IN VIVO AND MOLECULAR EVALUATION OF ARTESUNATE+SULFADOXINE-PYRIMETHAMINE EFFICACY FOR UNCOMPLICATED FALCIPARUM MALARIA IN TEHAMA REGION, YEMEN

WAHIB MOHAMMED MOHSEN ATROOSH

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

IN VIVO AND MOLECULAR EVALUATION OF ARTESUNATE+SULFADOXINE-PYRIMETHAMINE EFFICACY FOR UNCOMPLICATED FALCIPARUM MALARIA IN TEHAMA REGION, YEMEN

WAHIB MOHAMMED MOHSEN ATROOSH

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Wahib Mohammed Mohsen Atroosh

Matric No: MHA 120036

Name of Degree: PhD

Title of Thesis:

IN VIVO AND MOLECULAR EVALUATION OF ARTESUNATE + SULFADOXINE - PYRIMETHAMINE EFFICACY FOR UNCOMPLICATED FALCIPARUM MALARIA IN TEHAMA REGION, YEMEN

Field of Study: Molecular Parasitology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every right in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date: 27 April 2017

Subscribed and solemnly declared before,

Witness's Signature Date: 27 April 2017

Name:

Designation:

ABSTRACT

Yemen has updated its national malaria policies in 2009 to introduce histidine rich protein 2 – based rapid diagnostic test (HRP2-RDT) as a method of diagnosis in peripheral malaria-endemic areas, and to replace chloroquine (CQ) by artemisinin combination therapy (ACT) for treating uncomplicated falciparum malaria infection. The present study aimed to evaluate the HRP2-RDT and to assess the therapeutic efficacy of artesunate + sulfadoxine/pyrimethamine (AS+SP) as a first line treatment. A total of 622 febrile individuals from two malaria-endemic areas in Tehama region, Yemen (Hodeidah and Al-Mahwit governorates) were screened by *CareStart*^{TM} malaria HRP2-RDT and confirmed later by microscopy, followed by gene sequencing analysis of *Pfhrp2*. Evaluation of AS+SP therapeutic efficacy was performed through *in-vivo* evaluation of the clinical and parasitological response over 28 days of follow-up according to standard protocols. Moreover, frequency of mutations associated with drugs resistance was obtained for the *pfdhfr*, *pfdhps*, and *pfK13* genes for AS + SP, as well as *pfcrt* and *pfmdr1* genes for other antimalarials. A total of 188 (30.2%) participants were found positive for *P. falciparum* by the RDT compared to 189 (30.4%) by microscopy. The sensitivity and specificity of the RDT were 90.5% and 96.1%, respectively. Eighty-six patients completed the AS+SP *in-vivo* study, with a cure rate of 96.5% (94.2% PCR-uncorrected). However, the efficacy of gametocyte clearance was poor, with gametocytes persisting throughout the study in some patients. All the isolates sequenced had the pfk13 propeller domain wild-type allele, and mutations associated with SP failure were observed only for *pfdhfr*, with the double mutation (S108N+N51I) was reported in 65.4% of the isolates. *Pfcrt* gene showed wide prevalence of mutations, with the predominance of *pfcrt* 76T CQ resistance (97.7%). Mutated *pfcrt* haplotypes were highly prevalent (98.8%) with CVIET classic, old-world African/Southeast Asian haplotype being the most predominant, and was mostly found in the isolates from Khamis Bani Saad and AdDahi districts (93.1% and 88.9% respectively). Interestingly, the SVMNT new-world South American haplotype was exclusively detected in isolates from Bajil districts of Hodeidah (9.3%). Mutations at Y184F of *pfmdr1* were found at a fixation level (100%) in all districts, while mutations of codons 1034C and 86Y were only found in the isolates from AdDahi and Khamis Bani Saad districts. In conclusion, *CareStart*^{TM} Malaria HRP2-based RDT showed high level of sensitivity and specificity in malaria-endemic areas in Yemen. Moreover, AS+SP therapy remains effective for the treatment of uncomplicated falciparum malaria

with poor gametocidal activity. No polymorphism in *pfk13* was detected in all isolates studied. Adding a single dose primaquine, which minimizes transmission potential, to the current ACT drug policy is strongly recommended. The high prevalence of mutations in *pfcrt*, 5 years after official cessation of CQ suggests a sustained CQ pressure on *P. falciparum* isolates in the study area. Moreover, the low prevalence of mutations in the *pfmdr1* gene could be a good indicator of the high susceptibility of *P. falciparum* isolates to antimalarials other than CQ. Therefore, a new strategy to ensure the complete nationwide withdrawal of CQ from the private drug market is recommended.

ABSTRAK

Negara Yaman telah merombak dasar nasional malaria pada tahun 2009 dengan menggunakan RTD berdasarkan histidine rich protein 2 (HRP2-RTD), selaku kaedah diagnosis di kawasan periferi yang endemik malaria dan menggantikan Chloroquine (CQ) dengan terapi kombinasi Artemisinin untuk rawatan infeksi malaria falsiparum yang tiada komplikasi. Kajian masakini bertujuan menilai HRP2-RTD dan keberkesanan terapeutik Artesunate + Sulfadoxine/Pyrimethamine (AS+SP) selaku rawatan pilihan pertama. Sejumlah 622 individu yang febril daripada dua kawasan malaria endemik di wilayah Tehana, Yeman (Pemerintah Hodeidah dan Al-Mahwit) disaring dengan mengunakan teknik HRP2-RDT malaria *CareStart*^{TM}. Kemudian ini disahkan dengan teknik mikroskopi, diikuti dengan analisis penurutan gen *Pfhrp2*. Penilaian keberkesanan terapeutik AS+SP dilakukan melalui secara *in-vivo* bagi respon parasitological dan klinikal sepanjang 28 hari yang diikuti mengikut protokol standard. Tambahan, kekerapan mutasi yang berkaitan dengan ubat yang resistant diperolehi bagi *pfdhfr*, *pfdhps*, dan gen *pfK13* untuk AS+SP, dan juga gen *pfcrt* dan *pfmdr1* untuk antimalarial lain. Sejumlah 188 (30.2%) peserta didapati positif bagi *Plasmodium falcifarum* oleh RDT berbanding dengan 189 (30.4%) dengan mikroskopi. Peratusan sensitiviti dan spesifisiti dengan kaedah RTD tersebut amat tinggi, masing-masing 90.5 dan 96.1%. Seramai 86 pesakit melengkapi kajian *in-vivo* AS+SP, dengan mencapai kadar sembuh 96.5% (94.2% PCR tanpa pembetulan). Walau bagaimanapun, efikasi pemulihan gametosit amat rendah, diiringi dengan pengekalan gametosit dalam keseluruhan kajian dalam sesetengah pesakit. Kesemua pencilan yang diataturutkan mengandungi "propeller" *pfk13* domain allele tip liar. Mutasi yang berkaitan dengan kegagalan rawatan SP hanya kelihatan dengan *pfdhrf*. Bersama ini juga 65.4% di laporkan bahawa mutasi berganda (S108N + N511) terdapat dari pencilan tersebut. Gen *pfcrt* menunjukkan keprevalenan mutasi yang berleluasa bagi *pfcrt* 76T CQ resistant (97.7%). Haplotip *pfcrt* termutasi teramat prevalen (98.8%) manakala haplotip klasik CVIET, iaitu dunia lama Afrika/Asia Tenggara ialah yang paling pradominan dan sebahagian besarnya ialah dari pencilan daerah Khamis Bani Saad dan daerah Ad Dahi (93.1% dan 88.8%). Lebih menarik, ialah haplotip SVMNT dunia baru Amerika Selatan sepenuhnya dikesan dari pencilan berasal dari daerah Bajil, Hodeidah (9.3%). Mutasi pada Y184F *pfmdr1* terdapat di paras yang telah ditetapkan (100%) di semua daerah, manakala mutasi kodon 1034C dan 86Y hanya terdapat dari pencilan daerah AdDahi dan Khamis Bani Saad. Jelaslah bahawa *CareStart*^{TM} Malaria HRP2 yang berdasarkan RDT menunjukkan paras kesensitifan dan kespefikan yang tinggi di dalam wilayah

endemik bagi malaria di Yaman. Malahan, terapi AS+SP masih berkesan untuk rawatan malaria falsiparum tanpa komplikasi, tetapi kurang aktiviti gametosid. Polimorfisma bagi *pfk13* tidak dikesan dalam semua pencilan yang dikaji. Saranan yang kukuh kepada dasar dadah antimalarial terkini ialah dengan tambahan dos tunggal Primaquine yang akan menghasilkan potensi transmisi yang minimal. Keprevalenan pemmutasian *pfcrt* yang tinggi, setelah lima tahun CQ terhenti secara rasmi, menunjukkan tekanan CQ yang berterusan ke atas pencilan *P. falciparum* di kawasan kajian. Keprevalenan mutasi yang rendah bagi gen *pfmdr1* menandakan kerentanan yang tinggi bagi pencilan *P. falciparum* terhadap antimalarial selain dari CQ. Maka dengan ini, satu strategi baru untuk menentukan penarikan CQ yang menyeluruh di peringkat nasional daripada pasaran swasta disarankan.

ACKNOWLEDGEMENTS

First and above all, I praise Allah, the almighty for providing me this opportunity and granting me the capability to proceed this work successfully.

This thesis appears in its current form is due to the help, guidance, assistance and inspiration of several people. I would therefore like to offer my sincere thanks to all of them.

I am very grateful to all people, **children and their parents,** from the selected villages in Hodeidah and Al-Mahwait for their willingness to participate and cooperate in the period of follow-up of this study. All my hope is that this study has helped you at least alleviate the suffering of malaria and provided you with simple tools to fight malaria. Please use the bed nets and take care of your selves.

I would like to express my cordial and deepest thanks to my esteemed supervisors, **Associate Professor Dr. Hesham Mahyoub Sarhan Al-Mekhlafi** and **Professor Dr. Johari Bin Haji Surin** for accepting me as a PhD student, and for their full support during this journey. Your warm encouragement, thoughtful guidance, critical comments, and correction of the thesis are very much appreciated. I could not have imagined having a better supervisors and mentor for my PhD program. I am very grateful to them for enlightening the way of my PhD research. Really, the successful completion of this work depends largely on their support. Special thanks go to **Associate Professor Dr. Lau Yee Ling** for her kind cooperation, allowing me to use facilities at the Molecular laboratory 1 and for her essential advisory contribution to this work.

I am also indebted to the esteemed academic icon **Professor Dr. Georges Snounou** who actually did not spare any time or efforts throughout the period of this study. Thank you for the trust, the insightful discussion, offering valuable advices and support during the period of the study including the lab work guidance and the data analysis, and for revising a manuscript and responding to reviewer's comments. I am proud of having this type of international collaboration.

I would like also to express my greatest appreciation to **Dr. Adel Al-Jasari**, the general director of the National Malaria Control Program, Yemen for the full logistic support during field work activities. He willingly provides the study with all that we need for the field work including the supplying of the

AS + SP drug batch for *in vivo* study, malaria rapid diagnostic tests for malaria screening, slides, Giemsa stain and the mosquito nets (LLITNs) which were distributed as tokens of encouragement to participants. Dr. Adel has also arranged with well-trained malaria team in Bajil to achieve samples collection, malaria diagnosis, and malaria case management and follow up as well as to access easily to the health facilities and to selected villages.

Nabil Nasr and Hany Sady, I greatly appreciate your excellent assistance in the lab work and your spiritual supports during my PhD study. I will never forget the time that we were together and discussed about everything. The fun that we have had and sometimes the fight is the sweet taste of this period.

My grateful appreciation goes to the head of our department, department of Parasitology, University of Malaya (**Prof. Dr. Suresh Kumar** and the former head **Prof. Dr. Rohela Mahmud**) and for the staff, secretary and colleague, for providing a very nice atmosphere and harmony and for useful friendly cooperation.

I gratefully acknowledge the National Malaria Control Programme (NMCP) staff for their generous cooperation during this study especially Mr. Khaled Al-Mansab of the NMCP, Sana'a for providing the map of the malaria distribution in Yemen in 2012, and to Bajil malaria team represented by Dr. Yehea A. Aamer for the malaria case management, Fathya S. Bukair and Fatima Y. Hasan for their fruitful help in samples collection and malaria diagnosis.

Special thank is to Malaria Research and Reference Reagent Resource Centre (MR4), ATCC®, Manassas, VA, USA for providing us with *Plasmodium falciparum* parasite lines 3D7 (MRA-102G), HB3 (MRA-155G) and Dd2 (MRA150G) deposited by MR4 which are used as positive controls for *pfcrt* and *pfmdr1* wild and resistant *Plasmodium falciparum* strain, and as positive controls for *hrp2* and *hrp3* gene deletion.

I would like also to acknowledge University of Malaya for providing research fund to carry out this research through High Impact Research Grant (UM.C/625/1/HIR/MOHE/MED/18) and (UM.C/625/1/HIR/MOHE/MED/16) from the Ministry of Higher Education Malaysia. My great thanks go also to my university, University of Aden in my beautiful hometown "Aden" for granting me with the scholarship for Master and PhD.

Although we have lived far from our family, they are always with us. I thank my father and mother from the bottom of my heart for their love, wise counsel, sympathetic

ear, and spiritual and unconditional financial support throughout my life. My brothers and sisters were also there in my life, communication with them provided an emotional atmosphere for me. Hereby, I would like to thank them all for prayers and encouragements.

Finally, I want to express my greatest gratitude and deepest appreciation to my lovely wife, **Meram**, for her immeasurable patience and understandings during the stressful and difficult moments. Without her supports and encouragements, I could not have finished this work. I understand, it was so difficult for her to keep going in her PhD programme and to take care of our kids and me too, therefore, I can just say thanks for everything and may Allah give her all the best in her life. My lovely daughter **Raghad** and lovely son **Mohammed** are the best thing I have ever in my life, having you provides me with the power and stability to continue. I know that I did not give them enough time and optimal conditions to have fun, but hopefully the coming days will bring the best to them.

TABLE OF CONTENTS

CHAPTER 6: CONCLUSION AND RECOMMENDATION

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

P. falciparum	*Plasmodium falciparum*
P. vivax	*Plasmodium vivax*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. knowlesi	*Plasmodium knowlesi*
P. cynomolgi	*Plasmodium cynomolgi*
SNP	Single nucleotide mutation
PCR	Polymerase chain reaction
RBM	Roll Back Malaria
RBMP	Roll Back Malaria Program
NMCP	National Malaria Control Program
RDT	Rapid diagnostic test
pLDH	Pan lactate dehydrogenase
HRP2	Histidine rich protein 2
HRP3	Histidine rich protein 3
MSP1	Merozoite surface protein 1
MSP2	Merozoite surface protein 2
GLURP	Glutamate rich protein
PFCRT	*Plasmodium falciparum* chloroquine resistance transporter
PFMDR1	*Plasmodium falciparum* multidrug resistance 1
PFDHFR	*Plasmodium falciparum* dihydrofolate reductase
PFDHPS	*Plasmodium falciparum* dihydropteroate synthase
PFK13	*Plasmodium falciparum* kelch 13
TAE	Tris-acetate- ethylenediaminetetraacetic acid
RE	Restriction enzyme
MR4	Malaria Research and Reference Reagent Resource Center
TDR	Tropical Diseases Research
WHO	World Health Organization
PABA	Para-aminobenzoic acid
DNA	Deoxyribonucleic acid
IRS	Insecticide residual spray
ITN	Insecticide treated bed-net
LLITN	Long-lasting insecticide treated bed-net
IPT	Intermittent preventive treatment
IPTp	Intermittent preventive treatment in pregnancy
IPTi	Intermittent preventive treatment in infants

EMRO	Eastern Mediterranean Regional Office
AL	Artemether-lumefantrine
AM	Artemether
AQ	Amodiaquine
ART	Artemisinin
AS	Artesunate
AT	Atovaquone
CL	Clindamycin
CQ	Chloroquine
D	Doxycycline
DHA	Dihydroartemisinin
MQ	Mefloquine
NQ	Naphroquine
PG	Proguanil
PPQ	Piperaquine
PQ	Primaquine
PYR	Pyronaridine
QN	Quinine
SP	Sulfadoxine-pyrimethamine
T	Tetracycline
ACT	Artemisinin combination therapy
PEV	Pre-erythrocytic stages vaccine
BSV	Blood stage vaccine
TBV	Transmission blocking vaccine
ATV	Antitoxic vaccine
ECF	Early treatment failure
LCF	Late clinical failure
LPF	Late parasitological failure
ACPR	Adequate clinical and parasitological response
TTF	Total treatment failure
EDTA	Ethylene diamine tetra acetic acid
ACT	Artemisinin combination therapy
PCV	Packed cell volume
µM	Micromolar
µL	Microlitre
ELISA	Enzyme linked immunosorbent assay

LIST OF APPENDICES

CHAPTER I: GENERAL INTRODUCTION

1.1 BACKGROUND

Malaria is one of the most common life-threatening diseases and one of the most severe public health problems worldwide, particularly in sub-Saharan African countries which are home to 88% of malaria cases and 90% of malaria deaths. Almost half of the world population (3.2 billion) lives in areas at risk of malaria transmission in more than 90 countries and territories, and there were 214 million malaria cases and 438,000 malaria deaths as estimated in 2014 (WHO, 2015a).

Plasmodium falciparum is the predominant malaria parasite species in Africa and is responsible for most malaria-related deaths globally. For malaria diagnosis, microscopy is the gold standard technique based on the capacity of detecting low parasitaemia and the distinguishing capability of the different species of malaria parasites. However, variation among the microscopists; in addition to the problem of unavailability of electricity, logistics and qualified workers particularly in rural and remote areas are considered as disadvantages of microscopy method. Although its performance is limited in detection of low parasite density and in distinguishing all malaria parasite species, rapid diagnostic test (RDT) is found useful in areas with microscopy shortage, providing a quick and acceptable results especially in remote endemic areas. Hence, malaria diagnosis has been improved with the introduction of RDT, which has now become an indispensable tool especially in areas where the classical mode of identification, malaria microscopy, is not feasible (WHO, 2011b). In addition, polymerase chain reaction (PCR) and gene sequencing are excellent techniques for detecting different species of malaria parasites through amplifying target gene sequences. Besides, these methods require sophisticated instrumentations that are

expensive and well-trained personnel, therefore they are commonly used in research purposes.

With regards to drug resistance, *P. falciparum* is the leading cause of antimalarial drug resistance as this species started to develop resistance to chloroquine (CQ) in the 1950s in Southeast Asia, and this resistance later became widespread elsewhere. Furthermore, the emergence of *P. falciparum* resistance to other antimalarials such as mefloquine, amodiaquine and sulfadoxine-pyrimethamine has necessitated a switch to new drugs to ensure maximum effectiveness and to prevent further development of ongoing resistance. Also, the use of combination therapy rather than monotherapies has been suggested for falciparum malaria case management and malaria control. On the other hand, the World Health Organization (WHO) has adopted a strategy to fight against malaria that mainly depends on early diagnosis and prompt treatment (WHO, 1993). This strategy required updates on the diagnosis and therapeutic platforms of malaria control. From the therapeutic perspective, artemisinin combination therapy (ACT) has been adopted for uncomplicated falciparum malaria case management and malaria control in all the countries of Africa and Asia. However, the adoption of ACT in South America varies as there are some countries in the continent in which CQ is still effective (WHO, 2014a).

Susceptibility of live malaria parasite to antimalarials is monitored by either *in vivo* and/or *in vitro* assays. *In vivo* method of evaluation is achieved through monitoring the parasitaemia in patient's blood throughout a specific period of time after administration of standard dose of the antimalarial. This method has an advantage of enabling the linkage of assessment of the drug, the patient and the parasite together, but potential confounders such as malabsorption and immunity status of the patients may affect the *in vivo* test outcomes (WHO, 2009). The *in vitro* method for drug efficacy evaluation is useful in assessment of more than one drug and for more than one parasite

strain at the same time through artificial cultivation of the parasite against different drug concentration (WHO, 2001). However, the disadvantage of this test is the need of specific instrumentation and a higher level of training. In addition, molecular markers of drug resistance are another predicting tool for monitoring resistance to antimalarial drugs through the detection of mutation along genes of interest utilizing fresh and even archived blood samples. Different genes were reported having an association with low parasite susceptibility to antimalarials such as *P. falciparum* chloroquine resistance transporter (*pfcrt*) and multidrug resistance 1 (*pfmdr1*) genes for chloroquine, amodiaquine and lumefantrine, and dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*) genes for pyrimethamine and sulfadoxine resistance, respectively.

