55 cases (1958-2008). *Travel Medicine and Infectious Disease*, **7**: 147-159.

Ishengoma, D. S., F. Francis, B. P. Mmbando, J. P. A. Lusingu, P. Magistrado, M. Alifrangis, T. G. Theander, I. C. Bygbjerg and M. M. Lemnge (2011). Accuracy of malaria rapid diagnostic tests in community studies and their impact on treatment of malaria in an area with declining malaria burden in north-eastern Tanzania. *Malaria* Journal **10**: 176-188. http://www.malariajournal.com/ content/10/1/176

Jain, P., B. Chakma, S. Patra, and P. Goswami (2014). Potential Biomarkers and Their Applications for Rapid and Reliable Detection of Malaria. *BioMed Research International*, article ID 852645.

Janeway, C. A. Jr, P, Travers, M. Walport, and M. J. Shlomchik (2001). The Immune System in Health and Disease. Immunobiology, 5th edition; *Garland Science Publishers*; New York. Downloaded from "http://www.ncbi.nlm.nih.gov/books/NBK27144/" on 24 February, 2014.

Jerusalem, C. and W. Eling (1969). Active immunization against *Plasmodium berghei* malaria in mice, using different preparations of plasmodial antigen and different pathways of administration. *Bulletin of World Health Organization* **40:** 807-818.

Josic, D. and Y.-P. Lim (2001). Analytical and Preparative Methods for Purification of Antibodies. *Food Technology and Biotechnology*, **39** (3): 215–226.

University of Ghana http://ugspace.ug.edu.gh

Kakkilaya B. S. (2006). Malaria in Ancient Literature. *Dr. B.S. Kakkilaya's Malaria Web Site*, Retrieved from "http://www.malariasite.com/malaria/history_literature.htm on 23rd December, 2012.

Kattenberg, J. H., I. Versteeg, S. J. Migchelsen, I. J. González, M. D. Perkins, P. F. Mens and H. D. Schallig (2012). New developments in malaria diagnostics: monoclonal antibodies against Plasmodium dihydrofolate reductase-thymidylate synthase, heme detoxification protein and glutamate rich protein. *Monoclonal Antibodies*, **4**(1): 120-126.

Katzin, A. M., A. Marcipar, H. Freilij, R. Corral, J. F. Yanovsky (1989). Rapid determination of *Trypanosoma cruzi* urinary antigens in human chronic Chagas' disease by agglutination test. *Experimental Parasitology*, **68**: 208-215.

Katzin, A. M., C. O. Alexandre, E. A. Kimura, V. J. Peres (1988). Antigenuria in malaria. *Mem Inst Oswaldo (suppl)*, **83**: 208.

Katzin, A. M., E. S. Kimura, C. O. P. Alexandre and A. M. S. Val Ramos (1991). Detection of antigens in urine of patients with acute falciparum and vivax malaria infections. *American Journal of Tropical Medicine and Hygiene*, **45** (4): 453-462.

Kelly-Hope, L. A., J. Hemingway and E. F. McKenzie (2009). Environmental factors associated with the malaria vectors *Anopheles gambiae* and *Anopheles funestus* in Kenya. *Malaria Journal*, **2**: 268-275.

Kemna, E., H. Tjalsma, C. Laarakkers, E. Nemeth, H. Willems, D. Swinkels, (2005). Novel urine hepcidin assay by mass spectrometry. *Blood*, **106***:* 3268–3270.

Khor, S. M., P. Thordarson and J. J. Gooding (2013). The impact of antibody/epitope affinity strength on the sensitivity of electrochemical immunosensors for detecting small molecules. *Analytical and Bioanalytical Chemistry*, **405**(11):3889-3898. Doi: 10.1007/S00216-013-6782-8.

University of Ghana http://ugspace.ug.edu.gh

Kim, J. H., R. C. Stevens, M. J. MacCoss, D. R. Goodlett, A. Scherl, R. J. Richter, S. M. Suzuki, and C. E. Furlong (2010). Identification and Characterization of Biomarkers of Organophosphorus Exposures in Humans. *Advances in Experimental Medicine Biology*. **660**: 61–71. doi:10.1007/978-1-60761-350-3_7

Klee, G., G. d'Onofrio, B. S. Bull, B. H. Davis, K. Fujimoto, B. Houwen, W. Groner, J.A. Koepke, S. M. Lewis, R. M. Rowan, N. Tatsumi, O.W. van Assendelft, L. van Hove. and C. E. McLaren. (2000). Recommended Method for the Determination of Packed Cell Volume by Centrifugation. *The WHO Expert Panel on Cytometry of the International Council for Standardization in Haematology Report*, 2000.

Kofi, A. (1989). Epidemiology of malaria in Ghana. *Ghana Medical Journal*, **23**: 190-196.

Köhler G. and C. Milstein (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. **256** (5517): 495-497.

Kopel, E., G. Oren, Y. Sidi, and D. David (2012). Inadequate antibody response to rabies vaccine in immunocompromised patient. *Emerging Infectious Diseases*, **18** (9): 1493-1495. DOI: http://dx.doi.org/10.3201/eid1809.111833.

Laemmli, U. K. (1970). Cleavage of the structural proteins in the assembly of the head of the bacteriophage T4. *Nature* (London), **227**: 680-682.

Laupland, K. B. (2009). "Fever in the critically ill medical patient". *Crit. Care Med.* **37** (7 Suppl): S273–278.

Leenaars, M., and C. F. M. Hendriksen (2005). Critical Steps in the Production of Polyclonal and Monoclonal Antibodies: Evaluation and Recommendations, *ILAR Journal* **46** (3): 269-279.

Lipman, N. S., L. R. Jackson, L. J.Trudel and F. Weis-Garcia (2005). Monoclonal versus polyclonal antibodies: Distinguishing characteristics, applications, and information sources. *ILAR J*ournal **46 (**3**)**: 258-268.

University of Ghana http://ugspace.ug.edu.gh

Liu, D. and Y.-W. Tang (2012). Molecular detection of human parasitic pathogens. Dongyou Liu; CRC Press *pp*.203-214; Print ISBN: 978-1-4398-1242-6; eBook ISBN: 978-1-4398-1243-3.

Lopata, M. A. and D. W. Cleveland (1987). *In Vivo* Microtubules Are Copolymers of Available 13-Tubulin Isotypes: Localization of Each of Six Vertebrate [3-Tubulin Isotypes Using Polyclonal Antibodies Elicited by Synthetic Peptide Antigens. *The Journal of Cell Biology*, **105**: 1707-1720.

Mahajan, B., H. Zheng, P. T. Pham, M. Y. Sedegah, V. F. Majam, N. Akolkar, M. Rios, Isaac Ankrah, P. Madjitey, G. Amoah, E. Addison, I. A. Quakyi and S. Kumar (2012). Polymerase chain reaction-based tests for pan-species and species-specific detection of human *Plasmodium* parasites. *Transfusion*, **52**: 1949-1956.

Maier, A. G., M. Rug, M. T. O'Neill *et al.* (2008). Exported Proteins Required for Virulence and Rigidity of *Plasmodium falciparum*-Infected Human Erythrocytes. *Cell*, **134**: 48–61.

Martínez, M. J., A. Vilella, T. Pumarola, M. Roldan, V. G. Sequera, I. Vera and E. B. Hayes (2011). Persistence of yellow fever vaccine RNA in urine. *Vaccine (Impact Factor: 3.77)*, **29**(18): 3374-3376. DOI: 10.1016/j.vaccine.2011.02.075

Martinsen E. S., S. L. Perkins and J. J. Schall, (2008). A three-genome phylogeny of malaria parasites (Plasmodium and closely related genera): Evolution of life-history traits and host switches. *Molecular Phylogenetics and Evolution* **47** (1): 261-273.

Mathews, S. T., E. P. Plaisance and T. Kim (2009). Imaging systems for westerns: chemiluminescence vs. infrared detection. *Methods in Molecular Biology*, **536**: 499-513. doi: 10.1007/978-1-59745-542-8_51.

University of Ghana http://ugspace.ug.edu.gh

Mayer, G. (2009). Immunoglobulins - structure and function microbiology and Immunology On-line. "Retrieved http://pathmicro.med.sc.edu/mayer /igstruct2000.htm" on 24 July, 2014.

Mayer, G. (2010). Immunoglobulins- antigen-antibody reactions and selected tests. *Microbiology and Immunology On-line,* downloaded from http://pathmicro.med.sc.edu/ mayer/ab-ag-rx.htm on 3rd June 2014.

McAfee, K. J., D. T. Duncan, M. Assink and A. J. Link (2006). Analyzing Proteomes and Protein Function Using Graphical Comparative Analysis of Tandem Mass Spectrometry Results. *Molecular and Cellular Proteomics*, **5** (8): 1497-1513.

McGregor (1986). Retrieved on April 2, 2007, from http://malaria.wellcome.ac.uk/ doc_WTD023885.html

McKakpo, U. S., K. M. Bosompem, E. A. Addison, P. Madjitey, I. Ankrah, N. Amoah and I. A. Quakyi (2006, *Data unpublished*). Studies towards the development of a urine-based dipstick assay for diagnosis of malaria. A preliminary report submitted to the Department of Population, Family and Reproductive Health, School of Public Health, College of Health Sciences, University of Ghana, Legon.

Meeusen, E. N., R. J. Bischof and C. S. Lee (2001). Comparative T-cell responses during pregnancy in large animals and humans *American Journal of Reproductive Immunology* **46:** 169-179.

Midgett, C. R. and D. R. Madden (2007). Breaking the bottleneck: eukaryotic membrane protein expression for high-resolution structural studies. *Journal of Structural Biology*, **160**: 265-274.

Miller, L. H., and S. L. Hoffman (1998). Research toward vaccines against malaria. *National Medicine of Vaccine Supply*. **4:** 520–524.

University of Ghana http://ugspace.ug.edu.gh

Miller, L. H., D. I. Baruch, K. Marsh and O. K. Doumbo (2002). The pathogenic basis of malaria. *Nature*; **415** (7): 673-679.

Mohanan, D., B. Slütter, M. Henriksen-Lacey, W. Jiskoot, J. A. Bouwstra, Y. Perrie, T. M. Kündig, B. Gander, P. Johansen (2010). Administration routes affect the quality of immune responses: A cross-sectional evaluation of particulate antigen-delivery systems. *Journal of Controlled Release* **147**: 342–349.

Moody, A. (2002). Rapid diagnostic test for malaria parasites. *Clinical Microbiology Reviews*, **15**(1): 66-78.

Moormann, A. M. (2009). How might infant and paediatric immune responses influence malaria vaccine efficacy? Parasite Immunology 31 (9): 547–559.

Moorthy, V. S., M. F. Good and A. V. Hill. (2004). Malaria vaccine developments. *Lancet*, 2004, **363**: 150-156.

Morgan, U. M., and R. L. A. Thompson. **(**1998). Molecular detection of parasitic protozoa. *Parasitology* **117:**S73–S85.

Murray, C. K., R. A. Gasser, Jr., A. J. Magill, and R. Scott Miller (2008). Update on rapid diagnostic testing for malaria. *Clinical Microbiology Reviews*, **21**(1): 97–110. doi:10.1128/CMR.00035-07

Nadjm, B. and R. H. Behrens (2012). "Malaria: An update for physicians". *Infectious Disease Clinics of North America* **26** (2): 243–59.

Nakazawa, M., M. Mukumoto and K. Miyatake (2010). Production and purification of polyclonal antibodies. *Methods in Molecular Biology*, **657**: 63-74.

National Malaria Control Programme (GHS/MOH) 2004. Anti-Malaria Drug Treatment Policy.

University of Ghana http://ugspace.ug.edu.gh

Nayyar, G. M. L., B. G. Breman, P. N. Newton and J. Herrington (2012). "Poor-quality antimalarial drugs in southeast Asia and sub-Saharan Africa". *Lancet Infectious Diseases* **12** (6): 488–96. doi:10.1016/S1473-3099(12)70064-6. PMID 22632187

Nolen, B. M. L. S. Orlichenko, A. Marrangoni, L. Velikokhatnaya, D. Prosser, W. E. Grizzle, K. Ho, F. J. Jenkins, D. H. Bovbjerg and A. E. Lokshin (2013). An Extensive Targeted Proteomic Analysis of Disease-Related Protein Biomarkers in Urine from Healthy Donors. *Plos One* **8** (5): e63368. doi:10.1371/journal.pone.0063368.

O'Meara WP, Bejon P, Mwangi TW, Okiro EA, Peshu N, et al. (2008) Effect of a fall in malaria transmission on morbidity and mortality in Kilifi, Kenya. *Lancet* **372**: 1555–1562

O'Riordan, E., T. N. Orlova, V. N. Podust, P. N. Chander, S. Yanagi, M. Nakazato, R. Hu, K. Butt, V. Delaney and M. S. Goligorsky (2007). Characterization of Urinary Peptide Biomarkers of Acute Rejection in Renal Allografts. *American Journal of Transplantation*, **7**: 930–940.

Oguike, M. C., M. Betson, M. Burke, D. Nolder, J. R. Stothard, I. Kleinschmidt, C. Proietti, T. Bousema, M. Ndounga, K. Tanabe, E. Ntege,R. Culleton, and C. J. Sutherland (2011). *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* circulate simultaneously in African communities. *International Journal for Parasitology*, 41(6-10): 677–683. doi: 10.1016/j.ijpara.2011.01.004

Oguonu T., E. Shu, B. U. Ezeonwu, B. Lige, A. Derrick, R. E. Umeh, and E. Agbo (2014). The performance evaluation of a urine malaria test (UMT) kit for the diagnosis of malaria in individuals with fever in south-east Nigeria: cross-sectional analytical study. *Malaria Journal* **13**: 403-408.

University of Ghana http://ugspace.ug.edu.gh

Ohalete C. N., I. N. S Dozie, M. I. Nwachukwu and C. E. Obiukwu (2011). Epidemiology and socio-economic consequences of malaria in pregnant women in Imo State Nigeria. *African Journal of Microbiology Research* **5**(23): 3895-3900.

Ohrt, C., M. Purnomo, A. Sutamihardja, D. Tang, and K. C. Kain (2002). Impact of Microscopy Error on Estimates of Protective Efficacy in Malaria-Prevention Trials. *The Journal of Infectious Diseases*, **186**: 540–546.

Okell L. C., A. C. Ghani, E. Lyons and C. J. Drakeley (2009). Submicroscopic infection in *Plasmodium falciparum*-endemic populations: a systematic review and meta-analysis. *Journal of Infectious Disease*, **200**: 1509-1517.

Okiro, E. A. and R. W. Snow (2010). The relationship between reported fever and *Plasmodium falciparum* infection in African children. *Malaria Journal*, **9**: 99-104. doi: 10.1186/1475-2875-9-99-104.

Otabil, I. (1992). Notes on West African Traditional Religion Vol. 1 (3rd Edition). (*Manuscript unpublished*).

Palmer et al. (1998). Evaluation of the optimal test for rapid diagnosis of *Plasmodium vivax* and *Plasmodium falciparum* malaria. *Journal of Clinical Microbiology*, **36**: 203-206.

Pandey. S. (2010). Hybridoma technology for production of monoclonal antibodies. *International Journal of Pharmaceutical Sciences Review and Research*, **1**(2): 88-94.

Payne, D. (1988). Use and limitations of light microscopy for diagnosing malaria at the primary health care level. ***Bulletin*** *of the World Health Organization*, **66**: 621-626.

Phillips, R. S. **(**1994). Malaria vaccines—a problem solved or simply a promising start. *Protozoological Abstracts* **18:** 458–486.

Phillips, R. S. (2001). Current Status of Malaria and Potential for Control. *Clinical Microbiology Reviews* **14** (1): 208-226.

University of Ghana http://ugspace.ug.edu.gh

Pier, G. B., D. Boyer, M. Preston, F. T. Coleman, N. Llosa, S. Mueschenborn-Koglin, C. Theilacker, H. Goldenberg, J. Uchin, G. P. *et al*. (2004). Human monoclonal antibodies to *Pseudomonas aeruginosa alginate* that protect against infection by both mucoid and nonmucoid strains. *Journal of Immunology*, **173**: 5671–5678.