Furthermore, the molecular marker of artemisinin resistance was recently identified, and mutations in the Kelch 13 (K13)-propeller domain were shown to be associated with delayed falciparum parasite clearance *in vitro* (Ariey, et al., 2014). Analysis of the recently identified molecular marker for artemisinin resistance showed that the C580Y mutation is most prevalent in parts of the Greater Mekong sub-region, but other mutations in and near the K13-propeller domain region, such as in codons Y493H, R539T, I543T, were also found to be significantly associated with artemisinin resistance (WHO, 2014b). Unlike Asian countries, African and South American countries were found less affected by *P. falciparum* parasite resistance to artemisinin derivatives, with less than 10% resistance to ACT was reported in Africa and South America (WHO, 2015b). Moreover, *k13* gene mutation was rare in Africa as a non-synonymous propeller mutation A578S was the frequently reported mutation in some African countries which is not related to the phenotypic artemisinin clearance (WHO, 2014b; Torrentino-Madamet et al., 2014; Huang et al., 2015; Kamau et al., 2015;).

Yemen is the only country in the Arabian Peninsula that is highly affected by malaria with almost 60% of the population at risk, thereby creating a threat to the lives of the populations and the malaria control programmes of neighbouring countries. Most of the cases in Yemen originate in the northwest in the region of Tehama. However, some other areas in the southwest, middle and east of the country are also at risk, with *P. falciparum* being the most predominant species responsible for 99% of malaria cases (WHO, 2015a). Yemen falciparum malaria isolates have exhibited a gradual increase in the resistance to CQ since the first reported case of CQ-resistant *P. falciparum* in the country by Mamser (1989). Indeed, *in vivo* resistance to CQ has continued to emerge in all the malarious sites of Yemen, reaching 42% in Bajil districts of Hodeidah (western Yemen) in 2002, 46.5% in Odein, Ibb governorate (Central part of Yemen) in 2003 and 57% in Al-Musaimeer, Lahj (Southern Yemen) in 2003 (NMCP, unpublished data). Moreover, CQ resistance has later increased in Al-Musaimeer to 60.7% (Mubjer et al., 2011).

In an attempt to combat this growing resistance, the government of Yemen's National Malaria Control Programme (NMCP) updated its national malaria treatment policy in 2009 to include artesunate plus sulphadoxine-pyrimethamine (AS+SP) and artemether plus lumefantrine (AL) as a combination first-line and second-line drug treatment, respectively. In addition, a RDT based on histidine-rich protein 2 (HRP2) of *P. falciparum* has been introduced in Yemen for diagnosing malaria in peripheral malarious areas where there is a lack of personnel trained in malaria microscopy. The first RDT product to be procured by the NMCP was the First Response hrp2-RDT (*First Response*® Malaria Ag. *P. falciparum*, HRP2, Premier Med. Corp., India). This was later replaced by the CareStart hrp2-RDT (*CareStart*™ Malaria HRP2, Cat. no. G0141, Access Bio, Inc., USA) (NMCP, personal communication).

1.2 PROBLEM STATEMENT

From the academic side, several reports have been published over the last two decades that have aimed to evaluate the susceptibility of Yemen falciparum malaria isolates *in vivo* and/or *in vitro* to CQ, mefloquine, quinine, SP, or artemisinin (Alkadi et al., 2006; Al-Kabsi et al., 2009; Al-Maktari & Bassiouny, 2003; Al-Shamahy et al., 2007). Others have attempted to assess the effectiveness of CQ and SP from a molecular perspective through detecting whether there is mutation in the *pfcrt*, *pfmdr1*, *pfdhfr* and *pfdhps* genes of *P. falciparu*m isolates from Yemen (Abdul-Ghani et al., 2014; Al-Hamidhi et al., 2013; Al-Mekhlafi et al, 2011; Bamaga et al., 2015a; Bamaga et al., 2015b & Mubjer et al., 2011).

From the government side, the NMCP conducted five *in vivo* studies from 2009 to 2010 during the implementation of its new combination drug policy, which all aimed to evaluate the efficacy of AS+SP or AL against *P. falciparum* isolates from different malarious sites in Hodeidah, Dhamar, Hajjah and Ibb governorates (NMCP, unpublished data). Both of these artemisinin drug combinations were shown to have absolute effectiveness (100%) against the parasite. To the best of my knowledge, there have been no published works on the therapeutic efficacy of ACT in relation to delayed parasite clearance after the implementation of the ACT policy in Yemen in 2009. It should be noted that a recently published *in vivo* study of these two artemisinin-based combinations (AS+SP and AL) for falciparum malaria in Yemen (Adeel et al., 2015) was not available when we designed the present study, initiated the research and completed the data analysis. Nevertheless, neither the previously published studies nor this new resource tried to link the field *in vivo* evaluation outcome regarding the effectiveness of antimalarial drugs with the molecular existence of drug-related mutations, so there is a significant gap in knowledge with respect to this issue.

Within this context, the present study aims mainly to evaluate the AS+SP first-

line treatment based on the WHO-recommended 28-day *in vivo* protocol and to genetically analyse *k13* and *dhfr/dhps* genes as molecular markers of resistance to AS and SP antimalarial drugs, respectively. Malaria diagnosis in the study area, as in any other rural area in Yemen, is achieved mainly by using the RDT, CareStart^{TM} Malaria HRP2, which has not yet been evaluated for its capacity to diagnose falciparum malaria against Yemen isolates. The only study that has evaluated hrp2-RDT accuracy was published in 2012 and it examined the use of the former First Response hrp2-RDT product in a malaria outbreak in Hadramout governorate in the east of Yemen (Ghouth et al., 2012). That study reported that sensitivity and specificity were 74% and 94%, respectively. Thus, the present study is the first that aims to evaluate the performance of the *CareStart^{TM}* HRP2-based RDT currently in use.

1.3 OBJECTIVES OF THE STUDY

1.3.1 General objective

The present study aimed at evaluating the histidine rich protein 2 - based RDT (HRP2-RDT) in diagnosing malaria and the efficacy of the current first-line antimalarial drug treatment policy of artesunate plus sulfadoxine-pyrimethamine (AS+SP) against falciparum malaria in Yemen.

1.3.2 Specific objectives

i. To evaluate of the performance of HRP2-based RDT currently used for diagnosing malaria in peripheral districts in Yemen.

ii. To investigate the *pfhrp2* genetic variation and evaluate its impact on RDT performance in diagnosing malaria in the study areas.

iii. To evaluate the therapeutic efficacy of the current treatment policy (AS+SP) against uncomplicated falciparum malaria in the study areas.

iv. To investigate the presence of point mutations at *P. falciparum Kelch* 13 gene as a molecular marker of artemisinin resistance among the studied isolates.

v. To investigate the presence and frequency of point mutations at 6 codons: 16, 50, 51, 59, 108 and 164 of *P. falciparum* dihydrofolate reductase (*pfdhfr*) gene and at other 7 codons: 436, 437, 540, 581, 613, 640 and 645 of *P. falciparum* dihydropteroate synthase (*pfdhps*) gene as molecular markers of the partner drug, sulfadoxine-pyrimethamine among the studied isolates.

vi. To investigate the presence and frequency of single point polymorphisms (SNPs) in *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) at codons 72-76, 220, 271, 326, 356 and 371 (10 codons) and *P. falciparum* multidrug resistance 1 gene (*pfmdr1*) at 86, 184, 1034, 1042 and 1246 (5 codons).

1.4 HYPOTHESES

i. Using HRP2-based RDT to diagnose malaria is acceptable and fulfill the requirements for malaria diagnosis in peripheral malaria-endemic areas in Yemen (high sensitivity and specificity).

ii. The *pfhrp2* gene is highly polymorphic among the *P. falciparum* isolates from study areas which could have an impact on the performance of the RDT.

iii. The *P. falciparum* isolates from the study areas show a low level of treatment failure to AS+SP, mainly due to SP resistance.

iv. The *P. falciparum* isolates from the study areas are of wild type for *kelch 13* gene, reflecting a high efficacy of artesunate in the AS+SP combination.

v. There is a moderate rate of mutation in the *pfdhfr* and *pfdhps* conferring resistance to SP among the studied *P. falciparum* isolates.

vi. There is a high rate of mutation in the *pfcrt* and *pfmdr1* genes conferring high level of resistance to CQ and other antimalarials among the studied *P. falciparum* isolates.

1.5 SIGNIFICANCE OF THE STUDY

Due to the emergence of malaria parasites resistance to CQ as well as to other antimalarial drugs in Yemen, the Ministry of Health and Population, Yemen has endorsed the new malaria treatment policy in 2009, which involves the artesunate plus sulfadoxine-pyrimethamine (AS + SP) as the first line, and artemether plus lumefantrine (AL) as the second line treatment drug combination for treating uncomplicated falciparum malaria in the country (WHO, 2013). Concurrently, the HRP2-based RDT has been approved by the ministry to be used for malaria diagnosis by the National Malaria Control Programme (NMCP) and other health facilities, particularly in peripheral areas where the electricity as well as quality microscopy services are lacking (WHO, 2011c). Hence, the findings of the present study will provide important information about the performance of HRP2-based RDT in diagnosing malaria as well as therapeutic efficacy of the new drugs in treating uncomplicated falciparum malaria in Yemen. Such information is essential for the malaria control efforts in the country. Moreover, CQ and other antimalarial drugs are still available in Yemen, prescribed for falciparum malaria in some private health facilities, and is also used for self-administration (Bashrahil et al., 2010; Ghouth, 2013). Hence, the findings of the present study will provide important updates on the situation of antimalarial drug resistance in this continuing drug pressure. This would be useful in targeting interventions aimed at reducing inappropriate drug use in the country.

1.6 ORGANIZATION OF THIS THESIS

This thesis consists of six chapters and the format is "Article Style Format", according to the University of Malaya guidelines for the preparation of research reports dissertations and thesis (2015). According to this guideline, the main body of a research thesis in the "article style format" should contain the following chapters: "General Introduction", "Literature Review", "Article 1", "Article 2", "Article 3" (or more), and "Conclusion and Recommendation". Hence, the present thesis consists of the following chapters: -

Chapter 1 provides a general overview and the required justification for this study. The objectives of the study, problem statement, hypothesis as well as the significance of the study are stated in this chapter.

Chapter 2 provides extensive background information on previous studies and current knowledge pertaining to malaria. This chapter provides basic knowledge about the history, biology, epidemiology, diagnosis, treatment, prevention and control, and drug resistance situation and evaluation.

Chapters 3-5 comprise of published articles listed at the end of the thesis (list of a publications and presentations). These chapters are presented according to the objectives of the study. The format of these chapters is according to the university guidelines.

Chapter 3 describes the evaluation of PfHRP2-based RDT in diagnosing malaria as well as the genetic variation of the *pfhrp2* gene in Yemen and its impact on the performance of the RDT. This work was published in *Parasites & Vectors*.

C***hapter 4*** describes the *in vivo* therapeutic evaluation of the AS+SP in treating uncomplicated malaria among the study population in Yemen. This work was published in *Malaria Journal*.

Chapter 5 presents findings about the molecular analysis of the related mutations in the *pfcrt* and *pfmdr1* genes as molecular markers for the CQ and other antimalarial drugs among the study population in Yemen. This work was published in *PeerJ*.

Chapter 6 presents the general conclusion of this study and provides some recommendations as well as the limitations during candidature.

CHAPTER 2: LITERATURE REVIEW

2.1 MALARIA

2.1.1 Historical background

Malaria has occupied a great place in history throughout the millennia; victims have included Neolithic cave-dwellers, Chinese dynasties and ancient Greeks, and without any dispensation it has affected kings, princes and paupers. Ancient writings and artefacts attest to malaria's long reign; clay tablets from Mesopotamia from 2000 BC, papyri from 1570 BC during the era of the Pharaohs, Hindu texts from the sixth century BC and Chinese medical books, all have mentioned the periodic fever pattern which is specifically related to malaria infections. More recently, malaria has been detected in samples from the skin, lung and other remains of Egyptian mummies dating from 3200 and 1304 BC, and it has been suggested that *P. falciparum* parasite was most likely the cause of the death of the Egyptian Pharaoh, Tutankhamun (Miller et al., 1994a; Brier, 2004; Hawass et al., 2010; Nunn & Tapp, 2000).

In 270 BC, *Nei Chin*, the Chinese medical canon, which is considered the history book of Chinese internal medicine, linked malaria's tertian pattern (fever every 48 h) and quartan fever pattern (every 72 h) with the enlargement of the right side of the abdomen (splenomegaly), which are both common disease indications. *Nei Chin* also blamed the disease's symptoms on three demons: one carrying a hammer and responsible for headaches, another carrying a pail of water and causing chills and sweating and the third carrying a stove and responsible for fever (Bruce-Chwatt, 1988). Homer, an ancient Greek poet, mentioned malaria in *The Iliad* (750 BC), and later Hippocrates (450–370 BC) linked the appearance of the Dog Star Sirius in the summer and autumn with malarial fever and misery (Sherman, 1998).

It was later in eighteenth century in Italy that it acquired the name ‘malaria’, a combination of two words ‘mal’ & ‘aria’, which literally means ‘bad air’ that was particularly associated with the belief that the source of the disease was swampy, marshy areas which emitted foul-smelling air.

2.1.2 Malaria parasite

The causative agents of malaria remained obscure for a long time as the disease was thought to be restricted to low-lying humid plains near swamps. It was Charles Louis Alphonse Laveran (1845–1922), a French army doctor during the Franco-Prussian War, who noted the possibility of the occurrence of malaria in temperate zones too, and that it did not necessarily affect all tropical areas. Laveran was inspired by contemporary scientific articles that attributed many infectious diseases to a biological causative agent or ‘microbes’, which went against the prevailing belief that diseases were caused by miasmas, evil vapours or swampy air (Jarcho, 1984).

Later, Laveran was transferred to Algeria on the North African coast, and took with him his crude microscope. In 1888, he examined a blood specimen from a febrile soldier. Interestingly, he discovered crescent-like bodies that were almost transparent except for a small dot (malaria pigment). Laveran went on to examine a total of 192 febrile patients and reported the presence of these pigmented, crescent-shaped bodies in 148 of them (Laveran et al., 1982), thereby proving the existence of a protozoan parasite that he later named *Oscillaria malariae*. Laveran presented this finding at the French Academy of Medical Science in December 1880 (Cox, 2010). In addition, Laveran found that quinine had the capability to remove this type of body from the blood. Moreover, he reported four different forms of causative agent in human blood samples that were later proved to be different stages of the malaria parasite’s life cycle in humans. These stages are trophozoite, schizont, and male and female gametocytes.

Earlier, in 1890, the parasite names *Plasmodium vivax* and *P. malariae* were introduced by the Italian investigators Giovanni Batista Grassi and Raimondo Filetti. The name *Oscillaria malariae* of the parasite causing tertian malaria named by Laveran was no longer thought possible, and William Henry Welch, a US physician, renamed it in 1897 as *Plasmodium falciparum*. Later, in 1922, *P. ovale* was described by the British parasitologist John William Watson Stephens. A fifth species was described in detail by Robert Knowles and his assistant Biraj Mohan Das Gupta in 1932. Later in the same year, John Alexander Sinton and his colleague, Mulligan HW, were the first to call this parasite *P. knowlesi* in recognition of the efforts of Knowles (Sinton & Mulligan, 1933).

Camillo Golgi (1843–1926), the greatest neuroscientist and biologist of his era, linked the onset of the tertian fever due to *P. vivax* and the quartan fever caused by *P. malariae* infection with the rupture of blood schizonts and the release of merozoites. In 1906, Golgi was awarded the Nobel Prize for neuroscience, which was unrelated to his work on malaria. One year later, Laveran was awarded the Nobel Prize for his discovery of the unicellular protozoan parasite that causes malaria.

The role of the mosquito in transmitting malaria was recognized for the first time by Ronald Ross (1857–1932), a surgeon-major of the British Indian Medical Service. Ross spent more than one year studying the mosquito without obtaining any definitive results. Then, in 1897, on 20 August, he identified a clear, round body in a dotted-winged Anopheles mosquito that had previously been fed on malaria-infected blood. The body was clear but contained some pigments. Ross called that day ‘Mosquito Day’. The following day, he examined another Anopheles mosquito that had fed on the same day and from the same blood. On dissecting the mosquito, he discovered larger bodies with more malaria pigments inside them, which suggested the presence of a parasitic growth inside the mosquito. He published his observations in the

British Medical Journal in December 1897, in a paper entitled, 'On some peculiar *pigmented* cells found in two mosquitoes fed on malarial blood'.

Ross continued his work by focusing on *P. relictum* parasites that mainly infect sparrows and crows. He fed the mosquitoes on the blood of these infected birds and discovered sporozoites in the salivary gland of the infected mosquitoes (Sherman, 1998). His findings were shared by his teacher and mentor Sir Patrick Manson at a meeting of the British Medical Association at the University of Edinburgh in July 1898 (Harrison, 1978). Ross was awarded the Nobel Prize in 1902 for identifying the stages of malaria parasites in mosquitoes.

The story of the malaria parasite is one of a great history of 114 years of experimental research, scientific work and analysis that started in 1888 with the discovery of a crescent-shaped pigmented body in the blood of a febrile soldier in Algeria by a French army doctor using a crude microscope and since then the discovery of DNA has progressed, thus far, to the publication of the first *P. falciparum* malaria genome sequence in 2002 (Gardner et al., 2002).

2.1.3 Malaria vector

There are around 3,500 types of mosquito grouped into 41 genera. Only the female mosquito of the genus Anopheles contributes to the transmission of malaria. Out of approximately 400 recognized Anopheles mosquito species, only 40 have a natural capability of transmitting the malaria infection worldwide. The distribution of this malaria vector greatly depends on regional topography and environmental conditions (Macdonald, 1957; Balls et al., 2004; Minakawa et al., 2006).

In Yemen, 15 Anopheles species have been reported with *An. arabiensis* being predominant in all areas of Yemen and representing more than 90% of the malaria vector (NMCP, 2014; Al-Eryani et al., 2016). Moreover, it has been found that *An.*

sargentii is the second most common vector species after *An. arabiensis* in the country. In addition, *An. culicifacies* was reported as the principal vector in Socotra Island and in the coastal areas along the Arabian Sea, especially in Al-Mahra governorate in the eastern part of Yemen. Both *An. sargentii* and *An. fluviatilis* were reported in areas of higher altitude (500–1500 metres). Recently, *An. algeriensis* was reported for the first time in Yemen and was found to represent 3% of the malaria vector population in the southwest governorate, Taiz (Al-Eryani et al., 2016). Generally, the NMCP of Yemen classifies *An. arabiensis* and *An. culicifacies* as malaria vectors in areas with an altitude below 600 metres, while *An. arabiensis* and *An. sargentii* are designated as vectors at higher altitudes (NMCP, 2014). Data from two sentinel sites for the number of adult Anopheles mosquitoes in Yemen is provided in Table 2.1.

The susceptibility of Anopheles mosquitoes to insecticides has been monitored by the NMCP for some years; seven sentinel sites were established in 2009 followed by five additional sites in 2010, bringing the total number to 12 sentinel sites covering Socotra Island and other malaria-endemic areas within 17 districts in 10 governorates (NMCP, 2014). An investigation of the susceptibility of *An. arabiensis* to the organophosphate temephos was carried out in 2009 in all seven sentinel sites, the results of which showed that the species was 100% susceptible to this insecticide. In addition, the susceptibility of the species to other insecticides was investigated by the NMCP in 2010, and it was found that the species had very low levels of susceptibility to the concentration of 0.05% of lambda-cyhalothrin, 0.05% deltamethrin, 4% DDT, 0.75% permethrin and 0.1% of bendiocarb. The recent studies have reported that in the Wadi Sukhmal sentinel site in the western central governorate, Thamar, the resistance of *An. arabiensis* mosquitoes to lambda-cyhalothrin stands at 82.0% and for deltamethrin it is 90.5% and for DDT it is 92.0%; however, it is absolutely susceptible (100%) to bendiocarb (NMCP, 2014). Moreover, the *An. arabiensis* vectors in the Zabid sentinel

site in Hodeidah governorate in the west have been found to be resistant to lambda-cyhalothrin (48.6%) and deltamethrin (86%) (NMCP, 2014).