Pisitkun, T., R. Johnstone, and M. A. Knepper (2006). Discovery of Urinary Biomarkers. *Molecular and Cellular Proteomics*, **5** (10): 1760-1771.

Plotkin, S. L., and S. A. Plotkin (2008). A short history of vaccination. In Vaccines, S. A. Plotkin, W.A. Orenstein, and P.A. Offit, eds. (Philadelphia, PA: Elsevier Inc), pp. 1–16.

Ponnudurai, T., A. H. W. Lensen, G. J. A. Van Gemert, M. G. Bolmer and J. H. E. T. Meuwissen (1991). Feeding behaviour and sporozoite ejection by infected *Anopheles stephensi*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **85***:* 175-180.

Poovassery, J. S., D. Sarr, G. Smith, T. Nagy and J. M. Moore (2009). Malaria-Induced Murine Pregnancy Failure: Distinct Roles for IFN-γ and TNF. *The Journal of Immunology*, **183**: 5342-5349.

Quakyi I. A., A. E. Addison, K. M. Bosompem., M. D. Wilson, S. Kumar, N. Kumar, D. Boakye, A. A. Adjei, H. Armah, K. Koram, G. Quansah Asare, A. Dodoo, M. Appawu, G. Mensah ,U. S. McKakpo, D. Amarh, F. Lomotey, C. Brown, I. Ankrah, J. Bruce, P. Madjitey, R. Adjei, P. Amankwah, L. A. Kankponang, I. N. Soyiri, P. Koranchie, A. B. Tagoe (2004)**.** Malaria in Kpone-on-Sea: a Fishing Village in southern Ghana. (I) Prevalence Studies. Report to WHO/TDR, 2004.

Quakyi, I. A. (1980). Immunological investigation of malaria in Ghana Vol. I *Immunoepidemiology of malaria in Ghana*; Vol. II *Immunopathological sequelae of malaria*. Thesis University of London, England, 1980.

University of Ghana http://ugspace.ug.edu.gh

Ralston, A. (2008). Do transcription factors actually bind DNA? DNA foot printing and gel shift assays, *Nature Education* **1** (1)

Randell, H. F., K. L. Dickinson, E. H. Shayo, L. E. Mboera and R. A. Kramer (2010). Environmental management for malaria control: knowledge and practices in Mvomero, Tanzania. *Ecohealth* **7** (4): 507-516.

Reyburn, H., H. Mbakilwa, R. Mwangi, O. Mwerinde, R. Olomi, C. Drakeley and C. J. M. Whitty, (2007). Rapid diagnostic tests compared with malaria microscopy for guiding outpatient treatment of febrile illness in Tanzania: Randomized trial: BMJ, doi:10.1136/bmj.39073. 496829.AE.

Rodriguez-del Valle, M., I. A. Quakyi, J. Amuesi, J. T. Quaye, F. K. Nkrumah and D. W. Taylor (1991). Detection of antigens and antibodies in the urine of humans with *Plasmodium falciparum* malaria. *Journal of Clinical Microbiology*, **29** (6): 1236-1242.

Rodulfo, H., M. De Donato, R. Mora, L. González, and C.E. Contreras (2007). Comparison of the diagnosis of malaria by microscopy, immunochromatography and PCR in endemic areas of Venezuela. *Brazilian Journal of Medical and Biological Research*, **40**: 535-543.

Roll Back Malaria WHO partnership (2003). "Economic costs of malaria" (PDF). WHO

Rosenthal, P. J. (2012). How Do We Best Diagnose Malaria in Africa? *American Journal of Tropical Medicine and Hygiene,* **86**(2): 192–193. doi:10.4269/ajtmh.2012.11-0619

Sachs, J. and P. Malaney (2002). "The economic and social burden of malaria". *Nature* **415** (6872): 680–685. doi:10.1038/415680a. PMID 11832956.

Sallusto, F., A. Lanzavecchia, K. Araki and R. Ahmed (2010). From Vaccines to Memory and Back. *Immunity* **33**: 451-463.

University of Ghana http://ugspace.ug.edu.gh

Samuel, M. D., P. H. F. Hobbelen, F. Decastro, J. A. Ahumada, D. A. Lapointe, C. T. Atkinson, B. L. Woodworth, and P. J. Hart and D. C. Duffy (2011). The dynamics, transmission, and population impacts of avian malaria in native Hawaiian birds: a modeling approach. *Ecological Applications*, **21**(8): 2960–2973.

Santana-Morales, M., M. A. Quispe-Ricalde, R. N. Afonso-Lehmann, P. Berzosa, J. Lorenzo-morales, G. Tiziano, Fransisco Reyes Benitoa, B. Valladares and E. Martinez-Carretero (2013). Haemoglobin levels for population from Gambo, a rural area of Ethiopia, and their association with anaemia and malaria. *Malaria Journal*, **12**:435-441.

Schellenberg D, Menendez C, Aponte J, Guinovart C, Mshinda H, et al. (2004). The Changing Epidemiology of Malaria in Ifakara Town, southern Tanzania. *Tropical Medicine and International Health* 9: 68–76.

Schiff, M. J., M. M. Fisher, A. L. Jones and B. J. Underdown (1986). Human IgA as a heterovalent ligand: switching from the asialoglycoprotein receptor to secretory component during transport across the rat hepatocyte. *The Journal of Cell Biology*, **102**: 920-931.

Schwartlander, B. **(**1997). Global burden of disease. *Lancet* **350:** 141–142.

Sengupta, A., S. Ghosh, A. Basant, S. Malusare, P. Johri, S. Pathak, S. Sharma and H. M. Sonawat (2011). Global host metabolic response to *Plasmodium vivax* infection: a 1H NMR based urinary metabonomic study. *Malaria Journal*, **10**: 384-396.

Sermwittayawong, N., B. Singh, M. Nishibuchi, N. Sawangjaroen and V. Vuddhakul (2012). Human *Plasmodium knowlesi* infection in Ranong province, southwestern border of Thailand. *Malaria Journal* **11**: 36-41.

Seth, R. K., M. Saxena, A. A. Bhat, G.B.K.S. Prasad, K. Hajela, D. N. Rao and S. Biswas (2013). Antibodies against erythrocytic stages of *Plasmodium vivax* and use in

University of Ghana http://ugspace.ug.edu.gh

detection of malaria. *International Journal of Basic and Applied Medical Sciences*, **3** (1):76-86.

Sevier, E. D., G. S. David, J. Martinis, W. J. Desmond, R. M. Bartholomew, and R. Wang (1981). Monoclonal Antibodies in Clinical Immunology.. *Clinical Chemistry* **27** (11): 1797-1806.

Shiff, C. (2002). Integrated Approach to Malaria Control. *Clinical Microbiology Reviews*, **15**(2): 278–293. DOI: 10.1128/CMR.15.2.278–293.2002

Shimizu, Shinya (2004). Routes of Administration in *The Laboratory Mouse* Copyright 2004 Elsevier; ISBN 0-1233-6425-6

Siegrist, C. A. (2008). Vaccine immunology. In Vaccines, S.A. Plotkin, W.A. Orenstein, and P.A. Offit, eds. (Philadelphia, PA: Elsevier Inc), pp. 17–36.

Simmons, M.L. and Brick, J. O. (1970). In The Laboratory Mouse (ed. A. Hollaender), pp. 127–129. Prentice-Hall Inc., Englewood Cliffs

Smith, J. D., B. Gamain, D. I. Baruch and S. Kyes (2001). Decoding the language of var genes and *Plasmodium falciparum* sequestration. *Trends in Parasitology* **17**: 538-545.

Snow, R. W., C. A. Guerra, A. M. Noor, H. Y. Myint, and S. I. Hay (2005). The global distribution of clinical episodes of Plasmodium falciparum malaria. *Nature*, **434**: 214-217.

Stauffer, W. and P. R. Fischer (2003).Diagnosis and Treatment of Malaria in Children. Clinical Infectious Diseases; **37**: 1340–1348.

Stauga, S., A. Hahn, N. W Brattig, J. Fischer-Herr, S, Baldus, G. D. Burchard and J. P Cramer (2013). Clinical relevance of different biomarkers in imported *Plasmodium falciparum* malaria in adults: a case control study. Malaria Journal, 12:246 http://www.malariajournal.com/content/12/1/246.

University of Ghana http://ugspace.ug.edu.gh

Steenkeste, N., W. O. Rogers, L. Okell, I. Jeanne, S. Incardona, L. Duval, S. Chy, S. Hewitt, M. Chou, D. Socheat D, F. X. Babin, F. Ariey and C. Rogier (2010). Sub-microscopic malaria cases and mixed malaria infection in a remote area of high malaria endemicity in Rattanakiri province, Cambodia: implication for malaria elimination. *Malaria Journal* **9**:108.

Steketee, R.W. *et al*. (2001). The burden of malaria in pregnancy in malaria-endemic areas. *American Journal of Tropical Medicine and Hygiene*, **64**(Supple 1-2): 28-35.

Stills, H. F. Jr. (1994). Polyclonal antibody production. In: Manning PJ, Ringler DH, Newcomer CE, eds. The Biology of the Laboratory Rabbit, 2nd ed. San Diego: Academic Press Inc. p 435-448.

Sturm, A., R. Amino, C. Van De Sand, T. Regen, S. Retzlaff, A. Rennenberg, A. Krueger, J. M. Pollok, R. Menard and V. T. Heussler (2006). "Manipulation of Host Hepatocytes by the Malaria Parasite for Delivery into Liver Sinusoids". *Science* **313** (5791): 1287–1290.doi:10. 1126/science.1129720. PMID 16888102.

Sutherland, C. J., N. Tanomsing, D. Nolder, M. Oguike, C. Jennison, S. Pukrittayakamee, *et al*. (2010). Two Nonrecombining Sympatric Forms of the Human Malaria Parasite *Plasmodium ovale* Occur Globally. *The Journal of Infectious Diseases.* **201**(10): 1544–1550.

Thomas, C. E., W. Sexton, K. Benson, R. Sutphen and J. Koomen (2010). Urine Collection and Processing for Protein Biomarker Discovery and Quantification. *Cancer Epidemiology, Biomarkers and Prevention,* **19**: 953-959.

Tchouassi, D. P., I. A. Quakyi, E. A. Addison, K. M. Bosompem, M. D. Wilson, M. A. Appawu, C. A. Brown and D. A.Boakye (2012). Characterization of malaria transimission by vector populations for improved interventions during the dry season in the Kpone-on-Sea of coastal Ghana. *BioMed Central,* **5** 212.

University of Ghana http://ugspace.ug.edu.gh

Trager, W. and J. B. Jensen (1976). Human malaria parasites in continuous culture. *Science*, **193**: 673-675.

Trager, W. and J. B. Jensen (2005). Human malaria parasites in continuous culture. *Journal of Parasitology*, **91**(3):484-486. doi: http://dx.doi.org/10.1645/0022-3395(2005)091[0484: HMPICC]2.0.CO;2

Urban, B. C., D. J. Ferguson, A. Pain, N. Willcox, M. Plebanski, J. M. Austyn and D. J. Roberts (1999). *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature*, **400**: 73–77.

Van den Eede, P., H. N. Van, C. V. Overmeir, I. Vythilingam, T. N. Duc, L. X. Hung, H. N. Manh, J. Anné, U. D'Alessandro and A. Erhart (2009). Human *Plasmodium knowlesi* infections in young children in central Vietnam. *Malaria Journal*, **8**: 249-253.

Van der Heyde, H. C., J. Nolan, V. Combes, I. Gramaglia, and G. E. Grau (2006). A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. *Trends in Parasitology*, **22** (11): 503-508.

Vargas-Serrato, E., V. Corredor and M. R. Galinski (2003). Phylogenetic analysis of CSP and MSP-9 gene sequences demonstrates the close relationship of *Plasmodium* coatneyi to *Plasmodium knowlesi*. Infection, *Genetics and Evolution*, **3** (1):67-73.

Varun, G. (2012). What are the Factors that Influence Immunogenicity of an Antigen? Downloaded from "http://www.preservearticles.com/2012031827744/what-are-the-factors-that-influence-immunogenicity-of-an-antigen.html" on 11 May 2014.

Waller, R. F. and G. I. McFadden. (2005). The Apicoplast: A Review of the Derived Plastid of Apicomplexan Parasites. *Current Issue of Molecular Biology*, **7**: 57-80.

University of Ghana http://ugspace.ug.edu.gh

Weatherall, D. J., L. H. Miller, D. I. Baruch, K. Marsh, O. K. Doumbo, C. Casals-Pascual and D. J. Roberts (2002). Malaria and the red cell. *Hematology/ American Society of Hematology Education Program*, **1**: 35–57.

White, N. J. (1998). Malaria: In Manson's tropical diseases. International edition W. B. Saunders, London, 1779*p*.

White N. J. (2003). Malaria, pp. 1205-1296. In G. C. Cook and A. Zumla (ed.), Manson's Tropical Diseases, 21st ed. WB Saunders, Philadelphia.

WHO (1968). Nutritional Anemia: Report of a WHO Scientific Group. Geneva, Switzerland: World Health Organization.

WHO (1991). Management of severe and complicated malaria: a practical handbook.

WHO (1993). *A global strategy for malaria control*, Geneva.

WHO **(**1996). Investing in health research and development. Document TDR/Gen/96.1. Ad Hoc Committee of Health Research Relating to Future Intervention Options, World Health Organization, Geneva, Switzerland.

WHO (2008). World Malaria Report 2008. Geneva: WHO.

WHO (2010). Basic Malaria microscopy-2nd. Edition; Part 1: Learner's Guide.

WHO (2012). Guidelines on the quality, safety and efficacy of recombinant malaria vaccines targeting the pre-erythrocytic and blood stages of *Plasmodium falciparum*.

WHO /Malaria (2000). Report of a joint WHO/USAID informal consultation.

WHO Malaria Policy Advisory Committee and Secretariat Meeting Report (2012). Inaugural meeting of the malaria policy advisory committee to the WHO: conclusions and recommendations. *Malaria Journal*, **11**: 137-143.

WHO Malaria Report (2005). Minutes of the 11 Roll Back Malaria (RBM) Monitoring and Evaluation Reference Group (MERG) Task Force on Malaria-Related Anaemia

University of Ghana http://ugspace.ug.edu.gh

Meeting, held on 27-28 October, 2003. Retrieved from "http://whqlibdoc.who.int/ publications/2005/

WHO/ OMS (2002). Malaria. *WHO Fact Sheet No 94*.

WHO (2014). WHO Global Malaria Programme World Malaria Report 2014.

Wians, Jr., F. H. (2009). Clinical laboratory tests: which, why, and what do the results mean? *Labmedicine*, **40** (2): 105-113. doi: 10.1309/lm4o4l0hhutwwudd.

Wikipedia (2014). Fever. Downloaded from "http://en.wikipedia.org/wiki/Fever" on 5th May 2014.

Williamson, D. H., P. R. Preiser, P. W. Moore, S. McCready, M. Strath and R. J. M. Wilson (2002). The plastid DNA of the malaria parasite *Plasmodium falciparum* is replicated by two mechanisms. *Molecular Microbiology*, **45**: 533-542.

Wipasa, J., S. Elliott, H. Xu and M. F. Good (2002). Immunity to asexual blood stage malaria and vaccine approaches. *Immunology and Cell Biology*, **80**: 401–414.

Wogu, M N., F. O. Nduka and M. D. Wogu (2013). Prevalence of Malaria Parasite Infection among Pregnant Women Attending Antenatal Clinics in Port Harcourt, Rivers State, Nigeria. *International Journal of Tropical Disease & Health*, 3 (2): 126-132**.**

Wongsrichanalai, C., M. J. Barcus, S. Muth, A. Sutamihardja, and W. H. Wernsdorfer (2007). A Review of Malaria Diagnostic Tools: Microscopy and Rapid Diagnostic Test (RDT). *American Journal of Tropical Medicine and Hygiene,* **77** (6): 119–127.