Table 2.1: Adult female Anopheles vector in Yemen: collected during different months in 2012 from two sentinel sites *
(Methods of Collection: PKD[a], LT[b], ET[c] and Clay pot)

Anopheles species	April		June		September		Total	
	N	%	N	%	n	%	N	%
Anopheles arabiensis	137	62.8	185	77.1	748	85.6	1,070	80.3
Anopheles sergentii	68	31.2	27	11.3	118	13.5	213	16.0
Anopheles azaniae	1	0.5	18	7.5	5	0.6	24	1.8
Anopheles dthali	3	1.4	4	1.7	0	0	7	0.5
Anopheles pretoriensis	2	0.9	1	0.4	1	0.1	4	0.3
Anopheles rhodesiensi	0	0	1	0.4	0	0	1	0.1
Anopheles cinereus	0	0	0	0	1	0.1	1	0.1
Unidentified Anopheles	7	3.2	4	1.7	1	0.1	12	0.9
Total	**218**	**16.4**	**240**	**18.0**	**874**	**65.6**	**1,332**	**100**

*Pre-intervention data from Wadi Sukhmal and Wusab As-Safel sentinel sites, Republic of Yemen.

[a] PKD: pyrethrum knockdown
[b] LT : light trap
[c] ET : electric trap

2.1.4 Life cycle of Plasmodia

2.1.4.1 Overview

The malaria parasite is a unicellular protozoan belonging to the phylum Apicomlexa, the family Plasmodiidae and the genus Plasmodium. Around 250 species known can cause malaria to many different vertebral hosts including primates, rodents, bats, birds, lizards, carnivores, insectivores, and marsupials as well as humans. Generally, only four species have the capability of causing malaria in humans. These are *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. However, *P. knowlesi* has been reported to be the fifth species that can infect human beings, and is transmitted to humans from long-tail and pig-tail macaques in Southeast Asia (Jongwutiwes et al., 2004; Singh et al., 2004). This simian malaria parasite was previously often misdiagnosed as a malariae quartan malaria as it is morphologically similar to *P. malariae* (Cox-Singh et al., 2008; Jongwutiwes et al., 2004; Kantele & Jokiranta, 2011; Singh et al., 2004). Actually, *P. knowlesi* was identified earlier on as a zoonotic simian malaria in humans in the 1960s when it was isolated from a febrile white man visiting Peninsular Malaysia (Chin et al., 1965). However, *P. knowlesi* may not be the last to appear on the list as a sixth species of malaria parasite, *P. cynomolgi*, was reported for the first time in 2014 in a malaria-free area in Malaysia; it was isolated from a woman without any history of malaria or even a history of travelling to a malaria-endemic area (Ta et al., 2014). Although *P. cynomolgi* was found to be morphologically very similar to *P. vivax* and was misdiagnosed as vivax malaria, the reported *P. cynomolgi* case was interestingly microscopically misdiagnosed first as *P. malariae/P. knowlesi* infection. A polymerase chain reaction (PCR) and sequencing assay is the only method to confirm the diagnosis of *P. cynomolgi* infection (Ta et al., 2014). Based on genome sequencing analysis, both *P. knowlesi* and *P. cynomolgi* were both found to be very similar to *P. vivax*, sharing

many phenotypic, biological and genetic characteristics (Imwong et al., 2009; Tachibana et al., 2012).

2.1.4.2 Parasite's life cycle

The classical main mode of malaria transmission is through the bites of infected Anopheles mosquito (vector) that has previously fed on malaria patient's infected blood. However, other routes of transmission less commonly occur include infection from an infected blood transfusion, tissue transplantation and from an infected mother to her fetus during pregnancy (congenital malaria).

The life cycle for all human-infecting malaria species is essentially the same in which the parasite undergoes a series of developmental stages comprising an exogenous sexual development inside certain species of Anopheles mosquitoes (sporogony) and an endogenous asexual development (schizogony) in both liver and red blood cells of human host [Figure 2.1].

For the classical mode of transmission, the infection starts when an infected female Anopheles mosquito bites a healthy man and injects sporozoites with its saliva through his skin; usually, 20-30 sporozoites are injected by a single bite which are enough to initiate the infection (Satoskar, 2009). Within 30 minutes, all sporozoites reach the parenchymal hepatocytes of the liver where they mature into liver schizonts (exoerythrocytic schizogony cycle). Liver schizont contains thousands of merozoites (2,000 to 40,000) based parasite species. The liver schizogony takes 5-7 days in which the patient is completely asymptomatic. As the mature liver schizonts rupture, merozoites are released into the blood stream and rapidly invade red blood cells (erythrocytes). In the erythrocytes, the merozoites undergo several repeated asexual developmental processes (erythrocytic schizogony cycle) forming erythrocytic schizont containing a different number of merozoites (based on parasite species). The number of

merozoites in the mature schizont is maximum in *P. falciparum* (up to 36) and *P. vivax* (24 merozoites), while it is the least in *P. malariae* (6-8 merozoites). Once the erythrocytic schizont ruptures, merozoites are released and continuously infecting other red blood cells. The duration of this cycle is different from one species to another; it takes 48 hours in *P. vivax* and *P. ovale* (tertian malaria), 72 hours in *P. malariae* (quartan malaria), tertian and modified sub-tertian (36 hours) in *P. falciparum* and as short as 24 hours in *P. knowlesi* or what is called quotidian malaria (Satoskar, 2009). In *P. vivax* and *P. ovalae*, some of the sporozoites are kept dormant in the liver called hypnozoites which later reactivate in 1-2 years cause malaria relapse (Satoskar, 2009). After a few erythrocytic generations, some of the released merozoites differentiated into male and female gametocytes and wait in the patient's blood for its specific Anopheles mosquito vector.

When a female Anopheles mosquito bites malaria patient, it takes the gametocytes along with blood meal. Within the mosquito's gut, male and female gametocytes undergo a series of developmental stages to form sporozoites (sporogony cycle). Male and female gametocytes mature into microgamete and macrogamete, respectively. After fertilisation, a zygote is formed and developed into a mobile ookinete stage which is impeded into the basement membrane of the mosquito midgut and developed into an oocyst. The oocyst undergoes multiple multiplications process resulting in a formation of thousands of spindle-shaped sporozoites, the infective stage of humans. As oocyst bursts, sporozoites are liberated filling the abdominal cavity, migrate and invade the salivary glands from which they can be injected into the vertebral hosts. This process takes 10-18 days depending on *Plasmodium* species and on the ambient temperature (Paaijmans et al., 2009). The mosquito remains infectious for 1-2 months (Liljander, 2010).

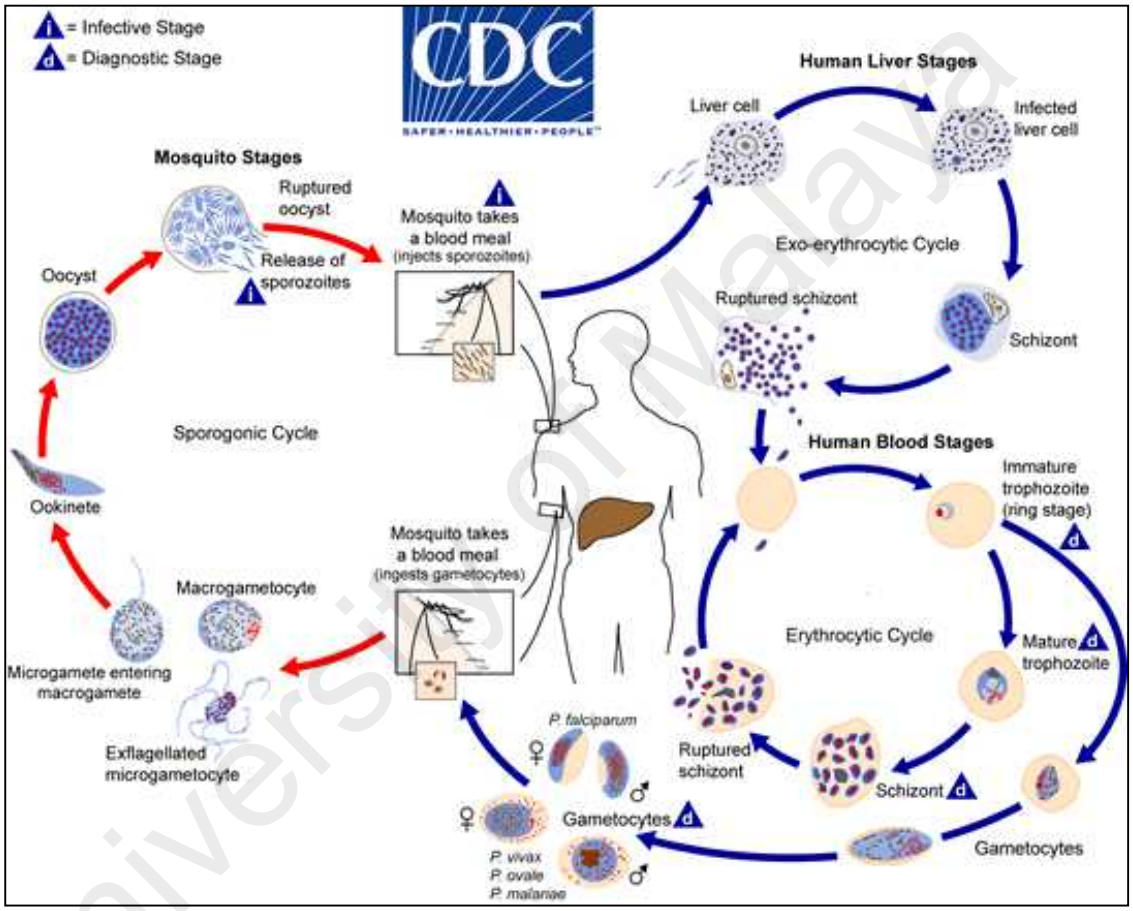


Figure 2.1: Life cycle of *Plasmodium* species
Source: Modified, Centre of Disease and Control (CDC)
http://www.cdc.gov/malaria/about/biology/index.html

2.1.5 Signs and symptoms of malaria

As the erythrocytic schizonts rupture, malaria antigens, pigments and malaria toxins are released and trigger a series of pathological effects including the production of cytokines, and particularly a tumour necrosis factor (TNFd) that is complemented by the action of other circulating endogenous pyrogens such as interleukins (IL-1 and IL-6). As a result, headache, fever, rigor and vomiting appear and the symptomatic phase of infection (malaria paroxysm) begins (Miller et al., 1994b; Pasvol et al., 1995). The clinical manifestations of malaria vary from mild symptoms to severe multi-organ dysfunction and life-threatening conditions including cerebral malaria (encephalopathy), pulmonary oedema and acute pulmonary distress, renal insufficiency and acute renal failure, severe anaemia and metabolic acidosis (Satoskar, 2009). However, asymptomatic infection is common in endemic areas in Africa and Asia (Dal-Bianco et al., 2007; Starzengruber et al., 2014; Vafa et al., 2008).

2.1.6 Diagnosis of malaria

The effective management of any disease basically depends on accurate diagnosis. Malaria microscopy has been the mainstay and the gold standard method for malaria diagnosis. Identification of the asexual and sexual parasite stages in a Giemsa-stained blood film has been the only way of diagnosing malaria infections for many decades. However, for malaria microscopy to be effective there need to be an adequate amount of suitable microscopes, a regular supply of materials including Giemsa stain and buffer and enough well-trained personnels, requirements which may not be feasible to meet in some malaria-endemic sites. In areas such as the rural areas of Yemen, a clinical indication of malaria was found to be extremely helpful for physicians in diagnosing the disease. However, a clinical indication has little specificity as malaria signs and

symptoms are nonspecific except for the synchronised pattern of fever. Thus, a clinical diagnosis of malaria may result in a hyperestimation of malaria cases. However, clinical manifestations are very important in cases of severe malaria.

The RDT was later introduced to aid malaria diagnosis, and it shortly entered into widespread use as an alternative indispensable tool for malaria case management, control and elimination worldwide, especially in areas that lacked well-trained microscopy personnel. Since 2010, WHO has recommended that all persons with suspected malaria undergo an examination by either malaria microscopy or malaria RDT before administration of any treatment (WHO, 2014a). In the WHO African region, the effectiveness of malaria diagnosis greatly increased from 36% of suspected cases of malaria in 2005 to 41% by 2010. In fact, this increase in the proportion of malaria diagnoses in the WHO African region was due to the increase in RDT usage and it continued to increase from 62% and 71% of suspected malaria cases in 2013 and 2014, respectively (WHO, 2014a, 2015a).

Other techniques have been proposed for use in the diagnosis of a malaria infection, including those that utilize an antigen or antibody, such as the enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IF). More recently, PCR has been introduced as an additional and advanced method of diagnosis. It utilizes the genomic DNA of malaria and represents a substantial leap forward in malaria diagnosis, but its use has so far been limited mainly to epidemiology and research studies.

2.1.6.1 Rapid diagnostic test (RDT)

The main technical component of a global malaria control strategy is based largely on the early diagnosis and prompt treatment of malaria cases in malaria-endemic areas. This strategy is highly dependent on two main points: the precision of the diagnostic

techniques used to confirm malaria cases and the relevance of antimalarial drugs including their efficacy, safety, availability, affordability and the accessibility.

Although malaria microscopy is the gold standard in malaria diagnosis, it seems that it is not feasible in peripheral areas that have limited access to high-quality malaria microscopy services, mostly due to a lack of well-qualified malaria microscopy personnel. The RDT is therefore a powerful alternative tool for quickly establishing a diagnosis of malaria infection in such areas.

An RDT for diagnosing malaria aims, as the name implies, to rapidly detect malaria. It does this by detecting specific antigens in the person's blood. The advantages of using an RDT is that it can provide a correct diagnosis in places where malaria microscopy is not feasible, and it does so in a very short time compared to microscopy. However, an RDT for malaria does not have the ability to differentiate all the human malaria species. Moreover, it has a limited capability of detecting low-density malaria parasitaemia. Thus, the RDT cannot eliminate the need for malaria microscopy as the latter is very important because it is used to distinguish the causative species. Microscopy is also used to quantify the parasite count and to calculate the number of parasitized RBCs, which are both very important prognostic indicators in malaria case management.

Commercially, there are three types of RDT available in the market, which are based on the type of malaria antigen they test for:

1. Histidine-rich protein 2 (HRP-2) is an abundant soluble, heat-stable protein that is produced by both the sexual and asexual stages of only the *P. falciparum* parasite. It is expressed on the cytoplasmic membrane of infected RBCs and it has been shown to remain in a patient's blood many days after initiation of treatment.
2. Lactate dehydrogenase (pLDH) is a soluble enzyme in the parasite glycolytic pathway that is produced by the sexual and asexual stages of the live parasites and

is released and contained inside the infected erythrocytes. Although LDH has been found in all of the main four human malaria species, different isomers of LDH for each of the four species exist. It is currently available mostly as a *P. falciparum*-specific, *P. vivax*-specific or pan-Plasmodium-based RDT (pLDH).

3. Aldolase is another enzyme of the parasite glycolytic pathway expressed by the blood stages of *P. falciparum* as well as other non-falciparum malaria parasites. It has been found that monoclonal antibodies against Plasmodium aldolase are pan-specific in their reaction (p-aldolase) and they have been used for detecting all four species of the malaria parasite targeting the pan-malarial antigen (PMA), and in a combination with Pfhrp2 to differentiate *Plasmodium falciparum* from others species in p-aldolase/Pfhrp2 immunochromatographic test.

2.1.7. Treatment of malaria

The clinical form of malaria varies depending on the species and the severe form of malaria is mainly due to *P. falciparum*. Due to low immunity, young children and pregnant women as well as travellers from non-endemic areas are more likely to contract the severe form of malaria. Thus, case management and the parasite elimination from the blood consequently require more effort to maintain the physiological functions of such patients and their need for tertiary healthcare services is greater. Similarly, malaria in areas with high and stable malaria transmission such as in Africa and Papua New Guinea is almost completely due to *P. falciparum*, and therefore the population in those areas is at high risk of getting severe malaria, and this is especially the case for high-risk groups such as young children and pregnant women. In these areas, patients must be given a high level of clinical care at a community level and there should be an optimal referring capability to tertiary healthcare services when required. In contrast, in

areas where vivax malaria is common, case management is quite simple, but more importantly, species differentiation is strongly required.

Uncomplicated malaria requires only the use of a schizonticidal antimalarial drug to clear parasitaemia from the patient's blood. However, the emergence of resistant strains of malaria parasites, mainly *P. falciparum*, makes the treatment and the selection of the drugs more complicated. Currently, combination therapy with one of the artemisinin derivatives is the cornerstone treatment strategy for treating uncomplicated falciparum malaria worldwide. Special attention needs to be given to selecting the correct drug(s) for treating pregnant women especially during the first trimester of pregnancy. On the other hand, CQ is still the drug of choice for treating malaria infections caused by *P. malariae*, *P. ovale* and the sensitive strains of *P. vivax*. Note that primaquine is added as a radical treatment drug to clear the tissue stage in the liver (hypnozoites) in the case of vivax and ovale malaria.

Complicated or severe malaria is always accompanied by one or more clinical manifestations such as prostration, impaired consciousness, multiple convulsions, circulatory collapse, bleeding, pulmonary oedema, jaundice and haemoglobinuria. However, laboratory investigations have often revealed hyperparasitaemia alongside a range of pathological changes such as severe anaemia, marked hypoglycaemia, acidosis, hyperlactataemia and renal insufficiency. Thus, the case management of severe malaria should include the provision of supportive treatment in tandem with the anti-parasitological drug(s). The treatment for severe malaria commonly starts with the administration of parenteral quinine or artesunate. In addition, it is essential to monitor the patient's vital signs and give other adjunctive treatment where required, such as antipyretic drugs for fever, intravenous or rectal diazepam or intramuscular paraldehyde for convulsions, correcting hypoglycaemia, and giving fresh blood transfusion in case of severe anaemia {haemoglobin < 5 g/dl or packed cell volume (PCV) < 15%}. It may

also be necessary to monitor renal function and administer diuretics and perform haemodialysis, as well as give oxygen and maintain the airway of a patient. Details of the malaria treatment policies of various countries within the WHO region are provided in Table 2.2.

2.1.7.1 Malaria treatment in Yemen

Chloroquine has been the drug of choice in Yemen for treating uncomplicated malaria, including falciparum malaria infection, for the last few decades. However, the emergence of CQ-resistant *P. falciparum* in Yemen prompted the adoption of ACT in 2009. The government introduced a new policy that provides for the use of AS+SP and AL as a first-line and second-line treatment, respectively, for uncomplicated falciparum malaria infection. Under the new policy, severe malaria is treated with quinine and artemether, while CQ has been retained as the drug of choice for treating vivax malaria followed by primaquine (for 14 days) as a radical tissue-stage treatment (WHO, 2015b).

Table 2.2: Antimalarial treatment policy in the countries within the WHO-regions, (WHO, 2014a)

WHO region /Country	Falciparum malaria treatment		Vivax malaria treatment
	Uncomplicated	Severe	
African			
Algeria	-	-	CQ
Angola	AL	AS; QN	-
Benin	AL	AS; QN	-
Botswana	AL	QN	-
Burkina Faso	AL; AS+AQ	AS; QN	-
Burundi	AS+AQ	AS; QN	-
Cabo Verde	AL	QN	-
Cameroon	AS+AQ	AS	-
Central African Republic	AL	AS	-
Chad	AL; AS+AQ	AS	-
Comoros	AL	QN	-
Congo	AS+AQ	QN	-
Côte d'Ivoire	AS+AQ	QN	-
Democratic Republic of the Congo	AS+AQ	AS	-
Equatorial Guinea	AS+AQ	AS	-
Eritrea	AS+AQ	QN	AS+AQ+PQ
Ethiopia	AL	AS; AM; QN	CQ
Gabon	AS+AQ	AS; AM; QN	-
Gambia	AL	QN	-
Ghana	AL; AS+AQ	AS; AM; QN	-
Guinea	AS+AQ	AS	-
Guinea-Bissau	AL	AS; QN	-
Kenya	AL	AS; AM; QN	-
Liberia	AS+AQ	AS; AM; QN	-
Madagascar	AS+AQ	QN	-
Malawi	AL	AS; QN	-

Table 2.2, continued

Mali	AL; AS+AQ	QN	-
Mauritania	AL; AS+AQ	QN	-
Mayotte, France	AL	QN; AS; QN+AS; AS+D; QN+D	CQ+PQ
Mozambique	AL	AS	-
Namibia	AL	QN	AL
Niger	AL	AS; QN	-
Nigeria	AL; AS+AQ	AS; AM; QN	-
Rwanda	AL	AS; QN	-
Sao Tome and Principe	AS+AQ	QN	-
Senegal	AL; AS+AQ	AS; QN	-
Sierra Leone	AL; AS+AQ	AS; AM; QN	-
South Africa	AL; QN+CL; QN+D	QN	AL+PQ; CQ+PQ
South Sudan*	AS+AQ	AM; AS; QN	AS+AQ+PQ
Swaziland	AL	AS	-
Togo	AL; AS+AQ	AS; AM; QN	-
Uganda	AL	AS	-
United Republic of Tanzania	AL; AS+AQ	AS	-
Mainland	AL	AS	-
Zanzibar	AS+AQ	AS; QN	-
Zambia	AL	AS; AM; QN	-
Zimbabwe	AL	QN	-
Eastern Mediterranean			
Afghanistan	AS+SP+PQ	AM; AS; QN	CQ+PQ (8w)
Djibouti	AL+PQ	QN	CQ+PQ (14 d)
Iran (Islamic Republic of)	AS+SP; AS+SP+PQ	AS; QN+D	CQ+PQ (14d & 8w)
Pakistan	AS+SP+PQ	AS; QN	CQ+PQ (14d)
Saudi Arabia	AS+SP+PQ	AS; AM; QN	CQ+PQ (14d)
Somalia	AS+SP	AS; QN	-
Sudan	AS+SP	AM; QN	AL+PQ (14d)
Yemen	AS+SP	AM; QN	CQ+PQ (14d)

Table 2.2, continued

European			
Azerbaijan	AS+SP	AS; QN	CQ+PQ (14d)
Kyrgyzstan	-	-	-
Tajikistan	-	AL	-
Turkey	-	-	-
Uzbekistan	-	-	-
Region of the Americas			
Argentina	AL+PQ	-	CQ+PQ
Belize	CQ+PQ (1d)	AL; QN	CQ+PQ (14d)
Bolivia (Plurinational State of)	AS+MQ+PQ	QN	CQ+PQ (7d)
Brazil	AL+PQ (1d); AS+MQ+PQ (1d)	AM+CL; AS+CL; QN+CL	CQ+PQ (7d)
Colombia	AL	AS+AL	CQ+PQ (14d)
Costa Rica	CQ+PQ (1d)	QN	CQ+PQ(7d); CQ+PQ (14d)
Dominican Republic	CQ+PQ (1d)	CQ; QN	CQ+PQ (14d)
Ecuador	AL+PQ	QN	CQ+PQ (14d)
El Salvador	CQ+PQ (1d)	QN	CQ+PQ (14d)
French Guiana, France	AL	AS; AL	CQ+PQ
Guatemala	CQ+PQ (3d)	QN	CQ+PQ (14d)
Guyana	AL+PQ (1d)	AM	CQ+PQ (14d)
Haiti	CQ+PQ (1d)	QN	CQ+PQ (14d)
Honduras	CQ+PQ (1d)	QN	CQ+PQ (14d)
Mexico	CQ+PQ	AL	CQ+PQ
Nicaragua	CQ+PQ (1d)	QN	CQ+PQ (7d)
Panama	AL+PQ (1d)	QN	CQ+PQ(7d); CQ+PQ (14d)
Paraguay	AL+PQ	AS	CQ+PQ
Peru	AS+MQ	AS; MQ	CQ+PQ
Suriname	AL+PQ	AS	CQ+PQ (14d)
Venezuela (Bolivarian Republic)	AS+MQ+PQ	AM; QN	CQ+PQ (14d)
South-East Asia			
Bangladesh	AL	AM; QN	CQ+PQ (14d)

Table 2.2, continued

Bhutan	AL	AM; QN	CQ+PQ (14d)
Democratic People's Republic of Korea	-	-	CQ+PQ (14d)
India	AS+SP+PQ	AM; AS; QN	CQ+PQ (14d)
Indonesia	AS+AQ; DHA-PP+PQ	AM; AS; QN	AS+AQ; DHA-PP+PQ(14d)
Myanmar	AL; AM; AS+MQ; DHA-PPQ; PQ	AM; AS; QN	CQ+PQ (14d)
Nepal	AL+PQ	AS; QN	CQ+PQ (14d)
Sri Lanka	AL+PQ	AS	CQ+PQ (14d)
Thailand	AS+MQ	QN+D	CQ+PQ (14d)
Timor-Leste	AL	AM; AS; QN	CQ+PQ (14d)
Western Pacific			
Cambodia	AS+MQ; DHA-PPQ+PQ	AM; AS; QN	DHA-PPQ
China	ART+NQ; ART-PPQ; AS+AQ; DHA-PPQ	AM; AS; PYR	CQ+PQ(8d)
Lao People's Democratic Republic	AL	AS+AL	CQ+PQ (14d)
Malaysia	AS+MQ	QN+T	CQ+PQ (14d)
Papua New Guinea	AL	AM; AS	AL+PQ
Philippines	AL+PQ	QN+T; QN+D; QN+CL	CQ+PQ (14d)
Republic of Korea	-	-	CQ+PQ (14d)
Solomon Islands	AL	AL; AS	AL+PQ(14d)
Vanuatu	AL	AS	AL+PQ(14d)
Viet Nam	DHA-PPQ	AS; QN	CQ+PQ (14d)

AL = Artemether-lumefantrine
AM = Artemether
AQ = Amodiaquine
ART = Artemisinin
AS = Artesunate
AT = Atovaquone
CL = Clindamycin
CQ = Chloroquine
D = Doxycycline
DHA = Dihydroartemisinin
MQ = Mefloquine
NQ = Naphroquine
PG = Proguanil
PPQ = Piperaquine
PQ = Primaquine
PYR = Pyronaridine
QN = Quinine
SP = Sulfadoxine-pyrimethamine
T = Tetracycline
D = day
W = week

* In May 2013 South Sudan was reassigned to the WHO African Region.

2.1.8 Epidemiology of Malaria

The transmission of malaria is confined to geographical areas where the Anopheles mosquito can flourish, and this depends greatly on the presence of a favourable climate, especially with respect to temperature and humidity, in tropical and sub-tropical regions. Malaria can be either endemic or epidemic. Endemic transmission is characterized by firm transmission over a long period of time while epidemic transmission occurs when a sharp increase in the frequency of malaria transmission that exceeds the normal inter-seasonal variation is reported (Kiszewski & Teklehaimanot, 2004). Moreover, transmission can be stable and continuous, i.e., it remains constant over many years with or without seasonal variability, or it can be unstable with notable fluctuations (Kiszewski & Teklehaimanot, 2004).

2.1.8.1 Classification of malaria endemicity

Malaria endemicity was first classified during a malaria conference in Kampala in 1950; and was based on spleen rate (degree of splenomegaly). The definition was later revised to take into account the parasite rate (Metselaar & Van Thiel, 1959). Both methods are applied to a specific age group, usually children aged 2–9 years old to classify malarious regions into four categories. An area is defined as hypoendemic if ≤ 10% of children have splenomegaly or if the malaria parasite is found in ≤ 10% of children of the same age group. A mesoendemic area such as Mali is characterized by 11–50% of children having splenomegaly. A hyperendemic area such as Liberia and Uganda is an area in which the spleen rate (splenomegaly) or parasite rate (presence of malaria parasite) is found constantly in more than 50% of the children. Lastly, a holoendemic area such as Nigeria, Senegal and Burkina Faso is an area with a constant more than 75% spleen rate or parasite rate in a specific age group, or more than 75% of parasite rate in infants aged 5–11 months (Kiszewski & Teklehaimanot, 2004; RBM, 2002).

2.1.8.2 Risk factors of malaria

The risk factors for malaria transmission have been extensively studied. These factors are either biological and are mainly related to humans, vectors or parasites, or they are external factors that are related to the environment. The human-related factors include age, housing conditions, occupation, human behavioural factors and malaria awareness among the general population. Generally, clinical malaria is more prevalent in children under 5 years of age than in adults; lack of immunity makes children in this age group more predisposed to the infection (Lusingu et al., 2004; Reyburn et al., 2005, Owusu-Agyei et al., 2002). Moreover, pregnant women are more likely to contract the disease than other adults, and interestingly, are more attractive to the *An. arabiensis* and *An. gambiae* mosquito vectors (Himeidan et al., 2004; Ansell et al., 2002). The reason for the increased chance of a predisposition to malaria during pregnancy is the increase in body heat and the amount of volatile substances released from the skin of pregnant women which attract mosquitoes (Lindsay et al., 2000) and the alteration in the immune response (Okoko et al., 2003). The type of housing is also an important risk factor; houses made of bamboo in Southeast Asia and houses made of mud brick in Yemen were found to be significantly associated with an increase in the incidence of malaria (Arasu, 1992; Butraporn et al., 1986; Oemijati, 1992; Bamaga et al., 2014). Moreover, if a house is located near water collections, such as a pond, river, stream or water dams, this significantly increases the size of the Anopheles mosquito population as these water bodies provide optimal mosquito breeding sites, which consequently increases the incidence of malaria (Trape et al., 1992; Ghebreyesus et al., 1999).

A person's occupation is another key risk factor; gem-mining, ore-digging, logging, agricultural and harvesting activities in Southeast Asia and fishing and farming in Yemen were found to increase the likelihood of acquiring malaria due to prolonged presence in a forest, farm or near water collections whereby workers are consequently

more exposed to vector bites (Sornmani et al., 1983; Butraporn et al., 1986; Luxemburger et al., 1997; Meek, 1988; Verdrager, 1995; Bamaga et al., 2014).

On the other hand, increasing knowledge of malaria aetiology, mode of transmission and methods of malaria prevention was found to be of high importance in reducing malaria burden. Some protective measures were found to play a very important role in reducing malaria infection as they decreased human–vector contact. These measures include the use of a mosquito bed-net while sleeping and the use of mosquito repellents or insecticides in addition to the screening of house doors and windows. In addition, use of prophylactic drugs by travellers during their time in high malaria-endemic areas was also found to be very useful (Arasu, 1992; Fungladda et al., 1987; WHO, 2015b). In the same context, delay in the prompt treatment of a malaria infection, mainly the falciparum strain, was reported to be a risk factor that predisposes patients to complicated or severe malaria. Notably, this delay is either due to difficulty in accessing healthcare facilities or to the tendency of the population to treat themselves traditionally (Arasu, 1992; Fungladda & Sornmani, 1986; Oemijati, 1992).

Population movement, especially to areas with less malaria endemicity, was shown to be related to an increase in the prevalence of malaria (Singhanetra-Renard, 1986; 1993). Population displacement due to war or natural disasters also affects prevalence; the movement of one million refugees of the Nagorno-Karabakh civil war in Southeastern Azerbaijan resulted in a malaria outbreak that produced around 3,000 autochthonous cases in 1996, and almost 30,000 cases were detected in Tajikistan the following year (Sabatinelli et al., 2000). Moreover, a malaria epidemic of around 62,000 cases was reported in central Europe after the breakup of the Soviet Union (Sabatinelli et al., 2000). On the other hand, travelling from malaria-free or non-endemic areas to visit friends or for business can also result in severe and fatal malaria; reports from the

UK have revealed that around 2,000 imported cases result in 5 to 16 deaths annually (Swales et al., 2007; Pinsent et al., 2014).

The characteristic features of the Anopheles mosquito differ from one vector to another. Malaria vectors in Asia have been described as zoophilic, i.e., the mosquitoes prefer to feed on animal blood. The presence of animal barriers such as cattle, sheep, goats or buffalo was found useful in decreasing human exposure to mosquito bites through mechanical competition (Bruce-Chwatt & Gockel, 1960; Yamamoto et al., 2009; Do Manh et al., 2010). In contrast, presence of pigs was found to be significantly associated with an increase in the mosquito population (Pålsson et al., 2004). In contrast, in Yemen, the vectors are predominantly anthropophilic (Al-Eryani et al., 2016). Another difference between vectors is the preference of the mosquito to rest indoors or outdoors. Vectors in Yemen are described as having a strong endophilic tendency (i.e. they prefer to rest indoors), a trait that resembles the African Anopheles mosquito vector which is of great interest in using indoor residual spraying (IRS) in malaria control (Al-Eryani et al., 2016).

External environmental factors also play a major role in the parasite's life cycle inside the vector as well as the aquatic stage of its life cycle (Craig et al., 1999). The developmental process (sporogony) is highly dependent on the ambient temperature. The time needed from infection of mosquito by gametocytes to the release of sporozoites is 9–11 days at 28^{o}C, but a temperature below 16^{o}C stops this development (Macdonald 1957, Bradley et al., 1987). Also, the vector feeding interval (gonotrophic cycle) is shorter under a high temperature as a high temperature increases the blood meal digestion rate which consequently results in more frequent vector bites. In addition, rainfall was found to be the main cause of fluctuation in malaria transmission in an area from the west coast of Africa to Sudan (Kiszewski & Teklehaimanot, 2004).

Similarly, a malaria outbreak in Colombia, which produced 3,822 malaria cases in 1999, occurred after 2 months of torrential rainfall (Gonzalez, 2000).

2.1.9 Prevention and Control

Many measures have been implemented to fight malaria at both the global and national levels, targeting both infected people and mosquitoes. Although the cornerstone of a malaria control programme is the early detection and prompt treatment of malaria cases, other protective parameters have been shown to be of great importance. These include indoor insecticide residual spraying, use of mosquito insecticide treated bed nets, chemoprophylaxis and preventive treatment during pregnancy.

2.1.9.1 Indoor residual spraying (IRS)

The Anopheles mosquito genus consists of various species that exhibit particular behaviours as regards their biting, resting and transmission activities. Indoor malaria vectors tend to bite people and rest inside human dwellings, especially those that are traditionally made of mud bricks or bamboo. Indoor residual spraying (IRS) was designed to target indoor mosquitoes by spraying insecticide onto the inner walls of dwellings. This intervention aims to reduce the lifespan of female mosquitoes as this stops them surviving long enough to transmit malaria parasites and to reduce human–vector contact. Some insecticides have been found to be effective against Anopheles mosquitoes, with DDT, lambda-cyhalothrin, deltamethrin, permethrin and bendiocarb being the most suitable and highly recommended insecticides by the WHO (WHO, 2010a). Protection coverage with IRS has increased throughout the years. Approximately 75 million people were probably protected by IRS in sub-Saharan Africa in 2009 compared to 13 million in 2005 (WHO, 2010a). In 2014, almost 116 million people were globally protected by IRS, of whom 50 million were in Africa (WHO,

2015a). However, it is also important to mention that Anopheles vector mosquitoes have developed resistance to some of the insecticides used in IRS.

2.1.9.2 Insecticide-treated bed nets (ITNs)

Mosquito bed nets help to protect people from mosquito bites, and this consequently decreases the malaria transmission rate. The mechanical function of mosquito bed nets has been improved to include chemical action by treating the nets with insecticide that selectively kills the mosquitoes. Pyrethroids constitute the only class of insecticides used in long-lasting insecticide-treated bed nets (ITNs). Although mosquitoes have developed resistance to the insecticides used in IRS, there has been no reported failure in the use of long-lasting insecticide-treated bed nets (LLITNs). In sub-Saharan Africa, use of ITNs has resulted in a reduction in the incidence of malaria and a decrease in malaria mortality by more than 50% (WHO, 2015a). The use of ITNs in sub-Saharan Africa, defined as the sleeping of an individual under a mosquito net, has increased gradually to 46% in 2014 and 55% in 2015 (68% for children < 5 years) as against only 2% in 2000 (WHO, 2015a). Although the use of ITNs has increased over the last 15 years in Africa, the target is to achieve universal 100% coverage.

2.1.9.3 Intermittent preventive treatment (IPT)

As pregnant women and children are more likely to get malaria, it is important to prevent them from being infected in the first place. One way to do this is through intermittent preventive treatment (IPT), which is basically achieved by giving antimalarials during periods of high risk. Intermittent preventive treatment in pregnancy (IPTp) involves the treatment of pregnant women with SP during the second and third trimesters of pregnancy and is usually administered during antenatal clinic visits. It was found that IPTp decreases the incidence of maternal anaemia, low birth weight and perinatal mortality (WHO, 2015a). On the other hand, the IPT measure has also been

applied to infants (IPTi), which involves administration of SP at 2, 3 and 9 months of age during visits to the childhood care and vaccination centres. The overall aim of the various forms of IPT is to maintain an adequate therapeutic level during the period of greatest risk as in seasonal malaria.

2.1.9.4 Malaria vaccine

The reduction in the global malaria burden is due to the increasing usage of the above-mentioned measures, especially the increase in the distribution coverage of mosquito insecticide treated bed nets, IRS-based vector control and the introduction of artemisinin derivatives in combination treatments. However, there is a pressing need for an efficacious malaria vaccine to complement the malaria elimination tools. The development of effective malaria vaccines has received a great deal of interest and made major progress in the last 15 years. As malaria parasite infects man, human immune system is stimulated which then induces immunity against specific stages of malaria parasite. In addition, most of the malaria antigens are not expressed in every parasitic stage. Thus, the immunity produced for a distinct parasite stage antigen does not protect against clinical malaria. Therefore, the ideal malaria vaccine has to be able to produce an immune response against all the different malaria antigens of the different malaria parasite stages, which is a major challenge (Taylor-Robinson, 2000). A few vaccines have been designed which grouped into the following four principal categories:

a. Pre-erythrocytic stages vaccines (PEVs) aim either to elicit the neutralizing antibodies to prevent sporozoites invading the liver or to induce cell-mediated immunity to prevent the invading sporozoites from growing and maturing inside liver cells (Girard et al., 2007). A commonly known PEV is RTS,S, which is based on the circumsporozoite protein (CSP).

b. Blood stage vaccines (BSVs) target the asexual-blood stages of the parasite and aims to prevent severe malaria disease but not the actual malaria infection; it aims at

decreasing parasitaemia and consequently malaria morbidity and mortality (The malERA, 2011). The leading candidate vaccines in this group are those based on merozoite surface proteins (MSPs), especially msp1 and msp2. Protection due to msp1 immunization was first reported in 1981 in immunized rodents. The antibodies against msp1 were found to block the invasion of malaria parasites into the RBCs of rodents. The protection works against reinvasion by erythrocytes through an antibody-based blocking mechanism (Holder et al., 1999; Holder, 2009; Ling et al., 1994; Woehlbier et al., 2006). Vaccines based on apical membrane antigen1 (ama1) and glutamate-rich protein (glurp) were also studied under this prevention pathway (Hermsen et al., 2007; Kocken et al., 2002). However, most of the parasite antigens belonging to this group show a high degree of genetic variation and polymorphism, which increases the challenges in developing effective malaria vaccines of this type (Girard, et al., 2007).

c. Transmission blocking vaccines (TBVs) were designed to work against the development of sporozoites in the Anopheles mosquito (Richard, 2001). The most commonly used vaccines in this group are those based on the ookinete of *P. falciparum* surface antigens Pfs25 and Pfs28 that were designed and developed by National Institutes of Health (NIH) (Girard, et al., 2007).

d. Antitoxic vaccines (ATVs) protect against malaria toxins and importantly, prevent the inflammatory reactions that can lead to complicated or severe malaria (Girard, et al., 2007; Good et al., 2005; Schofield et al., 2002).

A pre-erythrocytic vaccine, RTS, S, was designed through a recombination of CSP from the NF54 strain of *P. falciparum* clone 3D7 with a wild-type Hepatitis B surface antigen (HBsAg) (Olotu et al., 2011). Subsequently, the immunogenicity of the vaccine was improved by using AS01 adjuvant and the candidate vaccine was therefore named RTS, S/AS01 (Casares et al., 2010; Rutgers et al., 1988). An RTS,S/AS01 vaccination

requires the administration of three doses at 0, 1, and 2 months and a booster dose in month 20. The results of phase 3 trials at 11 sites in seven sub-Saharan African countries showed a reduction of clinical malaria by 36·3% and severe malaria by 32·2% in children. However, the reduction in the infant's group was lower at 25·9% and 17·3% for clinical malaria and severe malaria, respectively (RTS,S Clinical Trials Partnership, 2015; Aaby et al., 2015).

2.2 MALARIA SITUATION

2.2.1 Global situation

Malaria is a disease that occurs mainly in the poorest and most vulnerable countries of Africa, Asia and South America. In 2015, 97 countries were experiencing ongoing malaria transmission, with Africa being the area shouldering the greatest malaria burden especially among children below 5 years of age and pregnant women (WHO, 2015a).