Woodard, G. (1965). In Methods of Animal Experimentation, Vol. 1 (ed. W.J. Gay), pp. 343–359. Academic Press, New York

Worrall, E, S. Basu and K. Hanson (2005). "Is malaria a disease of poverty? A review of the literature". *Tropical Health and Medicine* **10** (10): 1047–1059. doi:10.1111/j.1365-3156.2005.01476.x. PMID 16185240.

University of Ghana http://ugspace.ug.edu.gh

Wykes, M. and M. F. Good (2007). A case for whole-parasite malaria vaccines. *International Journal for Parasitology* **37**: 705–712.

Yamauchi, L. M., A. Coppi, G. Snounou and P. Sinnis (2007). *Plasmodium* sporozoites trickle out of the injection site, *Cellular Microbiology* **9**(5): 1215–1222.

Yokoyama, W. M. (1999). Production of Monoclonal Antibodies. *Current Protocols in Cell Biology*, Section 16.1.1-16.1.17. John Wiley and Sons, Inc. pp 17.

Yzerman, E. P. F., J. W. den Boer, K. D. Lettinga, J. Schellekens, J. Dankert, and M. Peeters (2002). Sensitivity of Three Urinary Antigen Tests Associated with Clinical Severity in a Large Outbreak of Legionnaires' Disease in The Netherlands. *Journal of Clinical Microbiology*, **40**(9): 3232–3236. doi: 10.1128/JCM.40.9.3232-3236.2002

Zhang, S., J. Xiang, A. Cheng, M. Wang, X. Li, L. Li, X. Chen, D. Zhu, Q. Luo and X. Chen (2010). Production, purification and characterization of polyclonal antibody against the truncated gK of the duck enteritis virus. *Virololgy Journal*, 7:241-247.

Zhu, Y., Y. Li, S. Haraguchi, M. Yu, M. Ohira, T. Ozaki, A. Nakagawa, T. Ushijima, E. Isogai, H. Koseki, Y. Nakamura, C. Kong, P. Mehlen, H. Arakawa. and A. Nakagawara (2013). Dependence receptor UNC5D mediates nerve growth factor depletion–induced neuroblastoma regression. *Journal of Clinical Investigation*, **123** (7): 2935–2947. doi:10.1172/JCI65988

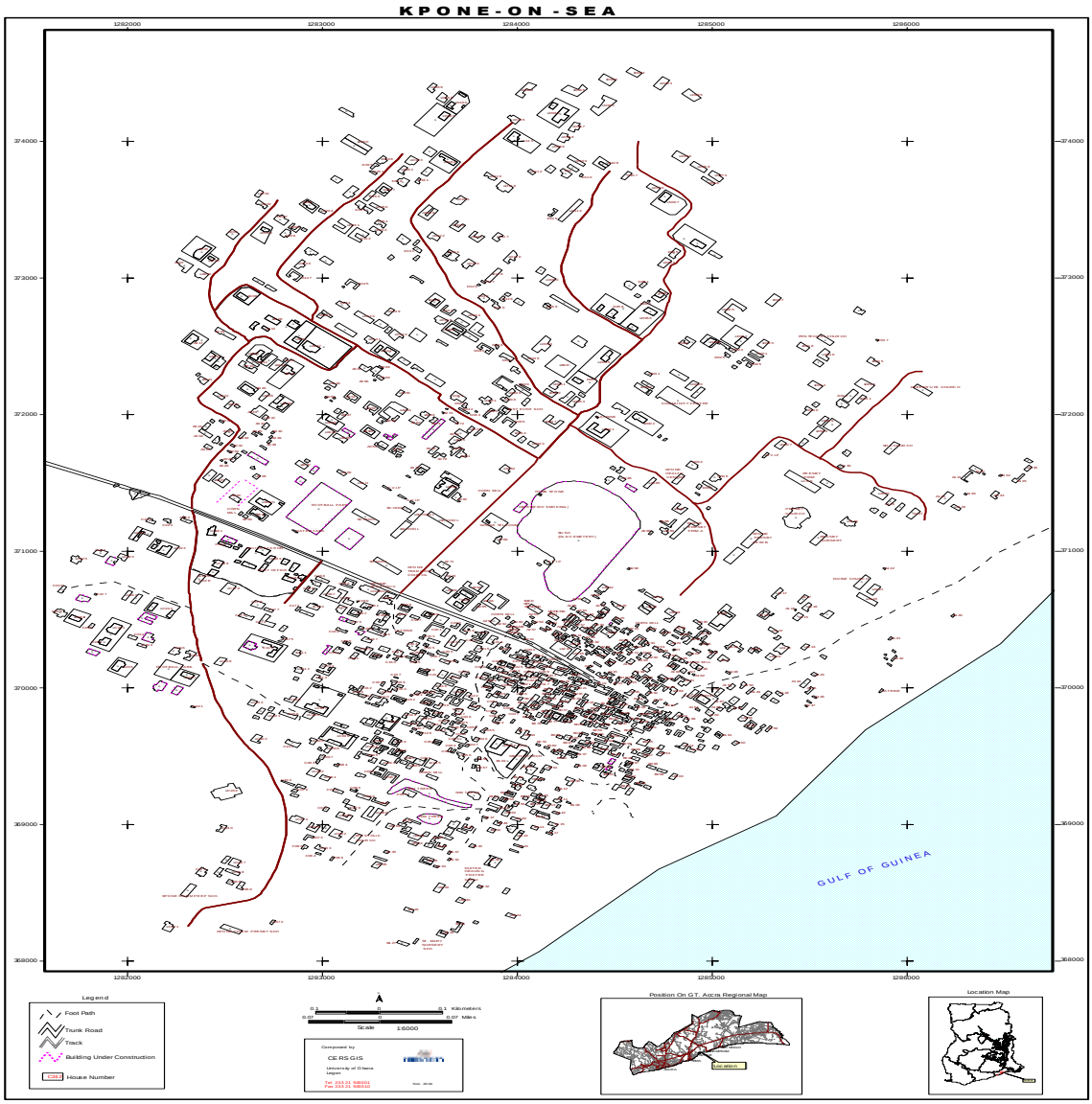
Zilversmit, M. and S. Perkins (2008). *Plasmodium*. Malaria Parasites. Version 05 May 2008. *http://tolweb.org/Plasmodium/68071/2008.05.05 in* The Tree of Life Web Project, http://tolweb.org.

Zola, H. and P. R. Thomson (2001). Monoclonal Antibodies: Diagnostic Uses. *Encyclopaedia of Life Sciences*, Nature Publishing Group / www.els.net. 9 pp. Retrieve from "http://www.avpivnik. ru/works/markers/markers_pdf_025.pdf" on 28 February, 2013.

University of Ghana http://ugspace.ug.edu.gh

APPENDICES

APPENDIX 1: MAP OF KPONE-ON-SEA STUDY AREA

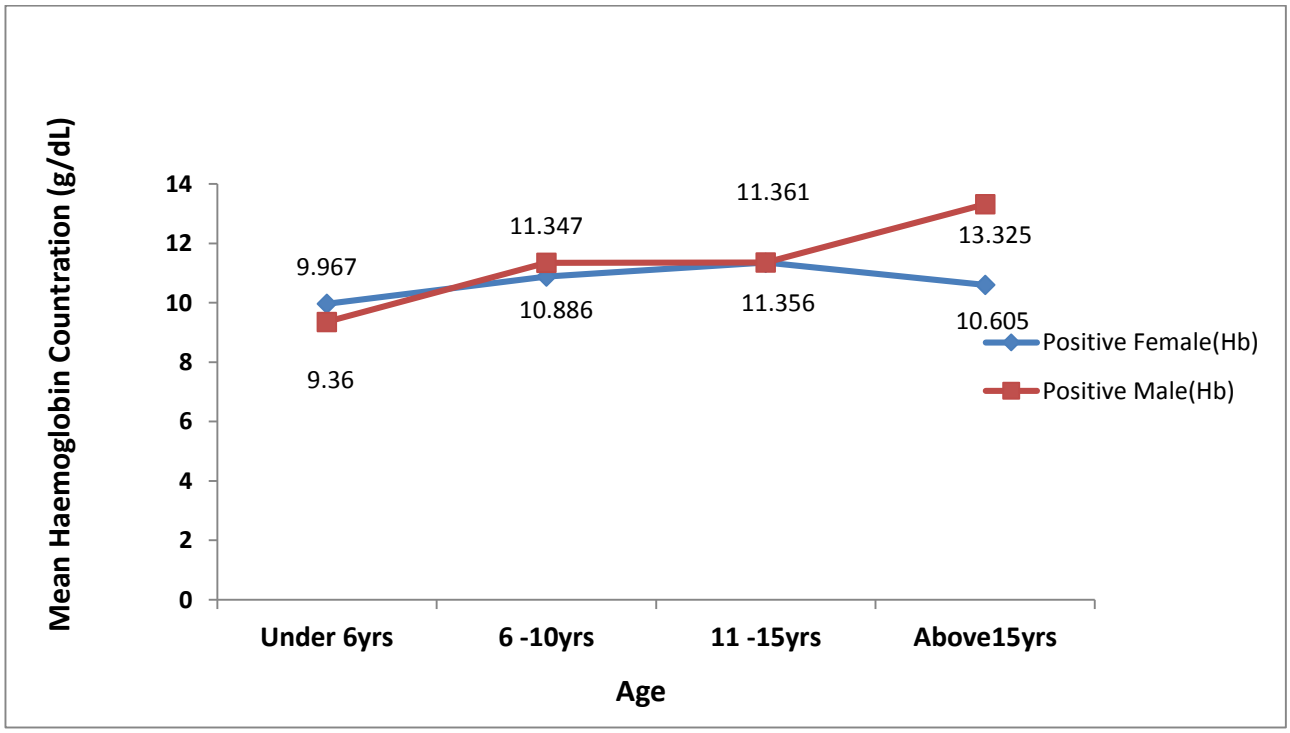


University of Ghana http://ugspace.ug.edu.gh

APPENDIX 2: ADDITIONAL DATA FROM CHAPTER FOUR

Appendix 2a Variation of mean haemoglobin concentration among malaria positive subjects of same sex relative to age

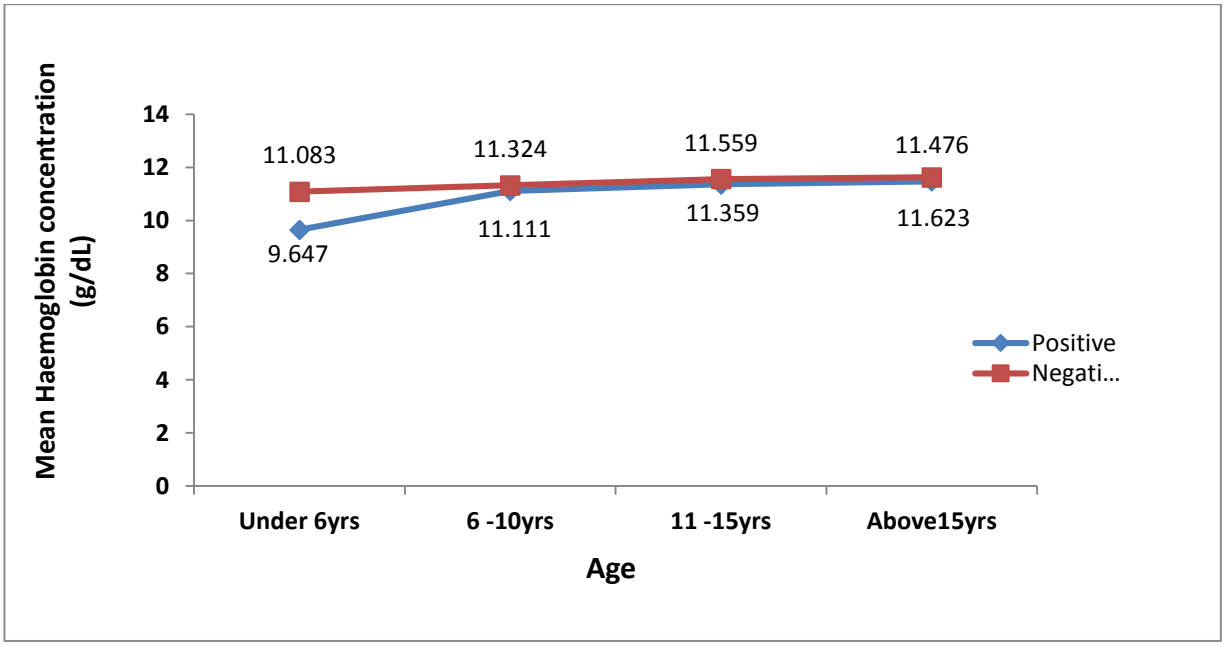
Appendix 2a summarizes the variation of the mean haemoglobin concentration (MHb) among malaria positive subjects of same sex with respect to age. According to these results all the female malaria positive subjects among the study community are anaemic, with a lower MHb than the normal value required for each category. The results also show that malaria positive female subjects above 15 years of age are the most severely anaemic (MHb = 10.605g/dL; normal MHb = 12 – 16 g/dL). Similarly, the male malaria positive subjects within the study community are anaemic, with MHb being lower than the normal range (children, MHb = 11 g/dL; adults, MHb = 14-18 g/dL). Comparably however, the male malaria positive subjects are more severely anaemic than their female counterparts. Generally, the 11 =15 year old positive male subjects are the most severely anaemic with a MHb being 2.6 g/dL lower than the normal range.



University of Ghana http://ugspace.ug.edu.gh

Appendix 2b Variation of malaria infection among study subjects relative to mean blood haemoglobin concentration and age

Upon analyzing the variation of malaria infection among the study subjects with respect to MHb and age however, the results (Appendix 2b) show that generally, apart from the uninfected subjects under age 6 who have normal blood MHb (MHb = 11.083 g/dL), the rest of the subjects made up of infected subjects below 6 years of age through to both infected and uninfected subjects above 15 years old were anaemic with their MHb below the standard level for each age and sex category.



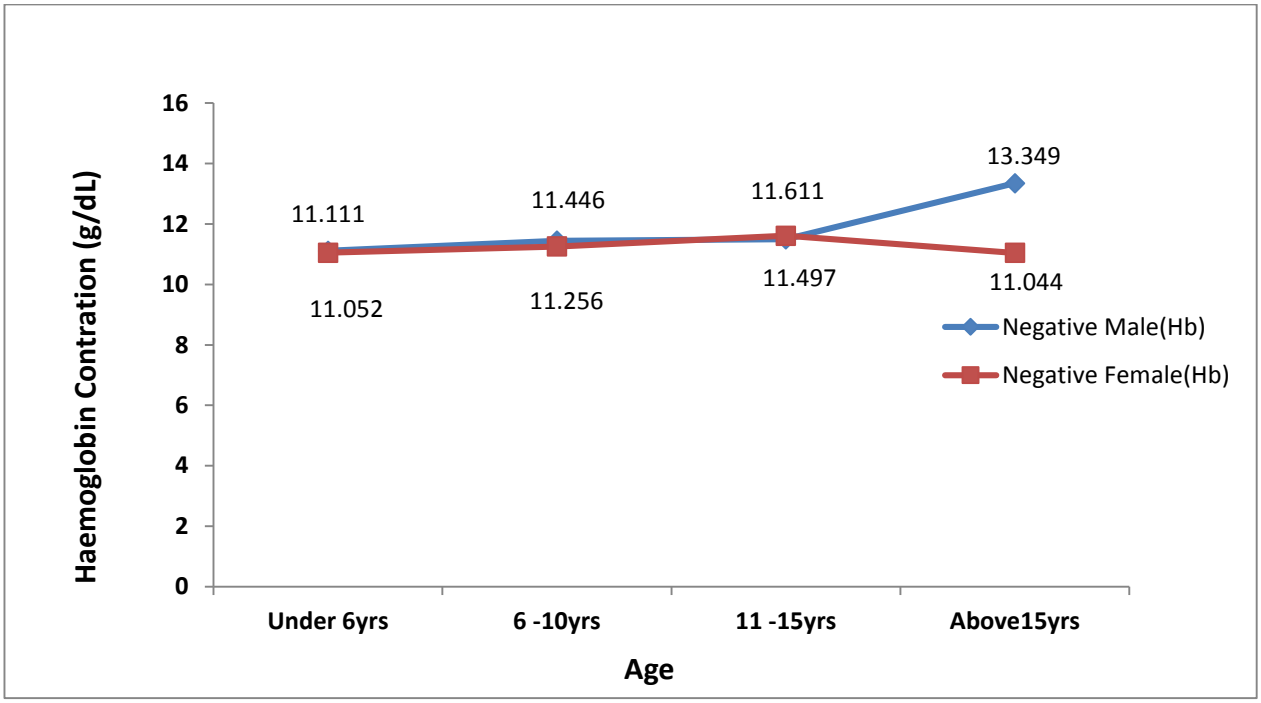
Variation of Malaria Infection Among Study Subjects Relative to Mean Blood Haemoglobin Concentration and Age

Appendix 2c Variation of mean haemoglobin concentration among malaria negative subjects of same sex relative to age

Unlike the case for malaria positive subjects, the MHb is generally similar among male and female malaria negative subjects, and ranges from 11.05 g/dL in female negative subjects below 6 years of age to 11.61 g/dL in female negative subjects within the 11-15 years age category. In both the positive and negative cases however, the MHb (11.3 d/gL) of male

University of Ghana http://ugspace.ug.edu.gh

subjects above 15 years old, even though it was lower than the standard range (14-18 g/dL) was not too far from the standard level. In totality, the two sets of results thus show that generally the MHb of both malaria positive and negative subjects within the study community follow a similar trend.

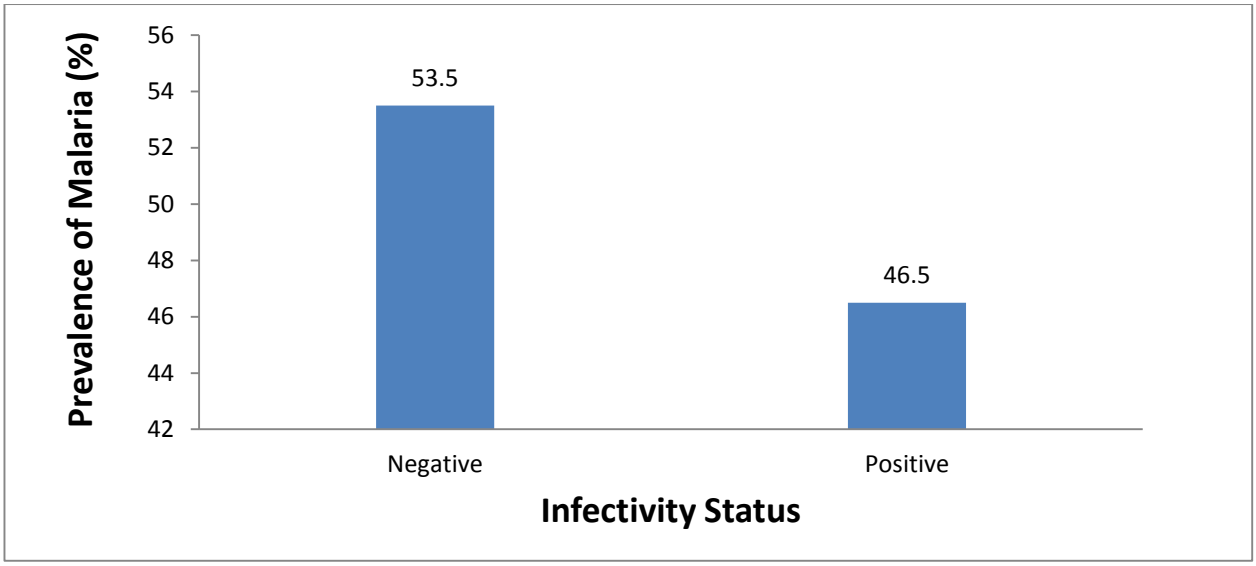


Variation of Mean Blood Haemoglobin Concentration Among Malaria Negative Subjects of Same Sex with Respect to Age

Appendix 2d Prevalence of Malaria Parasite Infection among Study Subjects as Determined by Commercial Rapid Diagnostic Test

Appendix 2d summarizes the prevalence of malaria infection among the study subjects as determined by the commercial rapid diagnostic test kit. According to the graph, approximately 47.0% of the study participants has malaria while the simple majority were malaria negative.

University of Ghana http://ugspace.ug.edu.gh

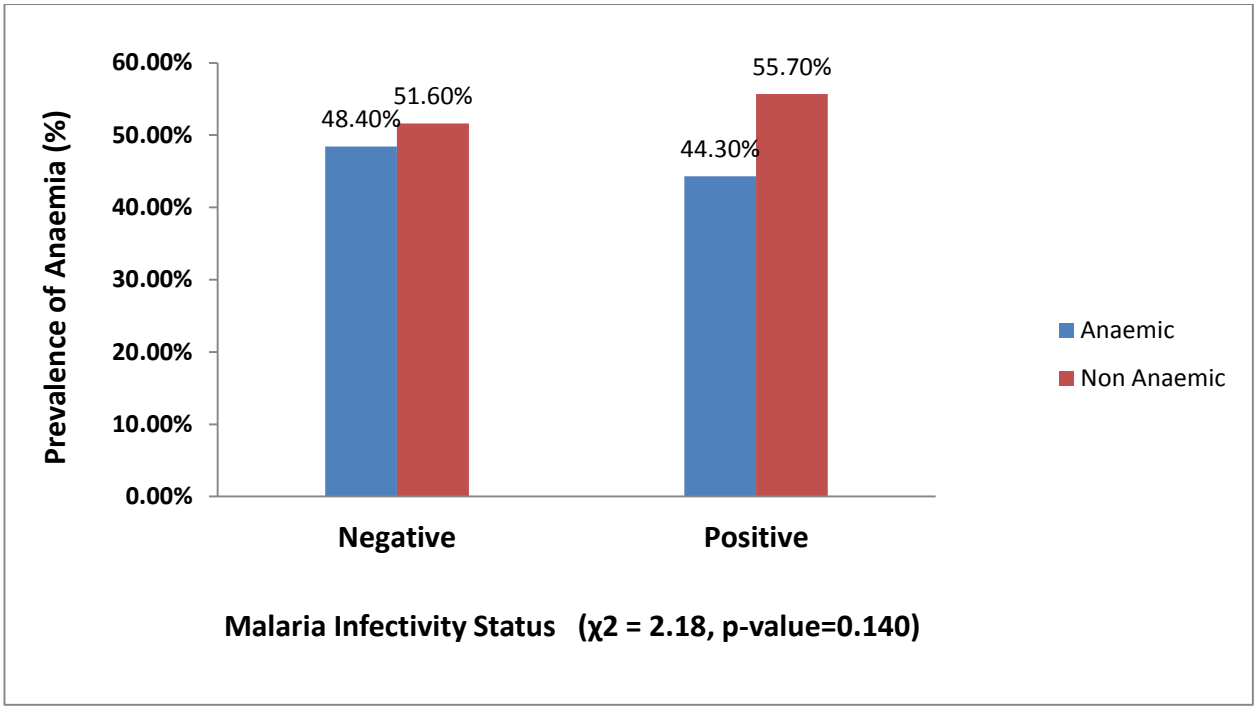


Prevalence of Malaria Parasite Infection among Study Subjects as Determined by Commercial Rapid Diagnostic Test

Appendix 2e Prevalence of Anaemia among Study Subjects in Relation to Malaria Infection Determined by RDT

Appendix 2e below, summarizes the prevalence of anaemia among the study subjects in relation to malaria infection as determined by the commercial rapid diagnostic test (RDT) kit. As shown, the prevalence of anaemia among malaria negative subjects (48.4%) is numerically higher than that (44.3%) among their malaria positive counterparts contrary to the normal expectation. Also even though the prevalence of anaemia among malaria negative subjects is numerically higher than that of malaria positive subjects, the difference is not statistically significant ($p>0.05$).

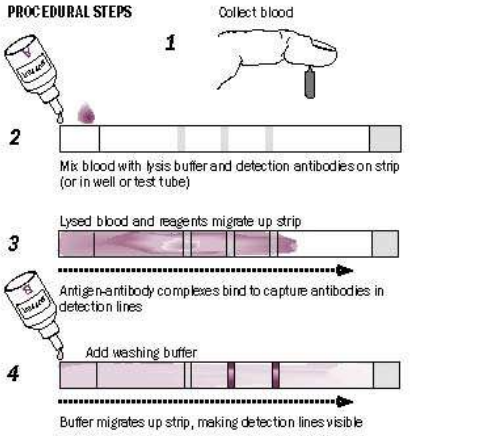
University of Ghana http://ugspace.ug.edu.gh

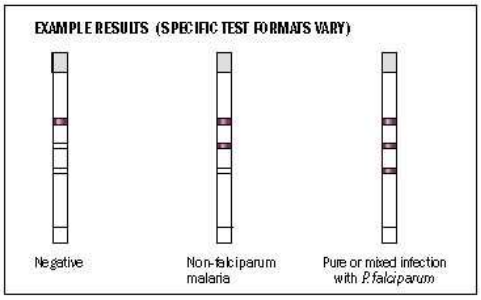


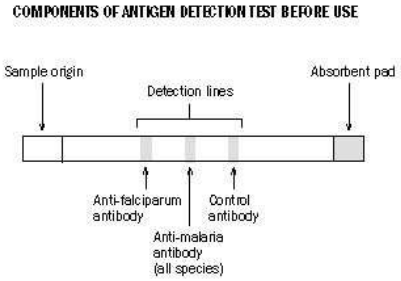
Prevalence of Anaemia among Study Subjects in Relation to Malaria Infectivity as Determined by RDT

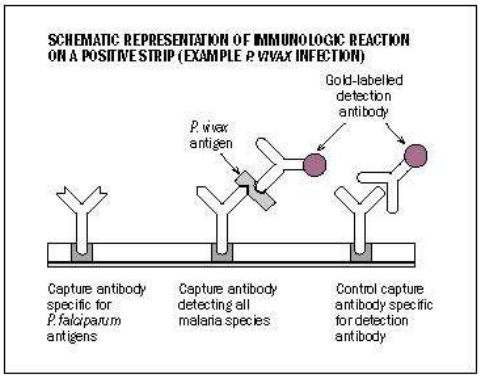
University of Ghana http://ugspace.ug.edu.gh

APPENDIX 3: SCHEMATIC REPRESENTATION OF THE RAPID DIAGNOSTIC TEST PROCEDURE AS SHOWN IN WHO (2000)









University of Ghana http://ugspace.ug.edu.gh

APPENDIX 4: RESULTS OF URINE ANALYSIS

Test Characteristic/Parameter	Test Results	Frequency	Percent
Leukocytes (WBC/µL)	Neg	1095	91.4
	±	9	.8
	+	64	5.3
	++	17	1.4
	+++	13	1.1
	Total	1198	100.0
Nitrite	Neg	1203	98.0
	Pos	24	2.0
	Total	1227	100.0
Urobilinogen (mg/dl; µmol/L)	Neg	1117	90.2
	1 [17]	60	4.8
	2 [35]	43	3.5
	4 [70]	11	.9
	8 [140]	7	.6
	Total	1238	100.0
Protein (mg/dl; (g/L)	Neg	781	69.4
	± [30; (0.15)]	43	3.8
	+ [30; (0.3)]	234	20.8
	++ [100; (1.0)]	52	4.6
	+++ [300; (3.0)]	12	1.1
	++++ [2000; (20)]	3	.3
	Total	1125	100.0
pH	5.0	491	40.3
	6.0	547	44.9
	7.0	94	7.7
	8.0	75	6.2
	9.0	12	1.0
	Total	1219	100.0
BLOOD (RBC/µL)	Neg	1115	90.1
	±	2	.2
	+	46	3.7
	++	29	2.3
	+++	32	2.6
	5 - 10(1)	8	.6
	5 - 10	6	.5
	Total	1238	100.0
Specific Gravity (SG)	None	8	.6
	1.000	427	34.6
	1.005	52	4.2
	1.010	146	11.8
	1.015	146	11.8
	1.020	207	16.8
	1.025	144	11.7

University of Ghana http://ugspace.ug.edu.gh

	1.030	104	8.4
	Total	1234	100.0
etones (mg/dl; mmol/L)	Neg	1065	86.4
	± [5 (0.5)]	54	4.4
	15 (1.5)	43	3.5
	40 (4.0)	11	.9
	80 (8.0)	43	3.5
	160 (16)	16	1.3
	Total	1232	100.0
Bilirubin (mg/dl; mmol/L)	Neg	1207	98.0
	+ [1(17)]	20	1.6
	++ [2(35)]	5	.4
	Total	1232	100.0
Glucose (mg/dl; mmol/L)	Neg	1216	98.5
	100(5)	10	.8
	250(15)	5	.4
	500(30)	2	.2
	1000(60)	2	.2
	Total	1235	100.0
Ascorbic Acid (mg/dl; mmol/L)	Neg	416	84.4
	+ [10(5.6)]	65	13.2
	++ [20(1.14)]	12	2.4
	Total	493	100.0

University of Ghana http://ugspace.ug.edu.gh

Urine Analysis with Respect to Age (Positive Cases)

		Under 6yrs		6 -10yrs		11 -15yrs		Above 15yrs	
		Count	Row N %	Count	Row N %	Count	Row N %	Count	Row N %
Leukocytes (WBC/µL)	**Neg**	98	18.8%	140	26.9%	81	15.5%	202	38.8%
	±	0	0.0%	2	50.0%	1	25.0%	1	25.0%
	+	6	30.0%	6	30.0%	2	10.0%	6	30.0%
	++	0	0.0%	0	0.0%	1	25.0%	3	75.0%
	+++	0	0.0%	0	0.0%	1	16.7%	5	83.3%
Nitrite	**Neg**	103	18.4%	151	27.0%	89	15.9%	217	38.8%
	Pos	2	16.7%	3	25.0%	0	0.0%	7	58.3%
Urobilinogen (mg/dl; µmol/L)	**Neg**	89	18.1%	142	28.9%	74	15.0%	187	38.0%
	1 [17]	4	10.8%	8	21.6%	5	13.5%	20	54.1%
	2 [35]	11	35.5%	3	9.7%	5	16.1%	12	38.7%
	4 [70]	0	0.0%	0	0.0%	4	44.4%	5	55.6%
	8 [140]	2	33.3%	1	16.7%	1	16.7%	2	33.3%
	12 [200]	0	0.0%	0	0.0%	0	0.0%	0	0.0%
	0.2 [3.5]	0	0.0%	0	0.0%	0	0.0%	0	0.0%
Protein (mg/dl; (g/L)	**Neg**	55	17.8%	88	28.5%	50	16.2%	116	37.5%
	± [30; (0.15)]	5	17.2%	9	31.0%	7	24.1%	8	27.6%
	+ [30; (0.3)]	25	18.0%	35	25.2%	22	15.8%	57	41.0%
	++ [100; (1.0)]	11	27.5%	11	27.5%	4	10.0%	14	35.0%
	+++ [300; (3.0)]	3	30.0%	1	10.0%	1	10.0%	5	50.0%
	++++ [2000; (20)]	0	0.0%	0	0.0%	0	0.0%	2	100.0%
pH	**Mean**	5.7		5.6		5.9			