The WHO has classified malarious countries and malaria-prone areas into six regions based on their geographical location. These are the African, Eastern Mediterranean, European, Southeast Asian, South American and Western Pacific regions. Africa, the continent which is greatly hit by malaria and which has the highest malaria morbidity and mortality, is further divided into three sub-regions: central Africa, west Africa, and east Africa and areas of high malaria transmission in southern Africa (WHO, 2015a). Of the 107 countries that experienced ongoing malaria transmission in 2000, 57 countries had reduced their malaria cases by more than 75% by 2015 and a further 18 countries had reduced their malaria burden to 50–75%, while some other countries reported zero indigenous cases for the first time in 2015.

Globally, around 3.2 billion people are at risk of being infected with malaria and 1.2 billion of these are at high risk (i.e. they have > 1 in 1,000 chance of getting malaria). Based on the latest report by WHO, there were around 214 million cases in

2015 compared to 262 million cases in 2000, which represents a decline of 18%. The WHO African region accounted for 88% of the total reported malaria cases followed by the WHO Southeast Asian (10%) and Eastern Mediterranean (2%) regions.

Deaths due to malaria have also fallen globally to 438,000 deaths in 2015 compared to 839,000 in 2000, which equates to a decline of 48%. The majority of deaths (90%) occurred mainly in the WHO African region followed by the WHO Southeast Asian (7%) and Eastern Mediterranean (2%) regions. Deaths among children under 5 years of age also fell from 723,000 in 2000 to only 306,000 cases in 2015. The decline in the number of deaths in 2015 was due to the reduction in the number of deaths in Africa, which fell from 694,000 to 292,000. Thus, malaria became the fourth biggest killer disease of children in sub-Saharan Africa responsible for only 10% of total deaths after neonatal infections (37%), pneumonia (19%) and diarrhoea (18%). Nevertheless, a child's life has been taken by malaria every 2 minutes in sub-Saharan Africa (WHO, 2015a).

However, countries in Africa have made remarkable progress in implementing effective prevention and treatment tools since 2000, which have reduced the mortality rate by 66% among all ages, and by 71% among children below 5 years of age. These impressive gains were made through preventing people from being exposed to malaria vectors; more than half of the population in sub-Saharan Africa is now covered by ITNs. Other tools included the wide expansion in malaria diagnosis testing mainly through RDTs, and the high accessibility to timely and effective antimalarials (WHO, 2015a). In the last 15 years (2001–2015), prevention and treatment measures have resulted in 1.2 billion fewer malaria cases and 6.2 million fewer deaths compared to 2000. Similarly, interventions in sub-Saharan African countries resulted in decreasing the malaria burden by 70% from 2001 compared to 2015, which is equivalent to preventing 663 million people from acquiring the infection. This reduction is estimated

to be due to the massive distribution of ITNs (69%), adoption of ACT (21%) and IRS activity (10%).

Among the countries with ongoing malaria transmission in 2015, 33 countries have achieved the target of having less than 1,000 confirmed malaria cases compared to only 13 countries in 2000. Moreover, three countries (Algeria, El Salvador and Mayotte) have reported fewer than 10 indigenous malaria cases. For the first time, countries in the WHO European region reported zero indigenous malaria cases, thereby achieving the target of declaring the elimination of malaria from the region by 2015. A total of 16 countries from different WHO regions (Argentina, Armenia, Azerbaijan, Costa Rica, Iraq, Georgia, Kyrgyzstan, Morocco, Oman, Paraguay, Sri Lanka, Tajikistan, Turkey, Turkmenistan, the United Arab Emirates and Uzbekistan) have succeeded in maintaining their status of being free of indigenous malaria (WHO, 2015a). However, the number of reported malaria cases is still only falling slowly in some other countries in Africa. A total of 15 countries still accounted for 80% and 78% of reported malaria cases and deaths, respectively in 2015. Globally, deaths due to malaria were particularly high in sub-Saharan Africa, especially in Nigeria and Democratic Republic of the Congo, representing more than 35% of deaths in 2015. Thus, the challenge facing those involved in the global fight against malaria is to permanently reduce the number of malaria cases and deaths in the most vulnerable countries.

The WHO has come up with a new global technical strategy for the period 2016–2030 to move toward a malaria-free world in conjunction with the Roll Back Malaria (RBM) partnership. The strategy was adopted in May 2015 the aim of which is to continue to reduce the global malaria burden and death by malaria through three consecutive steps. The first is to achieve universal accessibility to malaria prevention, diagnosis and treatment, the second is to accelerate the efforts to eliminate malaria and

attain a malaria-free environment and the third is to switch from a malaria surveillance to a core intervention approach. It is expected that the first objective will result in a 40% reduction of malaria morbidity and mortality by 2020, while the second and third objectives aim to achieve a reduction of 75% and 90% by 2025 and 2030, respectively (WHO, 2015c).

Although the number of worldwide clinical malaria episodes is huge, the rates of malaria morbidity and mortality were expected to be higher than those estimated by WHO based on national records. A study that re-analysed the epidemiological, geographical and demographical data from different malaria areas with different transmission intensities has revealed a double increase in clinical falciparum malaria cases, suggesting an underestimation in the clinical incidence of malaria in WHO records (Snow et al., 2005). The study model suggested that there were 515 million cases in 2002 and that 70% of those cases occurred in Africa and 25% in Southeast Asian countries, whereas WHO estimated 273 million cases and mostly (90%) in Africa (Snow et al., 2005).

2.2.2 Regional situation: WHO Eastern Mediterranean Region

The WHO Eastern Mediterranean region includes eight countries in which malaria transmission is still ongoing. Among the whole population of these eight countries, almost 279 million lives are at risk of being infected with malaria, 111 million of whom live at high risk. The malaria transmission in the region differs as six of the eight countries (Afghanistan, Djibouti, Pakistan, Somalia, Sudan and Yemen) are countries with areas of high malaria transmission and they are consequently in a control phase in WHO's global malaria programme. Saudi Arabia and Iran are in the elimination phase and the malaria transmission in these two countries is limited; only 376 and 51 locally acquired cases were reported in 2014 in Iran and Saudi Arabia, respectively. Malaria is

mostly due to *P. falciparum* in Djibouti, Saudi Arabia, Somalia, Sudan and Yemen, while it is predominantly due to *P. vivax* in Afghanistan, Iran and Pakistan.

In contrast, six other countries have achieved remarkable success in eliminating malaria, and they are now either in the phase of preventing reintroducing of malaria, (Egypt, since 1998; Oman, since 2004; Syria, since 2005 and Iraq, since 2011) or are certified as malaria-free (United Arab Emirates in 2007 and Morocco in 2010). A few cases have been reported from time to time: 22 cases were reported locally in a village 20 km from Aswan in Egypt in 2014, a few outbreaks of malaria importation have occurred in Oman reporting 984 imported cases since 2007 and 15 introduced cases in 2014, while Syria reported 21 *P. falciparum* imported cases in 2014.

According to WHO's 2015 assessment, seven countries of the Eastern Mediterranean region (Afghanistan, Iraq, Iran, Morocco, Oman, Saudi Arabia and Syria) succeeded in achieving a 75% reduction in malaria from 2000 to 2014. Actually, it is difficult to estimate the real number of malaria cases in politically troubled countries where there is instability, which currently refers mainly Iraq, Syria and Yemen that are experiencing a civil war, population displacement and the destruction of healthcare infrastructure, which very possibly exacerbates the malaria situation.

The confirmed malaria cases in the Eastern Mediterranean countries decreased from two million in 2000 to 1.5 million in 2014, and 91% of the total number of cases occurred in 2014 in just two countries which are Sudan (72%) and Pakistan (19%). The number of deaths due to malaria has also fallen from 2166 cases in 2000 to 960 cases in 2014. Similarly, the majority of deaths were reported in Sudan (86%) and Pakistan (6%).

The interventional measures against malaria in the Eastern Mediterranean region were basically based on the distribution of ITNs, the use of IRS and the provision of antimalarials mainly in the form of ACTs. In a period of 3 years (2012–2014), the total

population of malaria endemic areas in Afghanistan was covered by ITNs, while in the same period Yemen and Sudan were sufficiently covered by ITNs at 82% and 54% of the population, respectively. However, Iran and Saudi Arabia confined the distribution of mosquito bed nets to malaria foci only. National reports on the availability of antimalarials noted that Iran and Saudi Arabia have sufficiently delivered ACTs and other required antimalarials at the district level, while data from Afghanistan, Djibouti, Pakistan, Somalia, Sudan and Yemen is still incomplete (WHO, 2015a).

From the treatment point of view, all the countries of the WHO Eastern Mediterranean region have adopted and implemented ACTs as a first-line treatment. Seven countries (Afghanistan, Iran, Pakistan, Saudi Arabia, Somalia, Sudan and Yemen) have adopted AS+SP while Djibouti alone has adopted AL. The therapeutic clinical trials in the region have assured the international community and the national programmes of the high efficacy of AS+SP and AL except in Somalia and Sudan where a high rate of treatment failure was reported for the AS+SP combination; Somalia reported an alarming rate of AS+SP therapeutic failure (22.2%) in 2011, while Sudan reported an increasing rate of resistance to AS+SP from 5.3% in 2005 to 9.4% in 2011. In both Somalia and Sudan, the AS+SP therapeutic failure was found to be associated with quadruple and quintuple mutations in the *pfdhfr* and *pfdhps* genes, indicating a resistance of the *P. falciparum* isolates to the partner SP drug. Mutations in the *pfdhfr* and *pfdhps* are still rare in Afghanistan and Pakistan.

2.2.3 Malaria in Yemen

Yemen is located in the Middle East on the southern tip of the Arabian Peninsula, and is bordered to the west by the Red Sea and the Bab-el-Mandeb Strait which links the Red Sea to the Indian Ocean (via the Gulf of Aden), and which is one of the most active and strategic shipping lanes in the world. Yemen is bordered to the north by Saudi Arabia and to the northeast by Oman. Yemen also has maritime borders with Djibouti, Eritrea,

and Somalia. The population is 26 million and live in an area of 555,000 sq km including the islands of Perim at the southern end of the Red Sea and Socotra at the entrance to the Gulf of Aden (MOHP, 2014) (Figure 2.2).

The climate varies according to elevation and topography but generally has two main seasons, summer and winter. The highlands enjoy the very rainy weather in summer (temperature of 21-25°C) receiving 520–760 mm rainfall every year, while in winter it is cold and moderately dry and the temperature drops below 0°C. The climate is hot in the coastal areas where temperatures range from 37–40°C and humidity is in the region of 70–80%. The rain is irregular (130 mm) and often arrives in moderate to heavy torrents. The western mountainous hinterlands in the governorates of Ibb (in the centre) and Taiz (to the west) enjoy a mild rainy weather throughout the year with an average of 1000–1500 mm rainfall per year. In contrast, the eastern part of the country, notably Wadi Hadramout, is arid dessert and hot with a humidity ranging from 35% in summer to 65% in winter.

Yemen, is the country in the Arabian Peninsula that is most affected and the only country, along with Sudan, that has high malaria endemicity that threatens the control programmes of neighbouring countries, and especially Saudi Arabia (Al-Zanbagi, 2014). Malaria continues to be a major health problem in Yemen with almost 60% of the population at risk of contracting the infection. Around 25% of the population reside in areas with high malaria transmission, 53% live in areas with low malaria transmission and 22% live in malaria-free areas (WHO, 2015a). Pregnant women as well as children under 5 years of age are the most affected groups, but all age groups are at great risk in epidemic-prone areas (WHO, 2015a). *Plasmodium falciparum* is the most dominant species and is responsible for 99% of malaria cases annually, with *An. arabiensis*, *An. sargentii*, *An. culicifacies and An. azaniae* being the major mosquito vectors (WHO, 2015a; Al-Eryani, 2016). The peak period for malaria transmission in

the coastal areas including the islands is in winter (October to April), while the peak in mountainous areas occurs in summer (May to September), most commonly after rainfall.

2.2.4 Malaria control in Yemen, a story of success

Yemen, despite being the country with high malaria morbidity and mortality, has achieved noteworthy countrywide success in reducing the malaria burden; malaria cases have been reduced sevenfold since the late 1990s from nearly 1.4 million suspected malaria cases in 1997 to 900,000 cases by 2000 and remarkably to almost 200,000 cases in 2010 and 150,000 in 2013 (WHO, 2011a; WHO, 2013).

Another remarkable success story is that of Socotra Island, the main tourist island with 90,000 inhabitants living in an area of 3,600 sq. km. The government decided in 2002 to eliminate malaria from Socotra Island, and did so in just 3 years. The main elements of the Socotra elimination campaign were based principally on the reduction of human contact with the vectors through the use of IRS, LLITNs, and a larviciding biological control parameter through using larvivorous fish. Moreover, emphasis was also placed on early and correct diagnosis followed by prompt and correct treatment. All vector control staff, medical doctors and laboratory technicians and health workers were included, well trained and intensively supervised. Surveillance was strengthened to monitor the incidence of malaria, climatic changes, the occurrence of any outbreak, and the vector density and bionomics. Community participation, health education and intersectoral collaboration were promoted through strong political commitment, technical support was provided by WHO and the Government of Oman, a neighbouring country that successfully eliminated malaria in the 1990s (WHO, 2011a). As a result of all these efforts, Socotra Island reported its last four indigenous locally acquired malaria cases in 2005.

Figure 2.2: Geographic location of Republic of Yemen (Source: Maphill Map website). http://www.maphill.com/yemen/location-maps/shaded-relief-map/

2.3 ANTIMALARIAL DRUGS

Malaria chemotherapy is the most important cornerstone of malaria control efforts. An antimalarial drug can be described as effective only if it has the ability to eliminate malaria parasites and reduce malaria morbidity and mortality as well as to reduce the risk of the emergence of resistance in the parasites to the antimalarials.

Despite the remarkable developments in the global pharmaceuticals industry, the discovery of new antimalarials is still limited. Among the 1,393 entities registered between 1975 and 1996, only four were antimalarials: artemether, atovaquone, halofantrine and mefloquine (TDR, 2003). Therefore, the need to rationalize the antimalarial treatment policies is strongly recommended.

2.3.1. Types of antimalarial drugs

There are different antimalarial drugs available for malaria treatment, which are classified according to their antimalarial activity and/or chemical structure:

i. Classification according to antimalarial activity

Malaria parasites have a series of developmental stages in the human host which are morphologically and phenotypically different from each other. Antimalarial drugs have a selective action against different parasite stages:

- Tissue schizonticidal drug acts on the primary tissue form of the plasmodia. The tissue schizont grows within the liver, and its ruptures initiate the erythrocytic stage. Using this type of drug in prophylaxis was found to stop further development of the parasites *in vivo*. The schizonticidal drug acts on the latent stage of the parasite in the liver (hypnozoites) found in *P. vivax* and *P. ovale* infections to prevent relapse. Primaquine is the most effective tissue schizonticide.
- Blood schizonticidal drugs act on the blood forms of malaria parasites and thereby terminate clinical attacks of the disease. This group of drugs includes CQ, quinine,

mefloquine, halofantrine, pyrimethamine, sulfadoxine, sulphones, tetracycline etc. and are considered the most important drugs in malaria chemotherapy.

- Gametocidal drugs are used to destroy the sexual forms of the parasite in the blood and thereby prevent transmission of the infection to the mosquito. Chloroquine and quinine have gametocidal activity against only *P. vivax* and *P. malariae*, while primaquine has gametocidal activity against all plasmodia including *P. falciparum*.
- Sporontocidal drugs prevent the further development of young oocysts in the mosquito and thus stop the transmission. Examples of these drugs are primaquine and chloroguanide.

ii. Classification according to chemical structure

- Arylaminoalcohols are a group comprising quinolones such as quinine and quinidine (extracts from the bark of the cinchona tree), mefloquine, halofantrine and lumefantrine.
- 4-aminoquinolines are represented by CQ and amodiaquine.
- Folate synthesis inhibitors include type 1 antifolates such as sulphones and sulphonamides that are a competitive inhibitor of dihydropteroate synthase enzyme (DHPS), as well as type 2 antifolates such as the biguanides proguanil and chlorproguanil, and also diaminopyrimidine and pyrimethamine, that inhibit dihydrofolate reductase enzyme (DHFR).
- 8-aminoquinolines include primaquine and tafenoquine (Etaquine, WR238,605).
- Antimicrobials are antibiotics with antimalarial activity, such as tetracycline, doxycycline, clindamycin, azithromycin and fluoroquinolones.
- Peroxides consist of artemisinin (Qinghaosu) derivatives from the Chinese medicinal plant, *Artemisia annua*, and its semisynthetic analogues such as artemether, arteether, artesunate and artelinic acid.

- Naphthoquinones are represented by Atovaquone (BW566C80).
- Iron chelating agents include desferrioxamine B.

The following sub-sections describe the antimalarials that are most closely related to the present study, namely CQ, quinine, pyrimethamine-sulphonamide, artemisinin, and primaquine. The chemical structures of these antimalarial drugs are shown in Figure 2.3.

2.3.1.1 Chloroquine (CQ)

Chloroquine is a synthetic 4-aminoquinoline which was first made in 1934 and which by 1940 had already become an indispensable antimalarial agent because, in addition to its ease of use and low cost of commercial synthesis, it showed excellent clinical therapeutic efficacy and limited drug toxicity. Chloroquine is an erythrocytic schizonticidal drug, i.e., it is active against the erythrocytic stages of malaria parasites; it has no effects on the pre-erythrocytic, hypnozoite-liver or sexual (gametocytes) stages of the malaria parasite.

As malaria parasites grow, they obtain essential amino acids through the degradation of the haemoglobin within the host RBCs, which occurs inside the acidic digestive vacuole (PH 5) resulting in the generation of toxic soluble molecules called haem [ferriprotoporphyrin IX, FP Fe (II)]. The parasites detoxify the haem to a non-toxic byproduct called haemozoin through haem crystallization. Chloroquine inhibits FP crystallization by forming a highly toxic CQ complex with the haem (FP Fe (II) and/or hydroxyl complex with haematin (ferriprotoporphyrin IX, Fe (III) FP)), which is derived from the parasite's proteolysis mechanism acting on the host cells' haemoglobin. The CQ-haem complex becomes incorporated into the dimer chains and terminate chain extension, thereby blocking the sequestration of the haem, which then leads to disruption of the function of the digestive vacuole membrane. The effectiveness

of CQ has been shown to be dependent on the amount of drug accumulated inside the digestive vacuole. Chloroquine is a diprotic weak base, and in its unprotonated form it diffuses easily through the membrane of the parasitized RBCs and accumulates inside the vacuole. When CQ is inside the vacuole, it becomes protonated and membrane impermeable, then trapped in a high concentration inside the digestive vacuole.

2.3.1.2 Quinine

Quinine is a *cinchona* alkaloid and is the oldest antimalarial; it was first extracted from the bark of the cinchona tree in Peru in the 1600s. Although the cinchona tree originated in and is locally grown in the Andean region of the South America, plantations of this tree were established by Dutch and British colonialists in their Southeast Asian colonies quickly after they became aware of its antimalarial therapeutic potential. Due to World War II, *cinchona* tree plantation was lost in Japan, so scientists there looked for synthetic alternatives based on the chemical structure of quinine. Quinine has been found to be highly effective against malaria and has been used widely as a rescue drug treatment for treating severe malaria infections. The mechanism of its action is similar to that of CQ. However, quinine was found to increase insulin secretion from the pancreas which can lead to hyperinsulinemia and severe hypoglycaemia. Thus, it is important to give quinine to patients via an intravenous dextrose drip and to periodically monitor their glucose level. Cinchonism is another side-effect commonly associated with quinine treatment, which includes tinnitus, temporary deafness to high frequencies as well as headache, nausea and palpitations.

Other antimalarials were later synthesized based on the chemical structure of quinine and therefore classified as quinine-derivative drugs. These include 4-aminoquinoline CQ, as well as amodiaquine which is a side-chain analogue of CQ that has been found to play an important role in treating CQ-resistant falciparum malaria. Another quinine derivative is mefloquine, which is a 4-quinoline–methanol drug that is

widely used in ACT in many malarious areas in Southeast Asia. Primaquine is classified as an 8-aminoquinoline derivative of quinine. Lastly, quinidine, a dextro-diastereomer of quinine, has a similar antimalarial activity to quinine but it was found to act quite severely on the myocardium. Moreover, its therapeutic plasma level is close to a toxic level. Thus, it is not favoured; however, it has been used to treat malaria only in cases of emergency.