University of Ghana http://ugspace.ug.edu.gh

BLOOD (RBC/µL)	**Neg**	96	18.3%	148	28.2%	82	15.6%	198	37.8%
	±	0	0.0%	0	0.0%	0	0.0%	0	0.0%
	+	6	27.3%	3	13.6%	1	4.5%	12	54.5%
	++	1	6.7%	1	6.7%	6	40.0%	7	46.7%
	+++	3	25.0%	2	16.7%	0	0.0%	7	58.3%
	5 - 10(1)	0	0.0%	0	0.0%	0	0.0%	2	100.0%
	5 - 10	0	0.0%	0	0.0%	0	0.0%	0	0.0%
Specific Gravity (SG)	**None**	0	0.0%	1	25.0%	0	0.0%	3	75.0%
	1.000	40	20.5%	37	19.0%	25	12.8%	93	47.7%
	1.005	8	40.0%	4	20.0%	2	10.0%	6	30.0%
	1.010	12	19.7%	20	32.8%	13	21.3%	16	26.2%
	1.015	9	12.5%	23	31.9%	10	13.9%	30	41.7%
	1.020	18	16.5%	30	27.5%	21	19.3%	40	36.7%
	1.025	11	15.7%	23	32.9%	12	17.1%	24	34.3%
	1.030	7	17.1%	15	36.6%	6	14.6%	13	31.7%
Ketones (mg/dl; mmol/L)	**Neg**	71	15.1%	112	23.9%	77	16.4%	209	44.6%
	± [5 (0.5)]	8	23.5%	12	35.3%	5	14.7%	9	26.5%
	15 (1.5)	9	37.5%	7	29.2%	4	16.7%	4	16.7%
	40 (4.0)	4	66.7%	2	33.3%	0	0.0%	0	0.0%
	80 (8.0)	12	41.4%	14	48.3%	2	6.9%	1	3.4%
	160 (16)	1	10.0%	6	60.0%	1	10.0%	2	20.0%
Bilirubin (mg/dl; mmol/L)	**Neg**	102	18.5%	147	26.6%	87	15.8%	216	39.1%
	+ [1(17)]	2	14.3%	4	28.6%	2	14.3%	6	42.9%
	++ [2(35)]	0	0.0%	2	50.0%	0	0.0%	2	50.0%
	+++ [4(70)]	0	0.0%	0	0.0%	0	0.0%	0	0.0%

University of Ghana http://ugspace.ug.edu.gh

Glucose (mg/dl; mmol/L)	**Neg**	103	18.4%	152	27.1%	86	15.4%	219	39.1%
	100(5)	1	16.7%	1	16.7%	1	16.7%	3	50.0%
	250(15)	0	0.0%	0	0.0%	1	25.0%	3	75.0%
	500(30)	1	50.0%	0	0.0%	1	50.0%	0	0.0%
	1000(60)	0	0.0%	0	0.0%	0	0.0%	0	0.0%
	≥2000(110)	0	0.0%	0	0.0%	0	0.0%	0	0.0%
Ascorbic Acid (mg/dl; mmol/L)	**Neg**	35	21.0%	41	24.6%	23	13.8%	68	40.7%
	+ [10(5.6)]	5	18.5%	10	37.0%	5	18.5%	7	25.9%
	++ [20(1.14)]	0	0.0%	3	60.0%	1	20.0%	1	20.0%
	+++ [40(2.28)]	0	0.0%	0	0.0%	0	0.0%	0	0.0%

Urine Analysis with Respect to Age

Negative Cases

		Under 6yrs		6 -10yrs		11 -15yrs		Above15yrs	
		Count	**Row N %**	**Count**	**Row N %**	**Count**	**Row N %**	**Count**	**Row N %**
Leukocytes (WBC/µL)	**Neg**	96	16.7%	88	15.3%	58	10.1%	332	57.8%
	±	0	0.0%	0	0.0%	1	20.0%	4	80.0%
	+	4	9.1%	5	11.4%	2	4.5%	33	75.0%
	++	0	0.0%	2	15.4%	0	0.0%	11	84.6%
	+++	0	0.0%	1	14.3%	1	14.3%	5	71.4%
Nitrite	**Neg**	99	15.4%	99	15.4%	65	10.1%	380	59.1%
	Pos	2	16.7%	1	8.3%	1	8.3%	8	66.7%

University of Ghana http://ugspace.ug.edu.gh

Urobilinogen (mg/dl; µmol/L)	**Neg**	99	15.8%	95	15.2%	63	10.1%	368	58.9%
	1 [17]	2	8.7%	3	13.0%	4	17.4%	14	60.9%
	2 [35]	0	0.0%	0	0.0%	1	8.3%	11	91.7%
	4 [70]	0	0.0%	2	100.0%	0	0.0%	0	0.0%
	8 [140]	0	0.0%	0	0.0%	0	0.0%	1	100.0%
	12 [200]	0	0.0%	0	0.0%	0	0.0%	0	0.0%
	0.2 [3.5]	0	0.0%	0	0.0%	0	0.0%	0	0.0%
Protein (mg/dl; (g/L)	**Neg**	83	17.6%	78	16.5%	49	10.4%	262	55.5%
	± [30; (0.15)]	1	7.1%	5	35.7%	4	28.6%	4	28.6%
	+ [30; (0.3)]	9	9.5%	12	12.6%	10	10.5%	64	67.4%
	2+ [100; (1.0)]	0	0.0%	0	0.0%	0	0.0%	12	100.0%
	3+ [300; (3.0)]	0	0.0%	0	0.0%	0	0.0%	2	100.0%
	4+ [2000; (20)]	0	0.0%	0	0.0%	0	0.0%	1	100.0%
pH	**Mean**	5.7		5.8		5.8		6.0	
BLOOD (RBC/µL)	**Neg**	99	16.8%	98	16.6%	63	10.7%	331	56.0%
	±	0	0.0%	0	0.0%	1	50.0%	1	50.0%
	+	2	8.3%	2	8.3%	1	4.2%	19	79.2%
	++	0	0.0%	0	0.0%	1	7.1%	13	92.9%
	+++	0	0.0%	0	0.0%	1	5.0%	19	95.0%
	5 - 10(1)	0	0.0%	0	0.0%	0	0.0%	6	100.0%
	5 - 10	0	0.0%	0	0.0%	1	16.7%	5	83.3%
Specific Gravity (SG)	**None**	3	75.0%	0	0.0%	0	0.0%	1	25.0%
	1.000	31	13.4%	28	12.1%	18	7.8%	155	66.8%
	1.005	6	18.8%	5	15.6%	3	9.4%	18	56.2%
	1.010	17	20.0%	15	17.6%	10	11.8%	43	50.6%
	1.015	7	9.5%	15	20.3%	11	14.9%	41	55.4%
	1.020	20	20.4%	13	13.3%	13	13.3%	52	53.1%
	1.025	5	6.8%	12	16.2%	8	10.8%	49	66.2%
	1.030	12	19.0%	12	19.0%	4	6.3%	35	55.6%
	Neg	77	12.9%	85	14.3%	61	10.2%	373	62.6%

University of Ghana http://ugspace.ug.edu.gh

Ketones (mg/dl; mmol/L)	**± [5 (0.5)]**	5	25.0%	4	20.0%	3	15.0%	8	40.0%
	15 (1.5)	7	36.8%	3	15.8%	1	5.3%	8	42.1%
	40 (4.0)	3	60.0%	1	20.0%	1	20.0%	0	0.0%
	80 (8.0)	4	28.6%	6	42.9%	2	14.3%	2	14.3%
	160 (16)	4	66.7%	1	16.7%	0	0.0%	1	16.7%
Bilirubin (mg/dl; mmol/L)	**Neg**	99	15.1%	100	15.3%	67	10.2%	389	59.4%
	+ [1(17)]	2	33.3%	0	0.0%	1	16.7%	3	50.0%
	++ [2(35)]	0	0.0%	0	0.0%	0	0.0%	1	100.0%
	+++ [4(70)]	0	0.0%	0	0.0%	0	0.0%	0	0.0%
Glucose (mg/dl; mmol/L)	**Neg**	98	14.9%	100	15.2%	68	10.4%	390	59.5%
	100(5)	2	50.0%	0	0.0%	0	0.0%	2	50.0%
	250(15)	1	100.0%	0	0.0%	0	0.0%	0	0.0%
	500(30)	0	0.0%	0	0.0%	0	0.0%	0	0.0%
	1000(60)	0	0.0%	0	0.0%	0	0.0%	2	100.0%
	≥ 2000(110)	0	0.0%	0	0.0%	0	0.0%	0	0.0%
Ascorbic Acid (mg/dl; mmol/L)	**Neg**	48	19.3%	35	14.1%	22	8.8%	144	57.8%
	+ [10(5.6)]	4	10.5%	9	23.7%	5	13.2%	20	52.6%
	++ [20(1.14)]	0	0.0%	2	28.6%	0	0.0%	5	71.4%
	+++ [40(2.28)]	0	0.0%	0	0.0%	0	0.0%	0	0.0%

University of Ghana http://ugspace.ug.edu.gh

Patient Urine Test Analysis Test against Microscopy Test and ICT Strip

Parameter	Multistix 10 SG Reagent Strip	Microscopy					ICT Strip	
		Positivity Grade (Level of Parasitaemia)						
		No MPS	1+ [40 - 4999]	2+ [5000 - 49999]	3+ [50000 - 99999]	4+ [>100000]	Negative	Positive
Leukocytes (WBC/µL)	Neg	52.4%	26.0%	12.1%	4.4%	5.1%	52.4%	47.6%
	±	55.6%	22.2%	11.1%	11.1%	0.0%	55.6%	44.4%
	+	68.8%	25.0%	4.7%	1.6%	0.0%	70.3%	29.7%
	++	76.5%	23.5%	0.0%	0.0%	0.0%	76.5%	23.5%
	+++	53.8%	46.2%	0.0%	0.0%	0.0%	46.2%	53.8%
Nitrite	Neg	53.4%	25.7%	12.1%	4.2%	4.7%	53.6%	46.4%
	Pos	50.0%	33.3%	8.3%	0.0%	8.3%	45.8%	54.2%
Urobilinogen (mg/dl; µmol/L)	Neg	56.0%	25.1%	10.7%	3.8%	4.5%	54.9%	45.1%
	1 [17]	38.3%	31.7%	21.7%	3.3%	5.0%	41.7%	58.3%
	2 [35]	27.9%	34.9%	16.3%	11.6%	9.3%	51.2%	48.8%
	4 [70]	18.2%	27.3%	36.4%	9.1%	9.1%	27.3%	72.7%
	8 [140]	14.3%	28.6%	42.9%	0.0%	14.3%	14.3%	85.7%
	12 [200]	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	0.2 [3.5]	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Protein (mg/dl; (g/L)	Neg	60.4%	23.3%	9.9%	3.5%	2.9%	58.4%	41.6%
	± [30; (0.15)]	32.6%	48.8%	9.3%	2.3%	7.0%	23.3%	76.7%
	+ [30; (0.3)]	40.6%	26.1%	20.5%	5.6%	7.3%	41.0%	59.0%
	++ [100; (1.0)]	23.1%	26.9%	21.2%	13.5%	15.4%	23.1%	76.9%
	+++ [300; (3.0)]	16.7%	66.7%	0.0%	8.3%	8.3%	16.7%	83.3%
	++++ [2000; (20)]	33.3%	66.7%	0.0%	0.0%	0.0%	33.3%	66.7%

University of Ghana http://ugspace.ug.edu.gh

pH	Mean	5.9	5.8	5.7	5.5	5.6	5.9	5.7
BLOOD (RBC/µL)	Neg	53.0%	26.2%	12.0%	4.1%	4.7%	52.4%	47.6%
	±	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%	0.0%
	+	52.2%	23.9%	13.0%	0.0%	10.9%	63.0%	37.0%
	++	48.3%	31.0%	17.2%	3.4%	0.0%	58.6%	41.4%
	+++	62.5%	18.8%	6.2%	6.2%	6.2%	62.5%	37.5%
	5 - 10(1)	75.0%	12.5%	0.0%	12.5%	0.0%	75.0%	25.0%
	5 - 10	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%	0.0%
Specific Gravity (SG)	None	50.0%	50.0%	0.0%	0.0%	0.0%	50.0%	50.0%
	1.000	54.3%	26.5%	12.2%	2.1%	4.9%	67.9%	32.1%
	1.005	61.5%	30.8%	3.8%	3.8%	0.0%	53.8%	46.2%
	1.010	58.2%	23.3%	11.0%	3.4%	4.1%	47.9%	52.1%
	1.015	50.7%	32.2%	8.9%	4.8%	3.4%	42.5%	57.5%
	1.020	47.3%	21.7%	17.4%	7.2%	6.3%	40.6%	59.4%
	1.025	51.4%	24.3%	12.5%	6.9%	4.9%	46.5%	53.5%
	1.030	60.6%	23.1%	9.6%	1.9%	4.8%	54.8%	45.2%
Ketones (mg/dl; mmol/L)	Neg	56.0%	25.7%	11.0%	3.7%	3.7%	55.6%	44.4%
	± [5 (0.5)]	37.0%	33.3%	16.7%	7.4%	5.6%	44.4%	55.6%
	15 (1.5)	44.2%	23.3%	16.3%	2.3%	14.0%	46.5%	53.5%
	40 (4.0)	45.5%	0.0%	0.0%	27.3%	27.3%	45.5%	54.5%
	80 (8.0)	32.6%	23.3%	27.9%	7.0%	9.3%	32.6%	67.4%
	160 (16)	37.5%	37.5%	12.5%	0.0%	12.5%	31.2%	68.8%

Bilirubin (mg/dl; mmol/L)	Neg	54.3%	25.4%	11.8%	4.0%	4.5%	53.8%	46.2%
	+ [1(17)]	30.0%	30.0%	20.0%	5.0%	15.0%	50.0%	50.0%
	++ [2(35)]	20.0%	60.0%	0.0%	20.0%	0.0%	20.0%	80.0%
	+++ [4(70)]	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Glucose	Neg	53.9%	25.7%	11.9%	3.9%	4.5%	53.8%	46.2%

University of Ghana http://ugspace.ug.edu.gh

(mg/dl; mmol/L)	100(5)	40.0%	30.0%	10.0%	0.0%	20.0%	60.0%	40.0%
	250(15)	20.0%	40.0%	20.0%	20.0%	0.0%	40.0%	60.0%
	500(30)	0.0%	50.0%	0.0%	50.0%	0.0%	0.0%	100.0%
	1000(60)	100.0%	0.0%	0.0%	0.0%	0.0%	50.0%	50.0%
	≥2000(110)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascorbic Acid (mg/dl; mmol/L)	Neg	59.9%	22.4%	7.9%	5.8%	4.1%	50.7%	49.3%
	+ [10(5.6)]	58.5%	15.4%	10.8%	6.2%	9.2%	43.1%	56.9%
	++ [20(1.14)]	58.3%	8.3%	16.7%	0.0%	16.7%	58.3%	41.7%
	+++ [40(2.28)]	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

University of Ghana http://ugspace.ug.edu.gh

Urine Test Analysis Test against Microscopy Test and ICT Strip Malaria POSITIVE Cases Only

Parameter	Multistix SG Reagent Strip	Microscopy				ICT Strip	
		Positivity Grade (Level of Parasitaemia)					
		1+ [40 - 4999]	2+ [5000 - 49999]	3+ [50000 - 99999]	4+ [>100000]	Negative	Positive
Leukocytes (WBC/µL)	Neg	54.7%	25.3%	9.2%	10.7%	13.4%	86.6%
	±	50.0%	25.0%	25.0%	0.0%	0.0%	100.0%
	+	80.0%	15.0%	5.0%	0.0%	10.0%	90.0%
	++	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	+++	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%
Nitrite	Neg	55.2%	25.9%	8.9%	10.0%	12.9%	87.1%
	Pos	66.7%	16.7%	0.0%	16.7%	0.0%	100.0%
Urobilinogen (mg/dl; µmol/L)	Neg	56.9%	24.4%	8.5%	10.2%	10.8%	89.2%
	1 [17]	51.4%	35.1%	5.4%	8.1%	21.6%	78.4%
	2 [35]	48.4%	22.6%	16.1%	12.9%	35.5%	64.5%
	4 [70]	33.3%	44.4%	11.1%	11.1%	11.1%	88.9%
	8 [140]	33.3%	50.0%	0.0%	16.7%	0.0%	100.0%
	12 [200]	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	0.2 [3.5]	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Protein (mg/dl; (g/L)	Neg	58.9%	24.9%	8.7%	7.4%	11.3%	88.7%
	± [30; (0.15)]	72.4%	13.8%	3.4%	10.3%	0.0%	100.0%
	+ [30; (0.3)]	43.9%	34.5%	9.4%	12.2%	11.5%	88.5%
	++ [100; (1.0)]	35.0%	27.5%	17.5%	20.0%	5.0%	95.0%
	+++ [300; (3.0)]	80.0%	0.0%	10.0%	10.0%	0.0%	100.0%
	++++ [2000; (20)]	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%

pH	Mean	5.8	5.7	5.5	5.6	6.2	5.7
BLOOD (RBC/µL)	Neg	55.7%	25.6%	8.8%	9.9%	11.1%	88.9%
	±	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

University of Ghana http://ugspace.ug.edu.gh

	+	50.0%	27.3%	0.0%	22.7%	40.9%	59.1%
	++	60.0%	33.3%	6.7%	0.0%	26.7%	73.3%
	+++	50.0%	16.7%	16.7%	16.7%	16.7%	83.3%
	5 - 10(1)	50.0%	0.0%	50.0%	0.0%	0.0%	100.0%
	5 - 10	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Specific Gravity (SG)	None	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	1.000	57.9%	26.7%	4.6%	10.8%	32.8%	67.2%
	1.005	80.0%	10.0%	10.0%	0.0%	0.0%	100.0%
	1.010	55.7%	26.2%	8.2%	9.8%	1.6%	98.4%
	1.015	65.3%	18.1%	9.7%	6.9%	1.4%	98.6%
	1.020	41.3%	33.0%	13.8%	11.9%	0.9%	99.1%
	1.025	50.0%	25.7%	14.3%	10.0%	5.7%	94.3%
	1.030	58.5%	24.4%	4.9%	12.2%	2.4%	97.6%
Ketones (mg/dl; mmol/L)	Neg	58.4%	24.9%	8.3%	8.3%	13.4%	86.6%
	± [5 (0.5)]	52.9%	26.5%	11.8%	8.8%	17.6%	82.4%
	15 (1.5)	41.7%	29.2%	4.2%	25.0%	8.3%	91.7%
	40 (4.0)	0.0%	0.0%	50.0%	50.0%	16.7%	83.3%
	80 (8.0)	34.5%	41.4%	10.3%	13.8%	0.0%	100.0%
	160 (16)	60.0%	20.0%	0.0%	20.0%	0.0%	100.0%

Bilirubin (mg/dl; mmol/L)	Neg	55.6%	25.9%	8.7%	9.8%	12.0%	88.0%
	+ [1(17)]	42.9%	28.6%	7.1%	21.4%	28.6%	71.4%
	++ [2(35)]	75.0%	0.0%	25.0%	0.0%	0.0%	100.0%
	+++ [4(70)]	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Glucose (mg/dl; mmol/L)	Neg	55.7%	25.9%	8.6%	9.8%	12.3%	87.7%
	100(5)	50.0%	16.7%	0.0%	33.3%	33.3%	66.7%
	250(15)	50.0%	25.0%	25.0%	0.0%	25.0%	75.0%
	500(30)	50.0%	0.0%	50.0%	0.0%	0.0%	100.0%
	1000(60)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

University of Ghana http://ugspace.ug.edu.gh

	≥2000(110)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascorbic Acid (mg/dl; mmol/L)	Neg	55.7%	19.8%	14.4%	10.2%	0.0%	100.0%
	+ [10(5.6)]	37.0%	25.9%	14.8%	22.2%	0.0%	100.0%
	++ [20(1.14)]	20.0%	40.0%	0.0%	40.0%	0.0%	100.0%
	+++ [40(2.28)]	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

University of Ghana http://ugspace.ug.edu.gh

Urine Test Analysis Test against Microscopy Test and ICT Strip Malaria Negative Cases Only

		MPS Grade	ICT Strip	
Parameter	**Test Results**	**No MPS**	**Negative**	**Positive**
Leukocytes (WBC/µL)	Neg	100.0%	88.0%	12.0%
	±	100.0%	100.0%	0.0%
	+	100.0%	97.7%	2.3%
	++	100.0%	100.0%	0.0%
	+++	100.0%	85.7%	14.3%
Nitrite	Neg	100.0%	89.1%	10.9%
	Pos	100.0%	91.7%	8.3%
Urobilinogen (mg/dl; µmol/L)	Neg	100.0%	89.6%	10.4%
	1 [17]	100.0%	73.9%	26.1%
	2 [35]	100.0%	91.7%	8.3%
	4 [70]	100.0%	100.0%	0.0%
	8 [140]	100.0%	100.0%	0.0%
	12 [200]	100.0%	0.0%	0.0%
	0.2 [3.5]	100.0%	0.0%	0.0%
Protein (mg/dl; (g/L)	Neg	100.0%	89.2%	10.8%
	± [30; (0.15)]	100.0%	71.4%	28.6%
	+ [30; (0.3)]	100.0%	84.2%	15.8%
	++ [100; (1.0)]	100.0%	83.3%	16.7%
	+++ [300; (3.0)]	100.0%	100.0%	0.0%
	++++ [2000; (20)]	100.0%	100.0%	0.0%

University of Ghana http://ugspace.ug.edu.gh

pH	5.9			
BLOOD (RBC/µL)	Neg	100.0%	89.0%	11.0%
	±	100.0%	100.0%	0.0%
	+	100.0%	83.3%	16.7%
	++	100.0%	92.9%	7.1%
	+++	100.0%	90.0%	10.0%
	5 - 10(1)	100.0%	100.0%	0.0%
	5 - 10	100.0%	100.0%	0.0%
Specific Gravity (SG)	None	100.0%	100.0%	0.0%
	1.000	100.0%	97.4%	2.6%
	1.005	100.0%	87.5%	12.5%
	1.010	100.0%	81.2%	18.8%
	1.015	100.0%	82.4%	17.6%
	1.020	100.0%	84.7%	15.3%
	1.025	100.0%	85.1%	14.9%
	1.030	100.0%	88.9%	11.1%
Ketones (mg/dl; mmol/L)	Neg	100.0%	88.8%	11.2%
	± [5 (0.5)]	100.0%	90.0%	10.0%
	15 (1.5)	100.0%	94.7%	5.3%
	40 (4.0)	100.0%	80.0%	20.0%
	80 (8.0)	100.0%	100.0%	0.0%
	160 (16)	100.0%	83.3%	16.7%
Bilirubin (mg/dl; mmol/L)	Neg	100.0%	89.0%	11.0%
	+ [1(17)]	100.0%	100.0%	0.0%
	++ [2(35)]	100.0%	100.0%	0.0%

University of Ghana http://ugspace.ug.edu.gh

	+++ [4(70)]	100.0%		
Glucose (mg/dl; mmol/L)	Neg	100.0%	89.2%	10.8%
	100(5)	100.0%	100.0%	0.0%
	250(15)	100.0%	100.0%	0.0%
	500(30)	0.0%	0.0%	0.0%
	1000(60)	100.0%	50.0%	50.0%
	≥2000(110)	0.0%	0.0%	0.0%
Ascorbic Acid (mg/dl; mmol/L)	Neg	100.0%	84.7%	15.3%
	+ [10(5.6)]	100.0%	73.7%	26.3%
	++ [20(1.14)]	100.0%	100.0%	0.0%
	+++ [40(2.28)]	0.0%	0.0%	0.0%

University of Ghana http://ugspace.ug.edu.gh

APPENDIX 5: PUBLICATIONS

Appendix 5a A poster presented at the First Annual College of Health Sciences Conference, held on 26th to 28th September, 2007

Studies Towards the Development of an Accurate Urine-Based Rapid Diagnostic Test (UBRDT) for Malaria

McKakpo U.S., K.M. Bosompem, E.A.K. Addison, D. Sullivan Jnr., P. Amankwah, I. Ankrah, P. Madjitey, F. Lomotey, N. Amoah, A.N. Noye, M. Mensah, W.A. Lomotey and I.A. Quakyi

Introduction, Background and Rationale

Rapid, safe, simple and accurate diagnosis is required for effective management and control of malaria (**2**), however, the major current diagnostic tools (clinical, blood smear microscopy and the immuno-chromatographic tests) lack these qualities. **T**hese limitations that beset diagnosis of malaria make management and control of the disease very difficult.

This study is therefore being carried out to develop a simple, accurate, non-invasive and rapid urine-based dipstick assay (UBRDT) for alternative diagnosis of malaria.

Patients, Materials and Methods

Study Site: **T**he study field site is Kpone On Sea, a coastal fishing village in south eastern Ghana.

Study Subjects: **P**atients visiting Kpone health centre and pregnant women on antenatal care.

Methodology: ***P****lasmodium* parasite infectivity and anaemia were determined by Blood-Smear Microscopy and the Quantitative Buffy Coat (QBC) test. Urinary malaria proteins for UBRDT development were identified and analyzed by one-dimensional SDS-PAGE, Western Blot Analysis, Mass Spectrometry Sequencing (MSS) and a urine-based malaria antigen-detection ELISA (UBMADE) being evaluated.

P*lasmodium* antigens extracted from urine samples were used to immunize Balb/C mice towards monoclonal antibody (Moab) production and UBRDT development.

Results

Microscopy of blood samples from 290 recruited patients showed that 151 (52.1%) were malaria positive (Table 1). This included 24/58 (41.4%) of pregnant women (Table 3). In all 23/290 (7.9%) had severe malaria with parasitaemia >100,000 parasites/µl blood and 82 (28.3%) had mild malaria with <5,000 parasites/µl blood (Table 2). Generally, the parasitaemia ranged from 40 - 394,480 (Median = 3,520 parasites/ µl blood).

The UBMADE and Western blot analysis detected malaria urinary antigens in 53 (35.1%) of the 151 malaria positive patients. Of the 151 malaria positive individuals, 43 (28.5%) were febrile and 108 afebrile. Also, 26/43 (60.5%) of the febrile individuals were antigen positive, whilst only (25.0%) of the 108 afebrile cases had the malaria urinary antigen. The geometric mean parasitaemia in febrile versus afebrile groups was 8,109:1320.

In the SDS-PAGE/Western Blot analyses, a *P. chabaudi* infected mouse serum detected a 30-35kDa band that was present in both purified *P. falciparum* antigen and malaria positive urine. This protein is undergoing MSS for identification. Production of anti-urinary-*Plasmodium* Moabs for development of the UBRDT is also underway.

Discussions and Comments

The observation that a high proportion (41.4%) of the asymptomatic pregnant women (Table 3) attending antenatal clinic were infected with *Plasmodium* parasites may raise questions about the effectiveness of intermittent preventive treatment (IPT), which could be influenced by compliance, and drug failure even though this is less likely. This observation therefore calls for further research to determine the reasons for high malaria prevalence among pregnant women; and reaffirms the importance of preventive treatment in pregnancy.

Generally, children aged 5 years and below who are reported to be more susceptible (**3**) to the disease had the highest proportion 40/57 (70.2%) of malaria cases (Table 3), of which 4/40 (10%) arrived at the health centre either comatose or convulsive, indicating the need for early diagnosis and treatment.

Even though it has been reported that malaria causes elevation in the amounts of proteins, bilirubin and blood excreted in urine (**1**), it is difficult to infer from this study (Table 1). Additional data are therefore required to make any conclusive deduction.

Findings from this study show that a majority of the individuals from the study community are anaemic (Table 2). However, there is no statistical difference between the *Plasmodium*-infected anaemic and the uninfected anaemic groups (p>0.05).

Conclusion

In view of the observed prevalence of *Plasmodium* infections in pregnant women and children <5 years who were anaemic, the development of a rapid and accurate diagnostic tool for malaria would promote timely detection of infections in vulnerable risk groups.

Table 1. Relative sensitivity and specificity of various tests in detecting *Plasmodium* parasite infection

Test	Number Tested	Number Positive	Number Negative	Relative Sensitivity (%)	Relative Specificity (%)
Combined Microscopy (Gold Standard) [1]	290	169	146	100	100
Blood Film Microscopy (BF)	290	151 (14)[a]	139 (14)[b]	81.1	85.6
QBC[2]	290	178 (40)[a]	112 (18)[b]	81.7	64.4
Antigen Detection ELISA	151	53	98	35.1	NA
Western Blot Analysis	151	53	98	35.1	NA
Micro-haematuria	290	47 (18)[a]	243 (107)[b]	17.2	93.2
Proteinuria	290	164 (62)[a]	126 (65)[b]	60.4	41.8
Bilirubinuria	290	17 (3)[a]	273 (140)[b]	8.3	91.1

1 Combined Test Results of Blood Film Microscopy and QBC.
2 Quantitative Buffy Coat Test.
A Number of samples tested negative by Combined Microscopy.
b Number of samples tested positive by Combined Microscopy.
NA Not applicable because only microscopy positive samples were tested for antigens.

Table 2. Relationship between clinical outcomes of malaria, parasitaemia and anaemia

Clinical Outcome	Number of Subjects	Parasites per microlitre of Blood	Number Anaemic
Severe	23	>100,000	23
Moderate	46	99,999-5000	45
Asymptomatic	82	4,999-40	68
Uninfected	139	0	117
Total	**151**	-	**253**

Table 3. *Plasmodium* infection rates in different risk groups among study subjects

Subjects	Number Tested	Malaria Positive No. (%)
Pregnant Women	58	24 (41.4)
5 years and below	57	40 (70.2)
> 5 years	175	87 (49.7)
Total	**290**	**151 (52.1)**

Acknowledgements

School of Public Health, College of Health Sciences, University of Ghana, Legon.
The Department of Population, Family and Reproductive Health, School of Public Health, University of Ghana, Legon.
Gates Institute for Population and Reproductive Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA.
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA.
Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, Legon.
MIM/TDR/WHO.
The Kpone Health Centre.
The staff of the above named institutions.
The Kpone study community

References

1. **Rodriguez-del Valle, M**. *et al.* (1991. *Journal of Clinical Microbiology*, **29** (6): 1236-1242.
2. **WHO** (1993). A global strategy for malaria control, Geneva.
3. **WHO/OMS** (2002). Malaria. *WHO Fact Sheet No 94*.

University of Ghana http://ugspace.ug.edu.gh

Appendix 5b A publication on the relationship of parasitemia and anemia among patients with Plasmodium falciparum malaria in Ghana

Am. J. Trop. Med. Hyg., 77(4), 2007, pp. 623–626
Copyright © 2007 by The American Society of Tropical Medicine and Hygiene

Relationship of Hepcidin with Parasitemia and Anemia among Patients with Uncomplicated *Plasmodium falciparum* Malaria in Ghana

Caitlin T. Howard, Uri S. McKakpo, Isabella A. Quakyi, Kwabena M. Bosompem, Ebenezer A. Addison, Kai Sun, David Sullivan, and Richard D. Semba*

Johns Hopkins Medical Institutions, Baltimore, Maryland; School of Public Health and Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra, Ghana

Abstract. The pathogenesis of malarial anemia is incompletely understood. Hepcidin, a recently discovered peptide hormone, is a major regulator of iron metabolism and is thought to play a central role in the anemia of chronic inflammation. The specific aim of the study was to characterize the association between urinary hepcidin, hemoglobin, and parasitemia in 199 patients presenting for evaluation of *Plasmodium falciparum* malaria in Ghana. Urinary hepcidin was semi-quantitatively assessed using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. Urinary hepcidin (intensity/mmol creatinine) was associated with log parasitemia in 86 children (beta = 0.086, standard error [SE] = 0.035, $P < 0.017$), 31 pregnant women (beta = 0.218, SE = 0.085, $P < 0.016$), and 82 adults (beta = 0.184, SE = 0.043, $P < 0.0001$). Urinary hepcidin was not significantly associated with hemoglobin or anemia. Urinary hepcidin is more strongly associated with parasitemia than hemoglobin or anemia among patients with acute *P. falciparum* malaria in Ghana.