2.3.1.3 Pyrimethamine-Sulphonamide

Sulfadoxine and pyrimethamine are antifolate antimalarials. Folate derivatives are intracellular compounds that are very important for the production of deoxythymidylate (dTMP) in DNA biosynthesis. The inhibition of folate is the main strategy of antifolate antimalarials such as sulphadoxine and pyrimethamine. The antifolate mechanism was first proved in the 1940s with the discovery of triazine proguanil, which is transformed into the active compound cycloguanil that acts as an inhibitor of the dihydrofolate reductase enzyme (DHFR) of *P. falciparum*. Pyrimethamine is a derivative of 2,4-diaminopyrimidine, and its schizonticidal activity is based on a structural similarity to cycloguanil. Pyrimethamine activity was found to become synergized in combination with sulfadoxine.

Sulfadoxine, a schizonticidal drug, is the most commonly used antimalarial of the sulfa group, inhibiting the *P. falciparum* dihydropteroate synthetase enzyme (DHPS). Structurally, sulfadoxine is an analogue of para-amino benzoic acid (PABA) and it acts competitively to block the formation of dihydrofolate from PABA. A combination of folate inhibitors, pyrimethamine plus sulphadoxine, was found to be effective in the treatment of CQ-resistant falciparum malaria infections as well as in malaria prophylaxis for travellers. However, the sulphonamide component of this combination is potentially dangerous especially for patients with known hypersensitivity to sulphonamides, and patients may develop systemic vasculitis,

Stevens-Johnson syndrome, or toxic epidermal necrolysis mostly with intramuscular administration. Both pyrimethamine and sulphadoxine can cross the placenta and are excreted in mother's milk. Moreover, sulphonamides can displace bilirubin from plasma protein binding sites and cause kernicterus in the foetus and neonates. Thus, its combination use is strongly contraindicated in pregnancy and lactation.

2.3.1.4 Artemisinin

Artemisinin or Qinghaosu is the active component of the Chinese medicinal herb *Artemisia annua* (sweet wormwood), which has been used in China for treating fever for more than 1,000 years. The active principal was first isolated in China in 1972 and called *qinghaosu* (essence of qinghao), and then later named artemisinin. It is a sesquiterpene lactone with a distinctive 1,2,4 triaxone ring structure. Although the triaxone backbone is remarkably varied among the drug group, all artemisinin derivatives retain the characteristic endoperoxide in their structure, which is responsible for the microbial activity of artemisinin and its derivatives.

Artemisinin is a powerful schizonticidal antimalarial which can destroy young trophozoites as well as other blood stages of malaria parasites. Beside its powerful antimalarial activity, artemisinin has many other advantages which make the drug of great interest. It has wide therapeutic coverage against different parasite stages of all malaria species including CQ-resistant *P. falciparum* strains. It is safe for treating pregnant women and children and has no reported toxicity. However, artemisinin is an expensive drug compared to other antimalarials. In addition, dihydroartemisinin, the active metabolite of the artemisinin derivative, acts quickly to eliminate parasites with a short half-life (4–6 h). This rapid action of the drug may explain why resistance to artemisinin is slow to develop. In the same vein, malaria recrudescence is found to be more common in artemisinin monotherapy, thus it is strongly recommended that it is administered in combination with a long-lasting antimalarial partner. Nowadays, ACT is

the cornerstone principal of treatment policies in most malaria-endemic countries worldwide for treating uncomplicated falciparum malaria infection.

Artemisinins have a unique chemical structure unlike that of any of the previously known antimalarials and thus it is suggested that it has a different mechanism of action accordingly. The mechanism of action of artemisinin is not fully elucidated yet, but it has been hypothesized that its antimalarial activity is related to the unique endoperoxide moiety group (Brossi et al., 1988; Krungkrai & Krungkrai, 2016). It is well known that peroxides are a good source of reactive oxygen such as hydroxyl radicals and superoxide, thus the idea arose that free radicals may play a role in the principal action of artemisinin. Although the role of free oxygen radicals in artemisinin action has been confirmed by many studies (Krungkrai and Yuthavong, 1987; Levander et al., 1989; Meshnick et al., 1989; Senok et al., 1997), the way in which the endoperoxide bridge breaks to release free radicals remains somewhat unclear. It has been reported that artemisinin interacts with intra-parasitic heme-iron which, it has been suggested, activates the transformation of artemisinin into toxic-free radicals inside the parasite (Meshnick et al., 1991; Meshnick et al., 1996; Rosenthal & Meshnick, 1996). When formed, the artemisinin-derived free radicals appear to damage specific intracellular targets, possibly via alkylation.

2.3.1.5 Primaquine

Primaquine is a quinine-related compound. It is an 8-aminoquinoline derivative specifically used for eliminating the late tissue stage (hypnozoites) of *P. vivax* and *P. ovale* that cause malaria relapse. Moreover, primaquine has been used in radical malaria treatment to eliminate the sexual gametocyte stage to block malaria transmission. However, use of primaquine in patients with a deficiency of glucose-6-phosphate dehydrogenase (G6PD) can result in clinical disorders including severe haemolytic anaemia, neonatal jaundice, and several cardiovascular diseases (Shekalaghe et al.,

2010). Yet, recently, a single and low dose of 0.25 mg/kg of primaquine has been found to be effective for malaria transmission blockage and unlikely to cause any clinical haemolytic complications in individuals with G6PD-deficiency (White et al., 2012; White, 2013; WHO, 2015b).

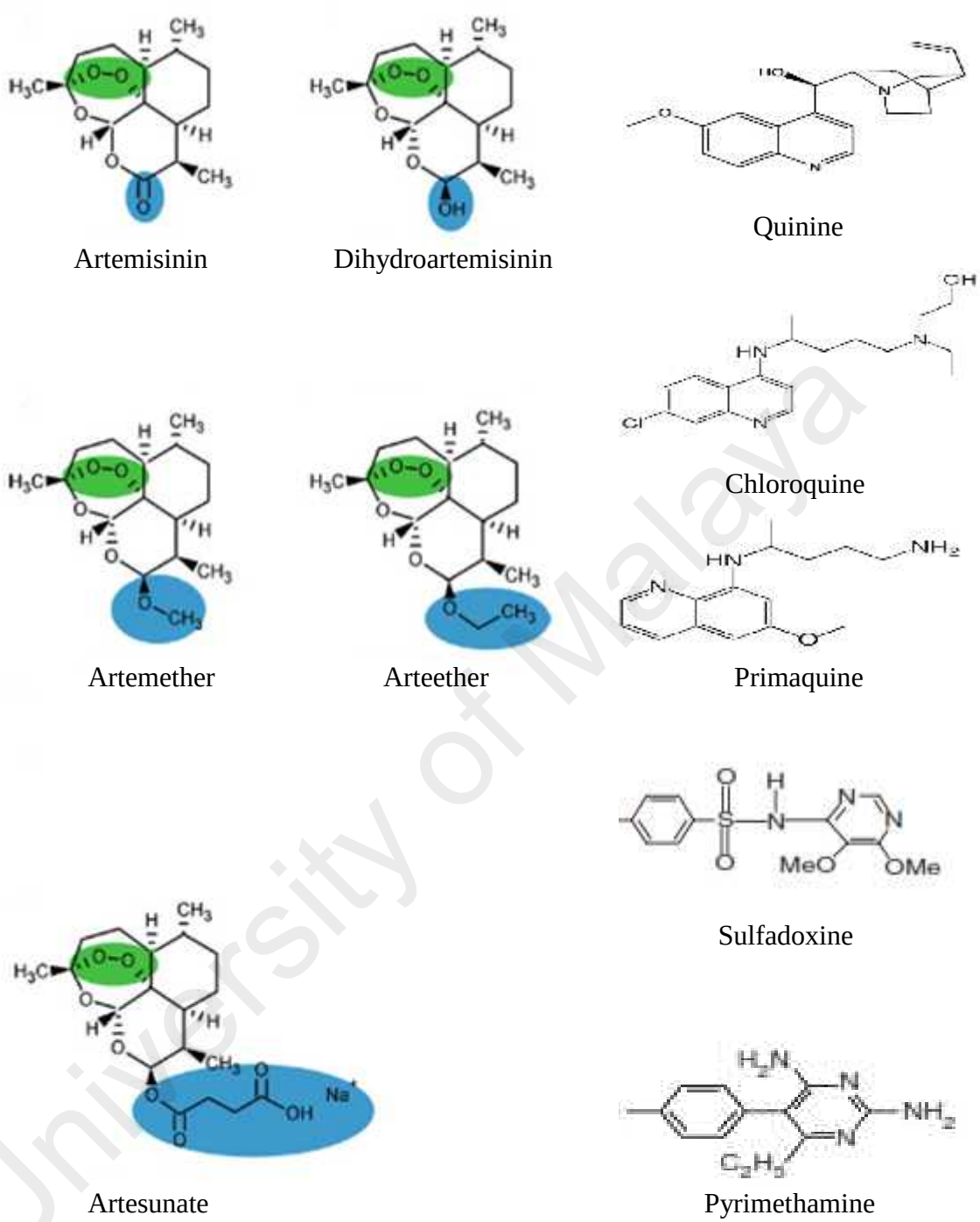


Figure 2.3: Chemical structures of common antimalarial drugs

2.4 ANTIMALARIAL DRUG RESISTANCE

Preventing and curing malaria infection depends predominantly on the selective administration of antimalarial drugs whose efficacy is continually threatened and eroded by the emergence of drug-resistant parasites especially *P. falciparum*. That there was resistance to the 'magic drug', CQ, was first suggested in 1957, and this was later confirmed in the 1960s on the Thai–Cambodian border (Eyles et al., 1963; Montgomery & Eyles, 1963; Young et al., 1963).

Resistance to antimalarial was defined by WHO early in 1967 as "*the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the tolerance of the subject*" (WHO, 2010b, p. 9). Actually, this definition of resistance did not consider the metabolism of the drug inside the patient. Later, the definition was modified as a result of gaining a better understanding of the pharmacokinetics of sulphonamide and other antimalarials to include the following text: "*The form of the drug active against the parasite must be able to gain access to the parasite or the infected erythrocyte for the duration of the time necessary for its normal action*" (WHO, 2010b, p. 9).

Drug-resistant malaria parasite can cause treatment failure, but not all treatment failure is in fact due to drug-resistant parasites. Many factors can contribute to the low or delayed susceptibility of parasites to antimalarials, such as inadequate drug dosage, incomplete duration of treatment regimen, poor quality of the used drug, drug antagonists, poor drug absorption, and rapid elimination of the given drug through diarrhoea and vomiting (White, 1999; White & Pongtavornpinyo, 2003). Just one of these factors may contribute to the development of antimalarial drug failure, but increasing the exposure of the parasites to a sub-optimal dose of a drug was found to play the major role in the parasites acquiring drug resistance (White &

Pongtavornpinyo, 2003). Interestingly, total cessation of CQ and the stopping of its pressure has resulted in the re-emergence of CQ-sensitive strains of *P. falciparum* (Kublin et al., 2003; Toshihiro Mita et al., 2003).

Four foci have been hypothesized for CQ resistance. The first was in Southeast Asia along the Cambodian–Thai border, which was discovered early in 1957 and is considered the first report of CQ resistance (Harinasuta et al., 1965). The second was on the Colombian–Venezuelan border in South America in 1960 (Moore & Lanier, 1961). The third focal point of CQ resistance was found in Papua New Guinea in 1976 (Grimmond et al., 1976). After that, a fourth spot appeared in Africa, in Kenya and Tanzania, in 1978 (Campbell et al., 1979; Fogh et al., 1979), which then extended to Madagascar 2–3 years later and thence to Sudan, Uganda, Zambia and Malawi late in 1980s (Ekvall et al., 1998; Fogh et al., 1984; Onori, 1984; Overbosch, 1984; Slatter et al., 1983).

Resistance to sulfadoxine/pyrimethamine was first reported in 1967 within the same year of its introduction (Peters, 1987; Wernsdorfer & Payne, 1991). Moreover, resistance to mefloquine was reported late in 1982, 5 years after its first usage (Nosten et al., 1991). On the other hand, resistance to atovaquone did not develop until 1996 (Looareesuwan et al., 1996). Finally and more recently, resistance to artemisinin was reported in Cambodian–Thai border in 2009, when a study revealed the existence of 30% resistance to artesunate monotherapy and 5% to artesunate-mefloquine combination therapy (Dondorp et al., 2009). In Africa and South America, a low treatment failure of less than 10% has been reported for AL and an artesunate+amodiaquine (AS+AQ) combination (WHO, 2015b).

2.4.1 Delaying resistance to new antimalarials

Southeast Asia has long been a hotspot for the emergence of parasite resistance to antimalarial drugs which forced malaria-prone countries in Southeast Asia to switch their first-line treatment to ACTs (Wongsrichanalai et al., 2002).

Both Thailand and Cambodia started to change their antimalarial treatment policies in 1995 and 2000, respectively, to include an ACT of artesunate plus mefloquine (AS+MQ). A few years later, a low susceptibility of *P. falciparum* isolates to the combination was again reported in the hotspot area of multidrug resistance, the Thai–Cambodian border (Dondorp et al., 2010). Resistance to ACTs has continued to emerge, and many reports have come out from different malarious areas in Asia. The efficacy of ACTs is still high in Africa and South America. Nowadays, WHO, investigators, national and international policy-makers and donors are working together in an effort to eradicate malaria globally and to ensure that antimalarial drugs, especially ACTs, are effective in areas hardly hit by malaria. It is well known that the use of a drug alone cannot achieve this aim; other strategies are required to enhance the effective life of antimalarial drugs and to prevent resistance from emerging. Such strategies include vector control, continuous monitoring of antimalarial drug efficacy, vaccine development and the selection of effective drugs for treating, preventing, and blocking the transmission of malaria, in addition to the supervision of private sector health facilities

2.4.2 Monitoring antimalarial drug resistance

Three methods are commonly used for monitoring antimalarial drug efficacy and to evaluate drug resistance. These are the *in vivo* test, the *in vitro* test and the detection of molecular markers. A method of measuring the drug concentration and analysing the drug pharmacokinetic in the patient's blood is added as another tool of monitoring the antimalarial drug resistance (WHO, 2010b).

2.4.2.1 *In vivo* test

The spread of resistance to CQ and other antimalarial drugs has made a lot of scientists and interested experts to adopt a standard method to evaluate the *in vivo* response of malaria parasites to such drugs. WHO formulated the first protocol for the *in vivo* test in 1964 (WHO, 2010b). The protocol was then revised in 1996, 2001 and 2009 to be used worldwide and to make it relevant to the changes in the resistant parasites and to the nature and pharmacokinetic of drugs and in accordance with malaria control programmes. This protocol can be achieved easily through administering the drug orally and then examining the patient's blood periodically to measure the parasitological and clinical outcome during the period of the study which commonly ranges from 14 days (short protocol) to 28 days (long protocol). For drugs with a longer half-life elimination such as mefloquine and piperaquine, a longer follow-up time of at least 42 days has been recommended for better evaluation (Stepniewska et al., 2004). One of the most important disadvantages of the in vivo test protocol is the overestimation of treatment failure as it is difficult to distinguish treatment failure from new infections which may occur during the long period of the follow-up (WHO, 2009).

2.4.2.2 *In vitro* test

Basically, the *in vitro* test is based on the cultivation of *P. falciparum* parasites (*in vitro*) in an artificial media with different known concentrations of the antimalarial drug. The result can be interpreted based on the number of parasites that grow into schizonts, which reflects the magnitude of the response of the parasite to the examined drug (Rieckmann et al., 1978). Unlike the *in vivo* test, this test is quantitative. Moreover, multiple tests can be performed and several drugs can be examined simultaneously using a single isolate. On the downside, this type of test requires more-expensive equipment and reagents, and in addition, personnel need to receive high level of training (WHO, 2010b).

2.4.2.3 Molecular markers

As a result of developments in the field of genetics, molecular tools have been introduced in many fields including the monitoring of antimalarial drug efficacy. Monitoring is performed through the detection of single nucleotide polymorphisms (SNPs) or point mutations in a specific gene that either modify the drug target enzyme or drug transporter (Vestergaard & Ringwald, 2007). Although molecular markers are good predictive tools for assessing drug effectiveness, the results are still controversial for most of the available markers.

Although molecular studies have had a clear and significant impact on progressing understanding and knowledge in all fields, they still are insufficiently advanced to evaluate the efficacy of some antimalarial drugs (WHO, 2010b). Molecular markers are considered to be tools that can predict the efficacy of an antimalarial drug which is helpful as they act as an early warning tool for monitoring the level of resistance of currently used drugs. Moreover, they serve as an indicator for the re-emergence of strains of the malaria parasite that are sensitive to drugs that were totally withdraw years ago from some areas (Kublin et al., 2003). But only a few markers can

prove the association with resistance to the selected drug. Data on the molecular markers of resistance to some other antimalarials including artemisinins are still controversial. Studies have reported an existence of wild strains of *P. falciparum* for a selected marker with a delayed susceptibility to related antimalarials. Moreover, molecular tools require high-tech laboratories with highly sophisticated and expensive equipment as well as trained personnel, an infrastructure that is not attainable in many countries with limited resources. Another disadvantage of the molecular marker method is the lack of standardized protocols for sample collection, DNA extraction and PCR amplification. The main molecular markers related to the present study are discussed below.

a. Plasmodium falciparum chloroquine resistance transporter (Pfcrt)

The *pfcrt* gene consisting of 13 exons, located near candidate gene 2 (cg2) on chromosome 7 and it encodes a digestive vacuole transmembrane protein (transporter protein). It comprises of a 424-amino acid of 48.6 kDa protein localized to the boundaries of the digestive vacuole of *P. falciparum* (Fidock et al., 2000).

Single nucleotide polymorphisms (SNPs) in the *Pfcrt* gene have been found to be widespread, and the association of these polymorphisms with *P. falciparum* resistance to different antimalarial drugs has been a subject of interest in recent studies. Nine SNPs in the *Pfcrt* gene have been found to have a significant linkage with CQ resistance in *P. falciparum*. Those SNPs are (1) codon C72S of *Pfcrt* in which cysteine is substituted by serine, (2) M74I in which isoleucine is replacing methionine at position 74, (3) substitution of glutamic acid or aspartic acid for asparagine at codon 75 (N75D/E), (4) threonine replacement of Lysine at 76 position (K76T), (5) substitution of serine for alanine at A220S, (6) glutamic acid replacement for glutamine at position 271 (Q271E), (7) serine replacement for asparagine at 326 (N326S), (8) threonine for isoleucine at position 356 (I356T) and (9) substitution of arginine by isoleucine at

codon 371 (R371I). They are all together capable of distinguishing CQ-resistant isolates from sensitive ones, with *Pfcrt* K76T being the most important pfcrt gene mutation which confers CQ resistance in the *P. falciparum* parasite (Djimdé et al., 2001; Fidock, et al., 2000).

b. Plasmodium falciparum multidrug resistance 1 (Pfmdr1)

The *pfmdr1* gene is a member of the ATP-binding cassette (ABC) transporter super-family and is located on chromosome 5. It is 4.2 kb in length and probably contains only one exon and encodes a 162 kDa P-glycoprotein homolog (*Pgh1*) which is localized in the digestive vacuole membrane of the asexual stages of the *P. falciparum* parasite and it has been suggested that it has a role in regulating the intracellular drug concentration (Cowman et al., 1991). Structurally, *pgh1* consists of two conserved domains of six transmembrane parts connected with a linker region (Cowman et al., 1991; Higgins, 1992; Wongsrichanalai, et al., 2002).

Sequence analysis of full-length *Pfmdr1* has revealed five polymorphic residues that appear to be dimorphic. The mutations of these codons have been found to be associated with resistance to different antimalarial drugs. Mutation at one of the codon nucleotides mostly results in the substitution of an amino acid with another one, causing alteration of the protein. These include substitution of tyrosine for asparagine at position 86 (N86Y), phenylalanine for tyrosine at 184 codon (Y184F), cysteine for serine at 1034 (S1034C), aspartic acid for asparagine at 1042 codon (N1042D) and replacement of aspartic acid by tyrosine at position 1246 (D1246Y) (Foote et al., 1990; Wellems et al., 1990). An association between *Pfmdr 1* and CQ resistance has been reported previously (Basco et al., 1995). However, many other studies have failed to prove this association (Wilson et al., 1993; Adagu et al., 1995; Bhattacharya et al., 1997; Chaiyaroj et al., 1999; Cojean et al., 2006). Moreover, mutation in the *Pfmdr1* gene has been reported as having an association with resistance to other antimalarial drugs

including amodiaquine, mefloquine, halofantrine, quinine, lumefantrine and artemisinin (Price et al., 1999; Reed et al., 2000; Sidhu et al., 2005; Holmgren et al., 2006; Alker et al., 2007; Danquah et al., 2010; Sisowath et al., 2007; Nwakanma et al., 2008; Malmberg et al., 2013; Cheruiyot et al., 2014; Gamo, 2014).

c. Plasmodium falciparum dihydrofolate reductase (Pfdhfr) and dihydropteroate synthase (Pfdhps)

Resistance to antifolates and sulphonamides is conferred by point mutations at specific codons in the gene coding for dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes, resulting in decreased affinity of the enzyme with the drug (Plowe et al., 1997). The risk of therapeutic failure when using pyrimethamine and sulfadoxine was found to increase with an increasing number of mutations in the *pfdhfr* and *pfdhps* gene, respectively. As an example, the odds ratio (OR) for triple mutations at 108, 51 and 59 was 3.9 compared to 2.1 for the 108 single mutation (Picot et al., 2009).

A single point mutation at S108N or multiple mutations with codons 50, 51, 59 and/or 164 of *dhfr* have been described as major keys to the resistance of *P. falciparum* to pyrimethamine drug (Mita et al., 2009; Plowe et al., 1997). Mutations in the *dhfr* gene occur in a stepwise fashion and a substitution of serine by asparagine at codon S108N was identified as the first mutation to play an important role in conferring resistance to pyrimethamine and increasing the IC_{50} to pyrimethamine up to 50-fold compared to the wild type (Sirawaraporn et al., 1997). Additional mutations to S108N along the *dhfr* were found to synergistically increase pyrimethamine resistance; a double mutations at N51I or C59R together with S108N has been found to result in a 2–16 fold increase in resistance compared to the single S108N mutation (Foote et al., 1990; Peterson et al., 1988).

Likewise, an accumulation of mutations in the *dhps* gene was found to be significantly associated with resistance to sulfadoxine (Triglia et al., 1997) with, more importantly, significant mutations at S436A, A437G, K540E and A581G (Mita et al., 2009). The OR for the *Pfdhps* A437G single mutation was 1.5, while it increased to 3.9 for the A437G+K540E double mutations (Picot et al., 2009).

Both the *pfdhps* and *pfdhfr* gene mutations were found to be very important in conferring resistance to SP, with quintuple mutants of *Pfdhfr* (codons 51, 59 and 108) plus *Pfdhps* (codons 437 and 540) being the mutations most significantly associated with SP resistance. The overall OR for quintuple mutations was 5.2, as estimated by Picot and others (2009).

Although mutations in the *dhfr* and *dhps* genes were found to be associated with SP resistance, they were also found to be significantly linked to resistance to AS+SP combination but not to AS monotherapy (Djimdé et al., 2008).

d. Plasmodium falciparum kelch 13

Although some reports have been published on the delayed susceptibility of falciparum malaria parasites to artemisinins in some areas particularly in Southeast Asia, ACTs remain the most effective treatment and the only right choice for treating uncomplicated falciparum malaria infections. The effectiveness of ACT depends highly on the efficiency of the partner drug in the ACT. The reported resistance to artemisinins was based on *in vivo* and *in vitro* assays which somehow was prone to variation. The best indicator for monitoring artemisinin therapeutic failure is the *in vivo* test through calculating the proportion of individuals who are parasitaemic from day 3 onward. Genetic involvement in artemisinin resistance was not known until 2014 when mutations in the kelch 13 (*k13*)-propeller domain in chromosome 13 of Cambodian *P. falciparum* isolates were found to be strongly associated with artemisinin resistance *in vitro* and *in vivo* (Ariey, et al., 2014). Analysis of the *k13* gene of Cambodian isolates

has shown that Y493H, R539T and C580Y mutations are associated with delayed artemisinin susceptibility. The parasite clearance half-life in patients with mutated alleles was found to be longer than that of the wild type (C580Y = 7.19 h, R539T = 6.64 h and Y493H = 6.28 h) compared to only 3.30 h for the wild allele. The clearance half-life has been shown to vary among the mutations, with the C580Y being the longest (Ariey, et al., 2014).

Currently, more than 50 SNPs within k13 propeller domain have been identified as being associated with artemisinin resistance (Straimer et al., 2015; Tun et al., 2015; Ashley et al., 2014; WHO, 2014b). The mutations C580Y and F446I were found to be the most prevalent mutations in Southeast Asia, particularly in Thailand, Cambodia and Myanmar (Tun et al., 2015; Huang et al., 2015; Nyunt et al., 2015; Takala-Harrison et al., 2015). The likelihood of *k13* mutations spreading to sub-Saharan Africa in a similar way to CQ and SP is high (Dondorp et al., 2010). Many studies have reported numerous non-synonymous k13 propeller polymorphisms circulating in Africa. However, none of them was found to be associated with artemisinin resistance (Conrad et al., 2014; Torrentino-Madamet et al., 2014; Kamau et al., 2015; Taylor et al., 2015).

2.4.3 Antimalarial drug resistance in Yemen

Malaria parasites, especially *P. falciparum*, show a high tendency to resist most antimalarial drugs as a result of increasing the selective drug pressure. In Yemen, the first CQ-resistant *P. falciparum* field isolates were reported by Mamser (1989) in a visit to the Taiz and Hodeidah governorates. Later on, it was reported that the resistance of *P. falciparum* parasites to CQ was gradually increasing (Berga, 1999). Since then, many studies have been conducted in different parts of Yemen which have almost all reported the *in vivo* or/and *in vitro* resistance of *P. falciparum* to CQ. For instance, a study conducted in Hodeidah from April 1998 to May 1999 reported resistance of 41% and

43% in an *in vi*vo and *in vitro* analysis, respectively (Al-Maktari et al., 2003). Al-Shamahy and colleagues (2007) conducted another study in Hodeidah (Bajil city) from August 2002 to March 2003, which aimed to make an *in vitro* assessment of the susceptibility of *P. falciparum* isolates to CQ, and they reported 47% resistance. Moreover, CQ resistance was also reported in 16.1% of isolates from Taiz governorate based on a 7-day *in vivo* study (Alkadi et al., 2006). The last report in this respect was an *in vivo* study conducted from October 2002 to January 2003 in Al-Musiemeer in Lahj governorate, a malaria-endemic district in Southeast Yemen. Based on follow-up data, only 39.3% of the participants had an adequate clinical and parasitological response (ACPR), while the rest (60.7%) showed CQ treatment failure with 23% classified as early treatment failure (ETF), 13.1% as late clinical failure, and 24.6% as late parasitological failure (Mubjer et al., 2011).

The efficacy of CQ in Yemen was also examined through analysing the CQ-resistant mutations, especially the mutation at position 76 in the CQ resistance transporter gene of *P. falciparum* (*pfcrt*). Several different studies all reported a high prevalence of *pfcrt* 76 mutation in the different governorates of Yemen, reflecting a low susceptibility of *P. falciparum* isolates to CQ. The prevalence of the mutated *pfcrt* 76T marker of CQ resistance varies: it was 71.8% in isolates from the central highlands of Rymah and Dhamar governorates and 90.5% in those from the coastal/foothill areas of Taiz and Hodeidah in the west (Al-Mekhlafi et al., 2011); 98% in Al-Musiemeer in Lahj (Mubjer et al., 2011), 85.2% for isolates from Hodeidah (Abdul-Ghani et al., 2013); 88%, 94.1% and 100% in Taiz, Hodeidah and Dhamar, respectively (Al-Hamidhi et al., 2013); and 74% for isolates from Hadramout (Bamaga et al., 2015a). The studies on antimalarial efficacy in Yemen are all described in Table 2.3 and Table 2.4.

Table 2.3: Previous studies on monitoring antimalarial drug efficacy in Yemen

Study	Type	Result
Adeel et al., 2015 - Eight *in vivo* studies - Site: five different governorates - Time 2009-2013 - Done by National Malaria Control Programme (NMCP), Yemen.	8 *in vivo* studies: (The only published artemisinin *in vivo* study) - 28-days protocol. - *Msp1*, *msp2* & *glurp* PCR-corrected <u>5 studies for AS+SP</u> - Al-Udayn, 2010 - Shares, 2010 - Tor-Bani-Qa'is, 2010 - Al-Qaflah, 2011 - Bajil, 2013 <u>3 studies for AL</u> - Bajil, 2009 - Jabal Al-Sharg, 2010 - Tor-Bani-Qa'is, 2013	Total parasite clearance in day 3 in all 8 studies. <u>AS+SP</u>: - Over all AS+SP efficacy exceeded 98-100% - Bajil 2013: 1 LCF, 2 LPF, ACPR 97%, Corrected 2LCF, ACPR 98%. - Tor-Bani-Qa'is 2010: 1 LCF, 1 LPF, ACPR 97.6%, Corrected ACPR 98%. - Others in Al-Udayn, Shares & Al-Qaflah were all 100% ACPR. <u>AL</u>: Cure rate in all sites 98-100%. - Jabal Al-Sharg 2013: 5 LCF, ACPR 94.3%, Corrected ACPR 100%. - Tor-Bani-Qa'is 2010: 1 LCF, 1 LPF, ACPR 97.6%, Corrected ACPR 98%. - Bajil was 100% ACPR.
Bamaga et al., 2015a - Site: Hadramout (Hajer & Al-Raydah-Qusyer districts) - 735 screened people - 138 *P. falciparum* samples - Time: July 2011- May 2012	Molecular study: *Pfcrt* : 76, 271, 326, 356 & 371 *Pfmdr1*: 86 & 1246	<u>*Pfcrt* mutation</u>: - 76 = 74% (Hajer 88%, Al Raydah-Qusayer 70.5%). - 271 = 58.7% (Hajer 61.5%, Al Raydah-Qusayer 58%). - 326 = 54.3% (Hajer 65.4%, Al Raydah-Qusayer 51.8%). - 356 = 0% - 371 = 44.9% (Hajer 69.2%, Al Raydah-Qusayer 39.3%). <u>*Pfmdr1* mutation</u> - 86 = 16.7% (Hajer 30.8%, Al Raydah-Qusayer = 13.4%). - 1246= 0%

Bamaga et al., 2015b - Site: Hadramout (Hajer & Al-Raydah-Qusyer districts) - 137 Pf samples - Time: July 2011-May 2012)	Molecular study: *Dhfr* : 51, 59, 108 & 164 *Dhps*: 436, 437 & 540	<u>*Dhfr*</u>: - 51I (84%) - 59R (0.8%) - 108N (84%) - 164L (0%) <u>*Dhps*</u>: - 436A (0%) - 437G (44.7%) - 540E (0%) <u>Haplotypes</u>: - *Dhfr*: <u>ICNI</u> = (82.8%) - *Pfdhfr–pfdhps*: - <u>ICNI</u>/SAK = (43%) - <u>ICNI</u>/S<u>G</u>K = (39.3%)
Al- Hamidhi et al., 2013 - Site: Dhamar, Hodeidah & Taiz - 108 Pf samples - Time: Unknown	Molecular study: *Pfcrt* (72-76) *Pfmdr1* (86, 184, 1034, 1042 & 1246) *Pfdhfr* (51, 59, 164 & 108) *Pfdhps* (436, 437, 540 & 613)	<u>*Pfcrt*:</u> - *Pfcrt76T*: 93% (100% Dhamar, 88% Taiz, 94% Hodeidah). - CVIET= 89% (100% Dhamar, 88% Taiz, 71% Hodeidah). <u>*Pfmdr1*:</u> - 86Y: 20% (10% Dhamar, 29% Taiz, 5.9% Hodeidah). - 184F 99% (97% Dhamar, 100% Taiz, 100% Hodeidah). - 1034 & 1042= 70% (69% Dhamar, 71% Taiz, 71% Hodeidah). - 1246 mutation: not detected. NFCDD = 57% NFSND = 21% YFCDD = 13% YFSND = 8% <u>*Pfdhfr*</u>: - 51I=54% (41% Dhamar, 65% Taiz, 35% Hodeidah). - 108N=54% (41% Dhamar, 65% Taiz, 35% Hodeidah). - 59R &164L mutations NOT DETECTED. NCSI = 46% (59% Dhamar, 36% Taiz, 65% Hodeidah). ICNI = 54% (41% Dhamar, 65% Taiz, 35% Hodeidah). - *Pfdhps*: No Mutation at all for 436, 437, 540 & 613.

Abdul-Ghani et al., 2014 - Site: Hodeidah - 90 Pf samples - Time: November 2012 - January 2013	Molecular study: *Pfdhfr* 51 & 108	*Pfdhfr* 108N = 61%
Abdul-Ghani et al., 2013 - Site: Hodeidah - 90 Pf samples - Time: Nov 2012-Jan 2013	Molecular study: *Pfcrt* 76 only	- *Pfcrt* T = 71/88 (80.7%) - *Pfcrt* K= 13/88 (14.8%) - Mixed= 4/88 (4.5%) So overall Mutated *Pfcrt* 76T= 75/88 (85.2%)
Mubjer, et al., 2011. - Site: Al-Musaimeer-Lahj - Samples: 124 P. falciparum isolates - Time: October 2002 - January 2003	- CQ *in vivo* efficacy trial - Molecular study: • *Pfcrt* (72-76, 163, 220,271,326,371) • *Pfdhfr* 59 • *Pfdhps*	<u>*In vivo*</u>: - 14-days protocol, *msp1*& *msp2* corrected. - ACPR= 48 (39.3%) - TF= 74 (60.7%): - ELF: 28 (23%), - LCF: 16 (13.1%), - LPF: 30 (24.6%). <u>Molecular</u>: - *Pfcrt* 76T = 98% - *Pfcrt* not associated with CQ efficacy. - Isolates carried CVIET-SESI for pfcrt (72-76,220,271,326,371) - No mutation reported for pfcrt S163R. - *Dhfr* C59R = 5% - *Dhps*: all Wild
Al-Mekhlafi et al., 2011 - Site: four governorates: • Taiz • Hodeidah • Rymah • Dhamar - 511 screened individuals - 81 Pf samples successfully amplified - Time: June 2008 to March 2009	Molecular study: *Pfcrt*	- Overall prevalence *pfcrt* 76T = 81.5% - Wild *Pfcrt* K76 = 18.5% - Mixed *Pfcrt* K76T = 10.3% So Corrected *Pfcrt* 76T = 93.8% - Coastal/foothills areas = 90.5% - Highlands = 71.8%

Al-Shamahi et al., 2007 - Site: Hodeidah, Bajil district - 219 Pf Samples - Time: August 2002-March 2003	*In vitro* evaluation of: - Chloroquine (CQ), - Mefloquine (MF), - Quinine (QN) and - Artemisinin (ART)	CQ-resistance = 47% MF-resistance = 5.2% QN-resistance = 0% ART-resistance = 0%
Al-Kabsi et al., 2009 - Site: Hodeidah - Time: March and May 2005	- SP *in vitro* & *in vivo*, PCR-uncorrected - 28-days protocol.	- SP- *in vivo* & *in vitro* efficiency = 100% - Total parasite clearance *in vivo* by day 3 onward
Al-Kadi et al., 2006 - Site: Taiz (Hethran & Al-Mafatch) - 447 screened participant • 83 enrolled cases • 56 continued follow-up - Time: unknown.	- CQ *in vivo* study, PCR-uncorrected - 7-days in vivo protocol.	Overall CQ- resistance = 16.1% In Hethran village = 19.4% In Al-Mafatch village = 10.0%
Al-Maktari et al., 2003 - Site: Hodeidah - 209 participants - Time: (April 1998-May 1999)	CQ *in vitro* & *in vivo*, PCR-uncorrected WHO 1973 *in vitro* protocol. WHO 1997 *in vivo* protocol.	<u>*In vivo*</u>: - CQR = 41.2% - RI=38.4%, RII=43%, RIII=18.6% - Zabid (45.3%), Azohrah (42.6), Bajil (34.7) and Azaidiah (30.8). <u>*In vitro*</u>: CQR = 43%
Berga, 1999	CQ resistance	Increasing the resistance to CQ
Mamser, 1989 - Site: Taiz & Hodiedah	First report of CQ-resistant *P. falciparum* in Yemen.	--

ACPR	:	Adequate Clinical and Parasitological Response	LPF	:	Late Parasitological Failure	R I	: Resistance Grade I
ELF	:	Early Treatment Failure	TF	:	Treatment Failure	R II	: Resistance Grade II
LCF	:	Late Clinical Failure	CQR	:	Chloroquine Resistance	R III	: Resistance Grade III

Table 2.4: Review on studies of monitoring antimalarial drug efficacy in Yemen: studies by National Malaria Control Program (NMCP), Yemen

Year	Site	Governorate	Drug/Protocol	Patients Enrolled	ECF		LCF		LPF		ACPR		TTF		Corrected
					No.	%	No.	%	No.	%	No.	%	No.	%	%
2002-03	Mesemeer	Lahaj	CQ:14 days *in vivo*	124	28	23.0	16	13.1	30	24.6	48	39.3	74	60.7	57.0
2002-03	Bajil	Hodeidah	CQ: 14 days *in vivo*	106	11	11.1	6	6.1	25	25.3	57	57.6	42	42.4	42.0
2003	Odein	Ibb	CQ: 14 days *in vivo*	106	15	14.9	2	2.0	30	29.7	54	53.5	47	46.5	46.5
2004	Harad	Hajja	SP: 14 days *in vivo*	102	0	0.0	0	0.0	0	0.0	102	100	0	0.0	0
2004	Odein	Ibb	SP: 28 days *in vivo*	53	0	0.0	0	0.0	0	0.0	51	100	0	0.0	0
2004	Mesemeer	Lahaj	SP: 28 days *in vivo*	60	0	0.0	0	0.0	3	5.0	57	95.0	3	5.0	5.0
2004-05	Mesemeer	Lahaj	AQ: 28 days *in vivo*	61	0	0.0	11	18.0	16	26.2	34	55.7	27	44.3	44.0
2004	Odein	Ibb	AST+AQ: 28 days *in vivo*	58	0	0.0	0	0.0	10	18.5	44	81.5	10	18.5	14.0
2009-10	Bajil	Hodaida	AL: 28 days *in vivo*	80	0	0.0	0	0.0	0	0.0	74	100	0	0.0	0
2010	Jabal Alsharq	Dhamar	AL: 28 days *in vivo*	95	0	0.0	0	0.0	2	2.2	88	97.8	2	2.2	0
2009-10	Tor Bani Qais	Hajjah	AS+SP: 28 days *in vivo*	95	0	0.0	1	1.2	1	1.2	81	97.6	2	2.4	0
2010	Sharia /Almaqarba	Hajjah	AS+SP: 28 days *in vivo*	93	0	0.0	0	0.0	0	0.0	82	100	0	0.0	0
2010	Odein	Ibb	AS+SP: 28 days *in vivo*	85	0	0.0	0	0.0	0	0.0	83	100	0	0.0	0

ECF: early treatment failure, LCF: late clinical failure, LPF: late parasitological failure, ACPR: adequate clinical & parasitological response, TTF: total treatment failure, AS + AQ: artesunate + amodiaquine, AS + SP: artesunate + sulfadoxine/pyrimethamine, AQ: amodiaquine, AL: artemether + lumefantrine, Q: chloroquine, SP: sulfadoxine/pyrimethamine.

CHAPTER 3: GENETIC VARIATION OF PFHRP2 IN PLASMODIUM FALCIPARUM ISOLATES FROM YEMEN AND THE PERFORMANCE OF HRP2-BASED MALARIA RAPID DIAGNOSTIC TEST

Wahib M. Atroosh, Hesham M. Al-Mekhlafi, Adel Al-Jasari, Hany Sady, Ahmed K. Al-Delaimy, Nabil A. Nasr, Salwa Dawaki, Awatif M. Abdulsalam, Init Ithoi, Yee-Ling Lau, Mun-Yik Fong, Johari Surin. Genetic variation of *pfhrp2* in *Plasmodium falciparum* isolates from Yemen and the performance of hrp2-based malaria rapid diagnostic test. ***Parasites & Vectors*** **2015; 8: 1-8**.

3.1 INTRODUCTION AND LITERATURE REVIEW

Malaria is still a major public health problem in Yemen, with almost 66% of the population living in areas that suffer from stable malaria transmission (WHO, 2014a). *Plasmodium falciparum* is the predominant species and was responsible for almost 99% of malaria cases in Yemen during 2012, a large number of which consisted of drug-resistant *P. falciparum* parasites (Al-Mekhlafi et al., 2011; Abdul-Ghani et al., 2014). Among 17 countries with malaria-endemic areas in the Middle East and Eurasia region; Pakistan, Afghanistan and Yemen account for more than 99% of the 56,000 regional deaths due to malaria (RBM Partnership, 2008).