INTRODUCTION

Malaria is a leading cause of morbidity and mortality and accounts for an estimated 500 million cases and 1 to 3 million deaths annually in developing countries.[1] Anemia is one of the most common and severe outcomes of *Plasmodium falciparum* malaria, and its pathogenesis is incompletely understood. Hepcidin, a recently discovered peptide hormone synthesized primarily by hepatocytes, is considered a major regulator of iron homeostasis.[2] Hepcidin regulates iron metabolism by inhibiting duodenal iron absorption at the intestinal epithelium and by reducing mobilization of iron from the liver and spleen.[2] The synthesis of hepcidin is modulated by iron status, hypoxia, and inflammation.[3–5] In the anemia of chronic inflammation, hepcidin is thought to block the release of iron from enterocytes, hepatocytes, and macrophages, leading to hypoferremia and limited iron availability for erythropoiesis.[2] Although studies suggest that hepcidin is a key regulator in the anemia of chronic inflammation, the role of hepcidin in the pathogenesis of malarial anemia has not been characterized. We hypothesized that urinary hepcidin levels would be associated with high parasitemia among patients with uncomplicated *P. falciparum* malaria. To address this hypothesis, we measured urinary hepcidin among patients being evaluated for malaria in Ghana.

MATERIALS AND METHODS

Study subjects. The study subjects consisted of a consecutive sample of 290 symptomatic individuals (187 adults, 58 pregnant women, 103 children) seen at Kpone Health Center in Kpone On Sea, Ghana, from July to August 2005 for evaluation of malaria and malarial antigens in urine. The Kpone Health Center serves Kpone On Sea, a village in eastern coastal Ghana, an area that is endemic for *Plasmodium falciparum* malaria. Temperature was measured using a digital axillary thermometer. A fingerprick blood sample was taken to prepare thick and thin blood films to determine the presence or absence of malaria parasites, level of parasitemia, and hemoglobin concentrations using a HemoCue machine (HemoCue Inc., Mission Viejo, CA). Subjects were eligible for the study if they were diagnosed with *P. falciparum* malaria, were not admitted for transfusion, had a hemoglobin level > 50 g/L, and had no evidence of cerebral malaria. Only one subject had received anti-malarial medication. Subjects were enrolled in the study after written informed consent was obtained, and for children, after written informed consent was obtained from the parent or guardian. Spot urine samples (5 mL) were collected, and urine samples were immediately aliquoted and frozen in cryotubes at −70°C. The study protocol was approved by the National Institutes of Health and the Noguchi Memorial Institute for Medical Research IRB CPN 025/-2-03. Analyses of urine were performed under approval by the Committee for Human Research of the Johns Hopkins Bloomberg School of Public Health.

Thick and thin Giemsa-stained blood films were analyzed for the number of parasites per 200 white blood cells. Slides were considered negative if no parasites were seen in 100 fields on the thick film. Urinary hepcidin was measured using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS).[6] Urine samples were thawed, vortexed, and centrifuged at 12,000 rpm for 25 minutes. The supernatant (7 uL) of each sample was applied in triplicate (three spots) on the NP20 Ciphergen ProteinChip® and incubated in a moist chamber for 30 min. Unbound proteins are removed by washing three times with HPLC grade water for 10 min. Chip arrays are air-dried, and 1.0 μL of alpha-cyano-4-hydroxy-cinnamic acid (CHCA, Ciphergen) in 50% acetonitrile and 0.25% trifluoroacetic acid was added to the array surface and allowed to air dry. The arrays were analyzed on a Ciphergen ProteinChip® Reader (Model PBS II, Ciphergen). Data was collected using the following settings: two warming shots at laser intensity 175 (not collected); 10 shots at laser intensity 170 at every five positions between 20 and 80; high mass 4500 Da; mass deflector 100 Da, detector sensitivity 7; acquired mass range from a mass-over-charge (m/z) ratio of 500 to 4500. Peak annotation was conducted using Ciphergen ProteinChip Software (version 3.2.0), after baseline subtraction and adjustment. A peak is recorded for

* Address correspondence to Richard Semba, 550 N. Broadway, Suite 700, Baltimore, MD 21205. E-mail: rdsemba@jhmi.edu

University of Ghana http://ugspace.ug.edu.gh

hepcidin at the characteristic m/v of 2790 when the signal-to-noise ratio is > 3:1. Although the molecular weight of hepcidin is 2789, it is ionized as a charged (protonated) species on SELDI-TOF MS; thus, the average peak on low-resolution mass spectrometry is one unit higher than the average molecular weight because of the added proton. Urinary creatinine was measured using a commercial ELISA (Quidel Corporation, San Diego, CA) in Baltimore. Between run coefficients of variation (CV) for urinary creatinine were 11.9% and 5.9% for high and low controls, respectively. Urinary hepcidin concentrations were expressed as intensity per mmol/L creatinine.

Means and standard deviations were calculated for descriptive statistics. Anemia was defined as hemoglobin < 11 g/dL for children and pregnant women, < 12 g/dL for non-pregnant adult women, and < 13 g/dL for men.[7] Both malaria parasitemia and urinary hepcidin (mmol/L creatinine) were continuous variables that were highly skewed and were transformed by $\log_e$ to normalize the distribution. Linear regression models were used to examine the relationship between urinary hepcidin and other variables such as parasitemia and hemoglobin.

RESULTS

Of the 290 subjects in the study series, urine samples were available from 199 patients (86 children, 82 adults, and 31 pregnant women) who were evaluated for uncomplicated *Plasmodium falciparum* malaria. Three subjects were excluded from the analysis because of a lack of age data. Characteristics of the study groups are shown in Table 1. The prevalence of anemia among children, adults, and pregnant women was 79.1%, 82.9%, and 90.3%, respectively. Linear regression models were used to examine the relationship between urinary hepcidin and parasitemia, hemoglobin, and anemia in each of the three groups and in all three groups combined, as shown in Table 2. In univariable linear regression models, log parasitemia was positively associated with urinary hepcidin (intensity/mmol creatinine) in 86 children (beta or regression coefficient = 0.086, standard error [SE] = 0.035, $P < 0.017$), 31 pregnant women (beta or regression coefficient = 0.218, SE = 0.085, $P < 0.016$), and 82 adults (beta or regression coefficient = 0.184, SE = 0.043, $P < 0.0001$). Hepcidin was not significantly associated with hemoglobin or anemia in any of the groups. In a multi-variable linear regression model, $\log_e$ urinary hepcidin was associated with $\log_e$ parasitemia (beta or regression coefficient = 0.174, SE = 0.026, $P < 0.0001$) but not hemoglobin (beta or regression coefficient = 0.022, SE = 0.058, $P = 0.71$). Anemia was not included in the same multi-variable model because the two variables are highly dependent. There were no significant interactions between $\log_e$ parasitemia and hemoglobin. We also ran an alternative model that included only subjects who had more severe anemia (hemoglobin < 9 g/dL). In a multivariable model, $\log_e$ urinary hepcidin was associated with $\log_e$ parasitemia (beta or regression coefficient = 0.248, SE = 0.056, $P < 0.0001$) but not hemoglobin (beta or regression coefficient = 0.211, SE = 0.192, $P = 0.28$).

DISCUSSION

This study shows that urinary hepcidin concentrations are more strongly associated with malaria parasitemia than hemoglobin levels or anemia among patients with acute, uncomplicated malaria in Ghana. To our knowledge, this is the first study to characterize urinary hepcidin levels in patients with malaria. Hepcidin is thought to play a central role in the anemia of chronic inflammation,[8] and factors that have been

TABLE 1
Characteristics of subjects evaluated for malaria in Ghana

Characteristic	Mean (SD) or %, unless noted
Children (n = 86)	
Age (years)	7.9 (5.1)
Female (%)	44.2
Hemoglobin (g/dL)*	9.5 (1.8)
Anemic (%)†	79.1
Log parasitemia	6.84 (4.36)
Geometric mean parasitemia (parasites/200 white blood cells)	934
Hepcidin (intensity/mmol creatinine)‡	4.2 (1.4, 9.5)
Adults (n = 82)	
Age (years)	31.3 (13.0)
Female (%)	56.1
Hemoglobin (g/dL)*	11.3 (1.6)
Anemic (%)†	82.9
Log parasitemia	3.45 (3.98)
Geometric mean parasitemia (parasites/200 white blood cells)	31.5
Hepcidin (intensity/mmol creatinine)‡	0.7 (0.3, 3.6)
Pregnant women (n = 31)	
Age (years)	24.4 (5.1)
Hemoglobin (g/dL)*	9.0 (1.7)
Anemic (%)†	90.3
Log parasitemia	3.95 (3.56)
Geometric mean parasitemia (parasites/200 white blood cells)	51.9
Hepcidin (intensity/mmol creatinine)‡	0.7 (0.2, 5.9)

* Minimum and maximum hemoglobin levels for children, adults, and pregnant women were 3.8 and 12.6, 7.9 and 17.2, and 6.2 and 12.6 g/dL, respectively.
† Anemia defined as hemoglobin < 11 g/dL for children, < 13 g/dL for adult men and < 12 g/dL for non-pregnant women, and < 11 g/dL for pregnant women.
‡ Median (25th and 75th percentiles).

TABLE 2
Results of univariable linear regression models of factors associated with levels of hepcidin for each patient group and all groups combined

Characteristic	Beta	95% CI*	SE*	P*
Children (n = 86)				
Log parasitemia	0.086	0.016–0.156	0.035	0.017
Hemoglobin	0.048	−0.123–0.221	0.086	0.58
Anemia	0.345	−0.426–1.112	0.388	0.38
Adults (n = 82)				
Log parasitemia	0.184	0.099–0.268	0.043	< 0.0001
Hemoglobin	−0.030	−0.262–0.201	0.116	0.79
Anemia	0.900	−0.068–1.869	0.487	0.07
Pregnant women (n = 31)				
Log parasitemia	0.218	0.043–0.392	0.085	0.016
Hemoglobin	−0.110	−0.516–0.276	0.193	0.54
Anemia	0.677	−1.541–2.986	1.083	0.54
All groups combined (n = 196)				
Log parasitemia	0.171	0.122–0.221	0.025	< 0.0001
Hemoglobin	−0.101	−0.222–0.020	0.061	0.10
Anemia	0.481	−0.143–1.106	0.317	0.13

* CI = confidence interval, SE = standard error.

University of Ghana http://ugspace.ug.edu.gh

implicated in the modulation of hepcidin expression include hypoxia[4] and inflammation.[9] In malaria, the destruction of parasitized and normal erythrocytes, as well as ineffective erythropoiesis contribute almost equally to anemia, but recent data suggest that these mechanisms alone or in combination do not adequately explain the anemia of malaria infection.[10]

The present study provides some initial insight into the pathogenesis of anemia during malaria. High levels of parasitemia were associated with high levels of hepcidin. Hepcidin inhibits duodenal iron absorption at the intestinal epithelium[11] and inhibits mobilization of iron from liver and spleen.[2] Hepcidin binds to the iron exporter, ferroportin, inducing its internalization and degradation.[12] Ferroportin is the only mammalian iron exporter identified to date and is necessary for matemofetal iron transfer and iron efflux from duodenal enterocytes, macrophages, and hepatocytes.[13] The finding from the present study suggests that during acute, uncomplicated malaria, the availability of iron for erythropoiesis may be limited through the upregulation of hepcidin.

Both inflammation and infection can increase hepcidin expression, as shown in different animal models, including mice challenged with turpentine[4] and lipopolysaccharide,[3] rats given Freund's complete adjuvant[11] and fish challenged with *Streptococcus iniae* infection.[14] Hepcidin expression is strongly upregulated by interleukin (IL)-6.[12] The present study is limited in that serum IL-6 concentrations were not measured; however, elevated serum IL-6 levels are known to be associated with high *P. falciparum* malaria parasitemia.[15] An acute episode of malaria induces inflammation and elevates IL-6 levels. IL-6 has been implicated in the upregulation of hepcidin, which blocks the release of iron from enterocytes, hepatocytes, and macrophages, leading to hypoferremia and limited iron availability for erythropoesis.[2]

Hepcidin expression can also be upregulated by an iron-replete state.[12] A limitation of this study is that indicators of iron status were not measured in the present study, but it is probably unlikely that an iron-replete state accounted for the high hepcidin levels in this population that included pregnant women and children, two high risk groups for iron deficiency. Although the study does not include a group of subjects without malaria, mean urinary hepcidin levels as measured by SELDI-TOF MS were 0.52 intensity/mmol Cr in healthy control subjects and 0.1 intensity/mmol Cr in subjects with iron deficiency anemia.[16] The urinary hepcidin levels of 3–6 intensity/mmol Cr in the present study are comparable to mean levels of 6 intensity/mmol Cr among adults challenged with lipopolysaccharide in a study of anemia and experimental endotoxemia.[16]

Hepcidin expression is upregulated by hypoxia,[4] but in the present study, low hemoglobin levels or anemia was not associated with urinary hepcidin. In multi-variate analyses, parasitemia was the strongest factor associated with hepcidin, suggesting that parasitemia, and possibly inflammation associated with parasitemia, are stronger modulators of hepcidin expression than hypoxia.

Hepcidin is best measured in urine because the active 25 AA form of hepcidin is readily filtered by the glomerulus and passes into the urine. In the present study, urinary hepcidin was measured by SELDI-TOF MS, a method that is semi-quantitative but has been shown to correlate well with an immunologic method developed by Ganz and colleagues for use in their own laboratory.[6] A commercially available immunoassay is available that measures the 64 AA prohormone form of hepcidin, but the prohormone does not have biologic activity and correlates poorly with indicators of iron metabolism[17–19] and inflammation.[20] The present study relied upon the use of a single Hemocue measurement, a method that has been used around the world in developing countries and has been extensively validated for hemoglobin measurement.[21–23]

Future studies are needed to examine longitudinal changes in hepcidin, hemoglobin, iron status indicators, and proinflammatory cytokines such as IL-6 among patients with malaria. The present study provides some initial insights and represents a first step in the elucidation of the role of hepcidin in the anemia that occurs in *P. falciparum* malaria. Such investigations are important because anemia remains a common but not well understood complication of malaria.

Received February 23, 2007. Accepted for publication July 5, 2007.

Acknowledgments: We thank Avindra Nath for the use of a Ciphergen Protein Chip Reader and Amanda Ray and Margaret Dayhoff-Brannigan for technical assistance.

Financial support: Supported in part by NIH R01 AG029148, WHO/MIM/TDR Project No. A30033, Bill and Melinda Gates Institute for Population and Reproductive Health, Johns Hopkins University.

Authors' addresses: Caitlin Howard, Kai Sun, David Sullivan, and Richard Semba, Johns Hopkins Medical Institutions, 550 N. Broadway, Suite 700, Baltimore, MD 21205. Uri McKakpo, Isabella Quakyi, Kwabena Bosompem, and Ebenezer Addison, School of Public Health and Noguchi Memorial Institute for Medical Research, University of Ghana. Legon, Accra, Ghana.

Reprint requests: Richard Semba, 550 N. Broadway, Suite 700, Baltimore, MD 21205. E-mail: rdsemba@jhmi.edu.

REFERENCES

1. Guinovart C, Navia MM, Tanner M, Alonso PL, 2006. Malaria: burden of disease. *Curr Mol Med 6:* 137–140.
2. Ganz T, 2005. Hepcidin—a regulator of intestinal iron absorption and iron recycling by macrophages. *Best Pract Res Clin Haematol 18:* 171–182.
3. Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loréal O, 2001. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem 276:* 7811–7819.
4. Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S, 2002. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J Clin Invest 110:* 1037–1044.
5. Weinstein DA, Roy CN, Fleming MD, Loda MF, Wolfsdorf JI, Andrews NC, 2002. Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. *Blood 100:* 3776–3781.
6. Kemna E, Tjalsma H, Laarakkers C, Nemeth E, Willems H, Swinkels D, 2005. Novel urine hepcidin assay by mass spectrometry. *Blood 106:* 3268–3270.
7. World Health Organization, 1968. *Nutritional Anemia: Report of a WHO Scientific Group*. Geneva, Switzerland: World Health Organization.
8. Roy CN, Andrews NC, 2005. Anemia of inflammation: the hepcidin link. *Curr Opin Hematol 12:* 107–111.
9. Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T, 2004. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Investig 113:* 1271–1276.
10. McDevitt MA, Xie J, Gordeuk V, Bucala R, 2004. The anemia of malaria infection: role of inflammatory cytokines. *Curr Hematol Rep 3:* 97–106.
11. Anderson GJ, Frazer DM, Wilkins SJ, Becker EM, Millard KN,

University of Ghana http://ugspace.ug.edu.gh

Murphy TL, McKie AT, Vulpe CD, 2002. Relationship between intestinal iron-transporter expression, hepatic hepcidin levels and the control of iron absorption. *Biochem Soc Trans 30:* 724–726.

12. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J, 2004. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science 306:* 2090–2093.
13. Donovan A, Lima CA, Pinkus JL, Pinkus GS, Zon LI, Robine S, Andrews NC, 2005. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell Metab 1:* 191–200.
14. Shike H, Lauth X, Westerman ME, Ostland VE, Carlberg JM, van Olst JC, Shimizu C, Bulet P, Burns JC, 2002. Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. *Eur J Biochem 269:* 2232–2237.
15. Aubouy A, Deloron P, Migot-Nabias F, 2002. Plasma and in vitro levels of cytokines during and after a *Plasmodium falciparum* malaria attack. *Acta Trop 83:* 195–203.
16. Kemna EHJM, Tjalsma H, Podust VN, Swinkels DW, 2007. Mass-spectrometry-based hepcidin measurements in serum and urine: analytical aspects and clinical implications. *Clin Chem 54:* 4.
17. Kulaksiz H, Gehrke SG, Janetzko A, Rost D, Bruckner T, Kallinowski B, Strennel W, 2004. Pro-hepcidin: expression and cell specific localization in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. *Gut 53:* 735–743.
18. Brookes MJ, Sharma NK, Tselepsis C, Iqbal TH, 2005. Serum pro-hepcidin: measuring active hepcidin or a non-functional precursor? *Gut 54:* 169–170.
19. Luukonen S, Punnonen K, 2006. Serum pro-hepcidin concentrations and their responses to oral iron supplementation in healthy subjects manifest considerable inter-individual variation. *Clin Chem Lab Med 44:* 1361–1362.
20. Oguz A, Uzunlulu M, Hekim N, 2006. Hepcidin is not a marker of chronic inflammation in atherosclerosis. *Anadolu Kardiyol Derg 6:* 239–242.
21. Sari M, de Pee S, Martini E, Herman S, Sugiatmi, Bloem MW, Yip R, 2001. Estimating the prevalence of anaemia: a comparison of three methods. *Bull World Health Organ 79:* 506–511.
22. Prakash S, Kapil U, Singh G, Dwivedi SN, Tandon M, 1999. Utility of HemoCue in estimation of hemoglobin against standard blood cell counter method. *J Assoc Physicians India 47:* 995–997.
23. Bhaskaram P, Balakrishna N, Radhakrishna KV, Krishnaswamy K, 2003. Validation of hemoglobin estimation using Hemocue. *Indian J Pediatr 7:* 25–28.

University of Ghana http://ugspace.ug.edu.gh

APPENDIX 6: ETHICAL CONSENT FORMS

Appendix 6a

ADULT CONSENT FORM FOR THE STUDY ENTITLED: RAPID URINE-BASED DIPSTICK TEST FOR DIAGNOSIS OF MALARIA IN INFANTS, PLACENTA AND LOW-RISK INDIVIDUALS

FOR SUBJECTS AGED 16 YEARS AND OLI
UNIVERSITY OF GHANA
KPONE ON SEA/GHANA

Identifying Number _____

Date _____

_____

Information: *to be read and translated to adults in their own mother tongue if necessary*

Adult's Name: _____ **Age:** _____
Last name *First name* *Middle initial*

Dear Sir/Madam,
We invite you to take part in a research study sponsored by the School of Public Health, College of Health Sciences, University of Ghana and the Bill and Melinda Gates Institute of Population and Reproductive Health, Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA. It is important that you understand several general principles that apply to everyone who takes part in this study:

1. Participation in the study is entirely voluntary.
2. Personal benefit to you may not result from taking part in the study, but knowledge gained may benefit others.
3. You may withdraw from the study at any time.

Malaria is a sickness caused by very small disease causing organisms that can enter the body when mosquitoes bite you. They can cause fevers, headaches, body aches and weakness, and if not treated, can progress to severe illness, especially in children and pregnant women. When malaria is treated with proper medicines, it can be cured completely; but before a patient is given medicines to cure malaria a proper test has to be done to be sure that he/she has malaria. Presently, there are a number of methods that can be used to test for malaria but none of them uses urine even though it is easier to obtain and simpler to use. The main purpose of this study is to come out with a new method of detecting the presence of malaria using urine as a test material. The reason why we are inviting you to participate in this study is because we would like to learn more about the malaria disease that occurs in this community so that we can produce a new tool for detecting the disease early enough to cure it so as to prevent the disease from becoming severe and leading to death. The alternative to participating in this study is to not participate.

If you agree to participate in this study, we will collect about seven dessertspoonful (50ml) of urine from you into a tube. In addition, we will stab, prick your finger with a

University of Ghana http://ugspace.ug.edu.gh

lancet, special blade and collect few drops of blood into one small (capillary) tube and also, onto two glass microscope slides. Proteins from the urine will be used to produce the tool for detecting malaria whilst the blood will be examined for malaria causing organisms and also be measured to see if you have enough blood. The malaria test results will be ready within thirty minutes, and if you are found to have the small organisms that cause malaria and/ or do not have enough blood, we will refer you to the doctor for treatment. It is our policy to not discuss such information unless it has direct medical implications for treatment of malaria. We will store a part of the samples we collect in case there are additional tests we wish to perform in the future. These stored samples will be used only for studies related to malaria. Samples will be labelled with a code and will be accessed by study investigators only. You have the right to request that your samples should not be saved, and you may ask that these samples be destroyed at any time.

The risks associated with this study are minimal. Drawing blood may cause discomfort and occasional bruising at the site; rarely, fainting or infection may occur. We will clean your finger with disinfectant before taking blood and will use a new lancet to prick your finger for the blood.

If you wish to withdraw from the study, you may do so at any time. Although you will not receive any monetary payment for your participation, you will be given a small amount of non-monetary compensation for your time. The findings of this study may be reported at meetings or in medical journals, but your name will not be used in the reports, and the specific information we learn about you will not be shared with anybody except the study investigators.

Do you have any questions about your participation in this study? If you have questions or concerns about your participation in this study at a later date, you may speak with one of our staff or you can ask the Chief of this area to send a message to Mr. Uri S. Markakpo at the School of Public Health, College of Health Sciences, University of Ghana, Legon. The doctors at the community clinic/ health centre can help you contact Mr. Markakpo.

Yours Sincerely,

Mr. Uri S. Markakpo
Research Fellow
Tel: 0246373898

If you agree to participate in this study, please put your signature or thumbprint below.

Adult Signature or Thumbprint	***Investigator Signature***	***Date***

University of Ghana http://ugspace.ug.edu.gh

Appendix 6b

PARENTAL CONSENT FORM FOR THE STUDY ENTITLED:
RAPID URINE-BASED DIPSTICK TEST FOR DIAGNOSIS OF MALARIA IN INFANTS, PLACENTA AND LOW-RISK INDIVIDUALS

FOR SUBJECTS AGED 15 YEARS AND YOUNGER
UNIVERSITY OF GHANA
KPONE ON SEA/GHANA

Identifying Number_____

_____

Child's Name: _____ **Age:** _____
Last name *First name* *Middle initial*

Adult's Name: _____ **Relationship to child:** _____
Last name *First name* *Middle initial*

Information: *to be read and translated to parents/ guardians in their own mother tongue if necessary.*

Dear Sir/Madam,
We invite your child to take part in a research study sponsored by the School of Public Health, College of Health Sciences, University of Ghana and the Bill and Melinda Gates Institute of Population and Reproductive Health, Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA. It is important that you understand several general principles that apply to everyone who takes part in this study:

1. Participation of your child in the study is entirely voluntary.
2. Personal benefit to your child may not result from taking part in the study, but knowledge gained may benefit others.
3. You may withdraw your child from the study at any time.

Malaria is a sickness caused by very small disease causing organisms that can enter the body when mosquitoes bite you. They can cause fevers, headaches, body aches and weakness, and if not treated, can progress to severe illness, especially in children and pregnant women. When malaria is treated with proper medicines, it can be cured completely; but before a patient is given medicines to cure malaria, a proper test has to be done to be sure that he/she has malaria. Presently, there are a number of methods that can be used to test for malaria but none of them uses urine even though it is easier to obtain and simpler to use. The main purpose of this study is to come out with a new method of detecting the presence of malaria using urine as a test material. The reason why we are inviting your child to participate in this study is because we would like to learn more about the malaria disease that occurs in this community so that we can produce a new tool for detecting the disease early enough to cure it so as to prevent the disease from becoming severe and leading to death. The alternative to participating in this study is to not participate.

University of Ghana http://ugspace.ug.edu.gh

If it is agreed that your child should participate in this study, we will collect about seven dessertspoonfuls (50ml) of urine from your child into a tube. In addition, we will prick your child's finger with a lancet (special blade) and collect few drops of blood into one small tube and also, onto two glass microscope slides. Proteins from the urine will be used to produce the tool for detecting malaria whilst the blood will be examined for small organisms that cause malaria and also measured to see if your child has enough blood (anaemia). The malaria test results will be ready within thirty minutes, and if your child is found to have malaria causing organisms and/ or does not have enough blood, we will refer him/her to the doctor for treatment. It is our policy to not discuss such information unless it has direct medical implications for treatment of malaria. We will store a part of the blood and urine samples we collect in case there are additional tests we wish to perform in the future. These stored samples will be used only for studies related to malaria. Samples will be labelled with a code and will be accessed by study investigators only. You have the right to request that your child's samples should not be saved, and you may ask that these samples be destroyed at any time.

The risks associated with this study are minimal. Drawing blood may cause discomfort and occasional bruising at the site; rarely, fainting or infection may occur. We will clean your child's finger with disinfectant before taking blood and will use a new lancet to draw the blood.

If you wish to stop your child from participating in the study, you may do so at any time. Although you will not receive any monetary payment for your child's participation, you will be given a small amount of non-monetary compensation for your time. The findings of this study may be reported at meetings or in medical journals, but your child's name will not be used in the report, and the specific information we learn about your child will not be shared with anybody except the study investigators**.**

Do you have any questions about your child's participation in this study? If you have questions or concerns about your child's participation in this study at a later date, you may speak with one of our staff or you can ask the Chief of this area to send a message to Mr. Uri S. Markakpo at the School of Public Health, College of Health Sciences, University of Ghana, Legon. The doctors at the community clinic/ health centre can help you contact Mr. Markakpo.

Yours Sincerely,

Mr. Uri S. Markakpo
Research Fellow
Tel: 0246373898

If you agree to have your child participate in this study, please put your signature or thumbprint below.

Adult Signature or Thumbprint	***Investigator Signature***	***Date***

University of Ghana http://ugspace.ug.edu.gh

Appendix 6c

CHILD INFORMATION FORM FOR THE STUDY ENTITLED: RAPID URINE-BASED DIPSTICK TEST FOR DIAGNOSIS OF MALARIA IN INFANTS, PLACENTA AND LOW-RISK INDIVIDUALS

Information: *to be read and translated to children in their own mother tongue if necessary*

Explanation of the study to children in few words

This study is to collect information about malaria in this community which has been selected as a model site for studies on diseases including malaria. The outcome of the studies will help to develop effective control methods to protect the people from these diseases, particularly, malaria.

You will be asked to donate about seven dessertspoonful (50ml) of urine. Also, a fresh sterile lancet (special blade) will be used to prick your finger to take a few drops of blood. The urine sample will be used to produce a new tool for determining whether you have malaria or not. If this new tool is produced urine may be used to check for malaria instead of blood. Also, qualified health personnel in the laboratory will examine the blood collected for small organisms that cause malaria and to see if you have enough blood. The malaria test results will be ready within thirty minutes, and if you are found to have the organisms that cause malaria and/ or do not have enough blood, we will refer you to the doctor for treatment. Apart from a minor pain at the point where your finger will be stabbed with the lancet, urine and blood sample collection, will not have any bad effect on you.

You will be assigned a code that will be used to identify you and all information gathered from you will be considered confidential.
Informed consent has been obtained from your parent/guardian, but you can decide not to take part in the study if you do not want to. Also, if at any time after the study has begun you decide not to participate or allow your samples to be used in the study anymore, you are free to withdraw or inform us immediately without any further discussion. Withdrawal from the study or refusal to participate will not have any adverse consequence on you or deny you with any rights or benefits you are already enjoying in this community or elsewhere.

If you agree to participate in this study, please allow your name to be written in the space provided below.

Name of Child: _____

Last name *First name* *Middle initial*

Translated by: _____

Translation witnessed by: _____

Signature: _____

Name: _____

Designation: _____

University of Ghana http://ugspace.ug.edu.gh

APPENDIX 7: ETHICAL CLEARANCE CERTIFICATE

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
INSTITUTIONAL REVIEW BOARD

(UNIVERSITY OF GHANA)

Phone: +(233) 21 500374 /501178
Fax: +(233) 21 502182
Email: Director@noguchi.mimcom.net
Telex No: 2556 UGL GH

P.O. Box LG 581
Legon
Ghana

My Ref. No: DF.22

2nd July, 2008

Your Ref. No:

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 0001276**

NMIMR-IRB CPN 053/07-08 **IORG 0000908**

On 2nd July, 2008, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB), at a full board meeting reviewed and approved your protocol titled:

TITLE OF PROTOCOL : **Rapid Urine-Based Dipstick Test for Diagnosis of Malaria In Infants Placenta and low-risk individuals**

PRINCIPAL INVESTIGATOR : **Uri Selorm Mckakpo (PhD Student)**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 1st July, 2009. You are to submit annual reports for continuing review.

Signature of Chairman: .....
Rev. Dr. Samuel Ayete-Nyampong
(NMIMR – IRB, Chairman)

cc: Professor Alexander K. Nyarko
Director, Noguchi Memorial Institute
for Medical Research, University of Ghana, Legon

University of Ghana http://ugspace.ug.edu.gh

APPENDIX 8: TECHNICAL CHALLENGES ENCOUNTERED IN THE COURSE OF THE STUDY AND HOW THEY WERE CIRCUMVENTED

Following the obtainment of funding, other problems encountered include:

- Lack of space for placement of freezers and refrigerators for storage of samples at NMIMR. **To overcome this problem, some broken down freezers occupying space in the laboratory were refurbished and used**.
- Removal of a voltage stabilizer from a storage freezer for my research by an unknown person which resulted in the breakdown of the freezer with associate loss of some reagents and processed samples which destabilized work for some time. **The stolen stabilizer has not been replaced. An alternative freezer belonging to Prof. I. A. Quakyi, containing left over reagents was evacuated and is now being used for storage of reagents**.
- Loss of a HAT selection reagent, a critical reagent for production of monoclonal antibodies (which are essential for development of the urine-based dipstick test) from my storage freezer at the Noguchi Memorial Institute for Medical Research (NMIMR) which took me about **three (3) months** to get a replacement. **To circumvent this challenge, a substitute for this reagent was procured for the work**.
- Breakdown of the two (2) carbon dioxide (CO_2) incubators for culture of hybridoma cells needed for production of monoclonal antibodies. This technical challenge caused cell fusion to be suspended for **more than 3 months**. **An alternative incubator belonging to Prof. Quakyi was later moved to Noguchi for continuation of work until the problem with the first incubator had been resolved.**
- Closure of my working laboratories at the NMIMR facility for refurbishment for **over 3 months.**
- Shut down of the biological safety cabinet for cell culture for maintenance which took **3 weeks** to complete.
- Breakdown of CO_2 cylinders for tissue culture which took **over 3 months** to repair and refilled by AirLiquide, Tema, before continuation of work.
- Failure of cell fusion to yield hybridoma cells which took **more than 2 months** to overcome.
- Loss of hybridoma cells in culture to bacterial contamination which took **2 months** to recover.
- Delay in the release of project funds. **This challenge has now minimized**