The national malaria control programme in Yemen (NMCP) has achieved substantial success in controlling local cases of malaria, achieving a significant reduction in the number of malaria cases, dropping from 900,000 cases in the early 2000s to around 150,000 cases by 2013 (WHO, 2014a). However, Yemen is still classified among areas of high malaria transmission, making it the only country in the

Arabian Peninsula and greater Middle Eastern region that is still plagued with malaria to the extent that residents still suffer from considerably high mortality and morbidity rates (Al-Mekhlafi et al., 2011). Imported malaria cases are still reported in neighbouring countries, threatening the malaria control and elimination programmes in the region. For instance, 2,788 malaria cases were diagnosed in southern Saudi Arabia between 2011 and 2012, with about 97% of the cases having been identified as originating outside the country, particularly from the Tehama region, a Yemen bordered area (Middle East Health, 2013).

Early and accurate diagnosis of malaria, along with prompt treatment, are essential to reduce the burden of the disease worldwide. Rapid diagnostic tests (RDTs) have been widely used for the diagnosis of malaria and become an indispensable tool for malaria case-management, control and elimination worldwide, especially in rural endemic areas without laboratory access (Murray & Bennett, 2009; WHO, 2011b). Besides the affordability and shorter turnover time of RDT-based diagnosis, significant reductions in the over-prescription of antimalarials have been reported when RDTs are introduced in presumptive treatment settings, especially with the new policy of using the expensive artemisinin-combination therapy (ACT) as the first line treatment for uncomplicated falciparum malaria infection (Bastiaens et al. 2014). However, recent studies revealed that the sensitivity of RDTs could be compromised due to genetic polymorphism of the parasite PfHRP2 antigen, particularly with regards to certain amino acid repeat types, causing false-negative results when using the HRP2-based RDT to diagnose *P. falciparum* malaria (Koita; 2012; Wurtz et al. 2013; Deme et al. 2014).

In Yemen, PfHRP2-based RDTs have been implemented by the NMCP in 2009, and are being used exclusively for malaria active case detection (ACD) targeting only falciparum malaria infections (WHO, 2011c). However, data on the genetic variation of

the *pfhrp2* are not available. Hence, the present study aims to investigate the genetic variations of the *pfhrp2* gene in malaria isolates from the Hodeidah and Al-Mahwit governorates, Yemen (areas with high malaria endemicity) and the possible impact of this variation on the efficacy of the currently used *pfhrp2*-based RDTs. The present study is the first to provide data on the genetic variation of PfHRP2 in Yemen, and therefore has the potential to impact on control strategies and efforts to eradicate malaria from Yemen and the Arabian Peninsula.

3.2 MATERIALS AND METHODS

3.2.1 Study area

An active case detection survey targeting individuals with fever suspected of having a malaria infection was carried out in some malaria endemic districts of the Tehama region in both the Hodeidah and Al-Mahwit governorates, Yemen. The survey was carried out from March to May 2014, during the malaria transmission season. Districts with high malaria endemicity; namely AdDahi, Al-Marawiah, and Bajil from Hodeidah, and Khamis Bani Saad from Al-Mahwit, were selected based on the national malaria records of 2010-2013 provided by NMCP.

The Hodeidah governorate (14.79° N, 42.97° E) is located in the Tehama region in the western part of Yemen, about 226 kilometers from Sana'a, the capital of Yemen. It is a coastal area located along the Red Sea, covering a total area of 117,145 km^2 with a total population of 2.16 million (NIC, 2014). Al-Mahwit governorate (16.25° N, 44.717° E) is located between Hodeidah and Sana'a (about 111 km west of Sana'a), covering a total area of 2,858 km^2 with a total population of 597,000 people (NIC, 2014). The climate of the selected districts is a combination of tropical monsoon with occasional rains in the summer and dry weather in winter, with a mean rainfall of 200

mm/year. The mean temperature is 37.5 ºC in summer and 24 ºC in winter, with humidity ranging between 70% and 90%. Malaria is highly prevalent in Tehama region with high transmission peaking between January and March each year. Figure 3.1 shows the study areas and the distribution of malaria burden in Yemen in 2012.

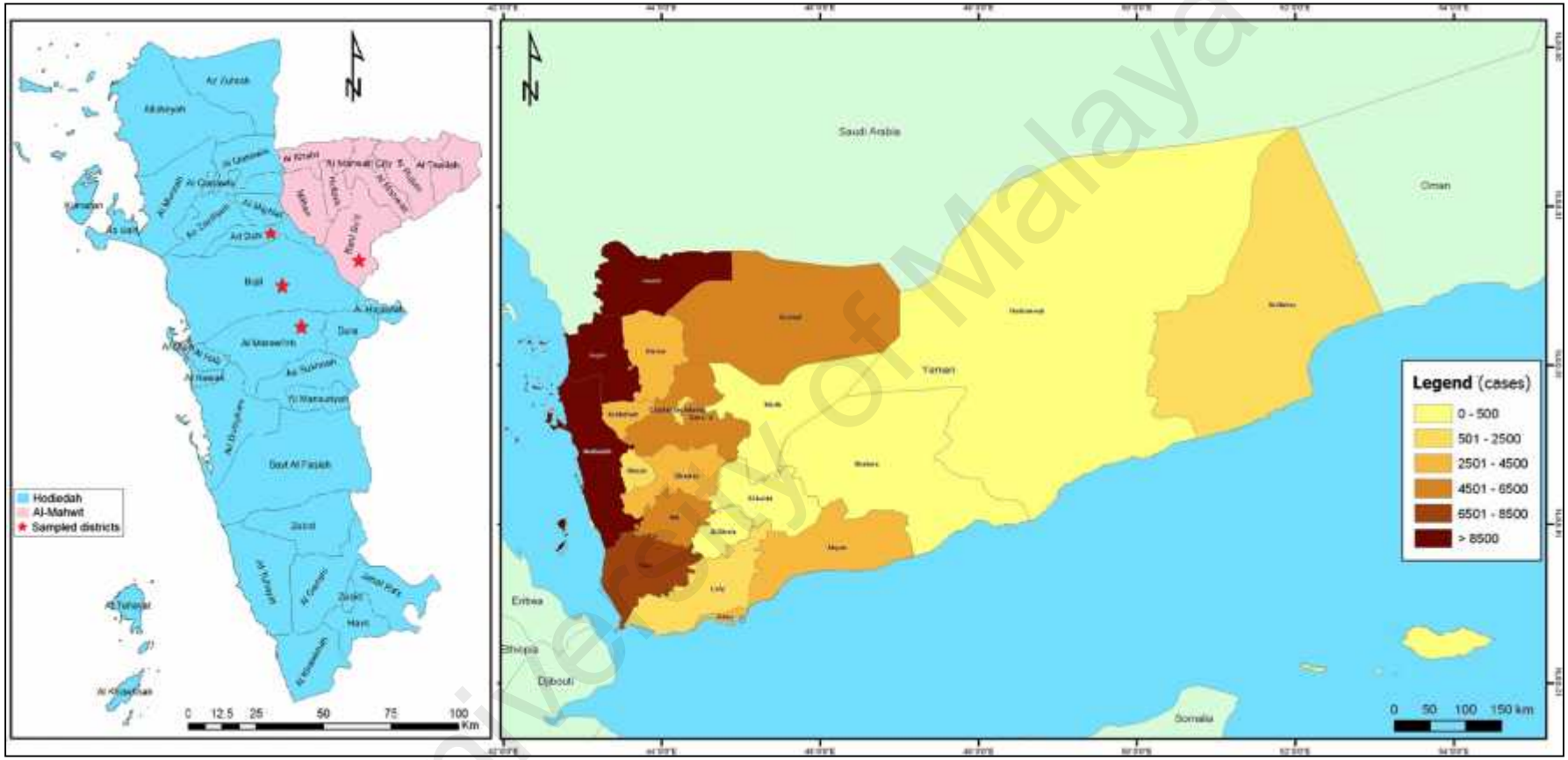


Figure 3.1: A geographic map showing study area (Hodeidah and Al-Mahwit governorates) and the distribution of malaria in Yemen according to incidence in 2012

3.2.2 *Plasmodium falciparum* isolates

A total of 622 individuals with fever were recruited to this study and examined for malaria. Finger prick blood samples were collected from participants and tested using the RDT (*CareStart*™ Malaria HRP2, Cat no. G0141, Access Bio, Inc, USA), and for preparing both thick and thin blood films. Filter paper blood spots were also collected from each participant on 3MM Whatman® filter paper (Whatman International Ltd., Maidstone, England), and kept in clean, dry, well-sealed aluminum pouches with desiccated silicon bags for molecular analysis. Blood films were stained with 5% of buffer-diluted Giemsa stain for 30 minutes and were examined microscopically for the presence of malaria parasites. All RDT tests were performed and interpreted by trained and skilled laboratory personnel from the NMCP following the manufacturer's instructions. Briefly, one purple line in the control line position interpreted negative for falciparum malaria and two purple lines at the test line position along with the control line were to be defined as positive. In case the control line did not appear, the result was considered invalid and the test was repeated.

For positive slides, parasite species and stages were reported and parasitaemia (parasite density) was determined by counting only the asexual stages against 300 white blood cells (WBC) and then multiplied by 25; assuming the average of total WBC count of individuals equal to 7500 cells per µL of blood. The level of parasitaemia was graded as low (< 1000 parasites/µL of blood), moderate (1000 - 9999 parasites/µL of blood) and severe (≥ 10,000 parasites/µL of blood). A double check for malaria microscopy was performed by two senior malaria microscopists; slides were examined twice and the average parasitemia per microlitre of blood was recorded for every microscopy positive slide. Genomic DNA was extracted from the filter paper blood spots and subjected to *pfhrp2* amplification using conventional single-run PCR.

3.2.3 DNA extraction

One or two discs (6 mm diameter) of 3MM Whatman's filter paper blood spot (cut by flamed-sterile punch) were used for DNA extraction using a Qiagen blood and tissue kit (QIAGEN, DNeasy® Blood & Tissue Kit, Cat. no. 69506, Germany) according to the manufacturer's instruction. DNA was eluted using 50 µL of AE elution buffer (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0), included in the kit, and kept at -20^{o}C until used.

3.2.4 Evaluation of HRP2-RDT performance

A malaria-positive blood sample, with a known parasite density (8500 asexual stage/µl) of *P. falciparum* mono-infection, was chosen to evaluate the *CareStart*^{TM} Malaria HRP2-RDT. The blood sample was also checked to make sure there were no sexual stage (gametocytes). Duplicate thick and thin blood films were prepared and stained with Giemsa stain as previously mentioned, and then examined microscopically by two senior malaria microscopists; the average of parasitaemia was recorded. The sample was then diluted in a ratio of 2:3 serial dilutions with healthy human O positive blood group. A total of 12 serial dilutions (labelled 1-12) were prepared in duplicates and the results of asexual-stage parasites density were recorded for each tube (Avila et al., 2002). Each tube dilution was then tested for PfHRP2-RDT according to manufacturer's instructions.

3.2.5 Molecular identification and *pfhrp2* sequencing

All DNA from malaria positive samples were confirmed by PCR (Singh et al., 1999), with *P. falciparum* samples then being considered for *pfhrp2* molecular characterization. Amplification of *hrp2* was carried out in a single-run PCR using a specifically designed oligonucleotide primer pair flanking the region of exon-2 of *pfhrp2* gene (Mariette et al. 2008). A 50 µL reaction mixture was made up containing

10 µM of each forward (PfHRP2-F 5'-TGTGTAGCAAAAATGCAAAAGG-3') and reverse primers (PfHRP2-R 5' TTAATGGCGTAGGCAATGTG-3'), along with 20 µL ExPrime Taq Premix ready-mix PCR reagent (Genet Bio, Korea) and 2 µL of the DNA extract. The amplification thermal conditions were initiated with DNA denaturation at 95 °C for 5 minutes, followed by 40 cycles of 95 °C/ 30 sec, 57 °C/ 40 sec and 72 °C/ 90 sec and a single extension step at 72 °C for 10 minutes. All PCR amplification reactions were amplified using thermal cycler (MyCycler- BioRad, Hercules, USA). Genomic DNA of *P. falciparum* lines 3D7 (MRA-102G), Dd2 (MRA-150G) and HB3 (MRA-155G) provided by Malaria Research and Reference Reagent Resource Centre (MR4), ATCC®, Manassas, VA, USA were used as positive and negative controls for PCR amplification of HRP2 and HRP3. A 3D7 was used as a positive control for both HRP2 and HRP3, and Dd2 (a laboratory line known to lack *pfhrp2*) and HB3 (a laboratory line known to lack *pfhrp3*) were used as negative controls for HRP2 and HRP3 respectively (Gamboa et al., 2010; Cheng et al., 2014).

The PCR products were then analysed using agarose-gel electrophoresis. Ten microliters of each amplicon were loaded into a 1.5% agarose gel and run in a TAE buffer (Tris acetate EDTA), stained with SYBR® safe DNA gel stain (Invitrogen, USA). The fragments size was visualized under UV compared to 100 bp DNA ladder.

The amplicons were sent for purification and sequencing and each amplicon was subjected to sequencing using the same forward and reverse primers as were used during PCR amplification. Both forward and reverse sequences were aligned using BioEdit Sequence Alignment Editor Software (version 7.1.9) and then translated into corresponding amino acids. Each sequence of amino acid repeats was identified and given a code from Type 1 to Type 14 based on the motif being repeated (Baker et al., 2005).

3.2.6 Data analysis

The performance of RDT was calculated based on the following indicators: sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) which were calculated with their corresponding 95% confidence intervals (CI) using Medcalc® online calculator. Moreover, Kappa statistics were used to assess the agreement between RDT and microscopy. This was calculated in IBM SPSS Statistics, version 18.0 (IBM Corporation, NY, USA) by creating a 2 × 2 contingency table. A *P*-value of < 0.05 was considered significant.

3.2.7 Ethical consideration

The protocol used in this study was approved by the Ethics Committee of the University of Malaya Medical Centre, Malaysia (Ref. 974.19). The protocol was also approved by the Ministry of Health and Population, in conjunction with the National Malaria Control Programme in Yemen (Appendix E). Written and signed or thumb-printed informed consents were taken from adult participants and parents or guardians on behalf of their children before starting the sample collection (Appendix C); these procedures were also approved by the ethics committees. RDT-positive participants were treated with artemisinin combination therapy (artemisinin + sulfadoxine/pyrimethamine) according to the national malaria treatment policy, Ministry of Health and Population, Yemen.

3.3 RESULTS

Of the 622 screened individuals, 188 (30.2%) were positive for malaria by the *CareStart*™ Malaria HRP2-RDT. Of these 188 RDT-positive, only 171 (91.0%) were confirmed microscopically by detecting either asexual or sexual stages or both. On the other hand, 18 (2.9%) microscopy-positive individuals were found to be negative with RDT. Similarly, 17 (2.7%) RDT-positive cases were found to be negative with

microscopy. Based on microscopy, the detection rate was the highest in Khamis Bani Saad (39.4%) followed by Bajil (23.5%), while Al-Marawiah has the lowest (5.3%). The level of asexual *P. falciparum* parasitaemia in positive cases ranged from 40 to 55,555 parasites/µl of blood with a geometric mean of 5,261 parasites/µl. High parasitaemic individuals (parasite count ≥ 10,000 parasites/µl of blood) represented 17.9%, while 41.9% and 40.2% of the malaria-positive individuals had moderate and low parasitaemia respectively.

The overall sensitivity and specificity of the used *CareStart*^{TM} malaria HRP2-RDT was rated as 90.5% (95% CI = 85.4, 94.3) and 96.1% (95% CI = 93.8, 97.7), respectively (Table 3.1). The PPV and NPV were 91.0% (95% CI = 85.9, 94.6) and 95.9% (95% CI = 93.5, 97.5), respectively. The agreement between the microscopy and HRP2-RDT was statistically significant by Kappa (K = 0.867; $P < 0.001$).

Table 3.1: The sensitivity and specificity of PfHRP2-based RDT against the reference technique (microscopy) using samples collected from Hodeidah and Al-Mahwit, Yemen (n = 622).

RDT	Microscopy		
	Negative	**Positive**	**Total**
Negative	416	18	434
Positive	17	171	188
Total	433	189	622

Sensitivity: true positive = 171/189 = 90.5%; false negative error rate = 18/189 = 9.5%. Specificity: true negative = 416/433 = 96.1%; false positive error rate = 17/433 = 3.9%. PPV: 171/188 = 91.0%; NPV: 416/434 = 95.9%.

In the same vein, HRP2-RDT was examined against serial dilutions of a blood sample with known *P. falciparum* parasite density. The test revealed a high detection rate of *CareStart*^{TM} malaria HRP2-RDT for falciparum malaria isolates; 10 out of the 12 tubes with parasitaemia levels ranged from 8500 to 147 asexual stage/µl were found positive (visible clear band), with a faint band appearing for tube no.11 (98 parasites/µl). The last tube, no. 12, with parasitaemia of 65 parasites/µl was found negative by HRP2-RDT (Table 3.2 and Figure 3.2). With our Yemeni isolates, it was found that performance of RDT increased with parasitaemia level, with the lowest detection rate (44.4%) reported with parasitaemia levels of 1-100 parasites/µl while it was ≥ 97.0% with parasitaemia levels of ≥ 1,000 parasites/µl. Although most of the negative RDT results were found in samples with low parasitaemia, but negative RDT results were also reported with two samples with moderate and high parasitaemia levels.

Overall, 180/189 cases were successfully amplified for *pfhrp2* and yielded good and quality sequences, and were therefore subjected to amino acid repeat type analysis. The sequence lengths of the isolates were found to vary, ranging from 477 bp to 879 bp (giving proteins of 159-293 amino acids), with a sequence of 540 bp (180 amino acids) being the most frequent genotype (43.9%). All isolates sequences were found to be similar in that they started with either a single or multiple Type 1 (AHHAHHVAD), and ended with a single Type 12 repeat (AHHAAAHHEAATH). Overall, Type 1 (AHHAHHVAD), Type 2 (AHHAHHAAD), Type 6 (AHHATD), Type 7 (AHHAAD), Type 10 (AHHAAAHHATD) and Type 12 (AHHAAAHHEAATH) were found in all sequenced isolates. On the other hand, type 9 (AAY) and Type 11 (AHN) were totally absent in all isolates collected from both governorates.

Other amino acid repeat types were varied in their frequencies. Type 8 (AHHAAY), Type 5 (AHHAHHASD) and Type 4 (AHH) ranged between moderate and low, with percentages of 49.4%, 47.2% and 15.6% respectively. Moreover, Type 13

(AHHASD) and Type 14 (AHHAHHATD) were rarely reported in the present study (5.6% and 2.8%, respectively). Further, the mean numbers for 2 and 7 repeat types in *pfhrp2* were the highest among the 12 types detected by this study (mean = 9.85 and 4.97, respectively). No association was found between HRP2 repeats and age, sex and districts of participants as well as parasitaemia level. The frequency and mean number of PfHRP2 repeat types from *P. falciparum* isolates from Hodeidah and Al-Mahwit, Yemen are shown in Table 3.3.

Interestingly, one isolate with low parasitaemia (184 parasites/µl) was found to be positive by both *CareStart*^{TM} malaria HRP2-RDT and microscopy but it was PCR-negative for *pfhrp2* gene. DNA of this isolate was processed for further PCR confirmation using three PCR protocols that aimed at amplifying *P. falciparum 18S rRNA* (Singh et al., 1999), *pfhrp2* and *pfhrp3* genes (Mariette et al., 2008). The DNA was successfully amplified for *18S rRNA* and *pfhrp3* genes while it was confirmed negative for *pfhrp2* gene.

Table 3.2: Evaluation of *CareStart*™ PfHRP2-based RDT against serial dilutions of *P. falciparum* parasitaemia.

Tube No.	**Blood Volume**	**Diluent** (human-O+ Blood)	**Parasite Density** (Parasites/µL)	**RDT Result**
0	Whole Blood	0	8500	Positive
1	200 µL of Tube No. 0	100 µL	5666	Positive
2	200 µL of Tube No. 1	100 µL	3777	Positive
3	200 µL of Tube No. 2	100 µL	2518	Positive
4	200 µL of Tube No. 3	100 µL	1679	Positive
5	200 µL of Tube No. 4	100 µL	1119	Positive
6	200 µL of Tube No. 5	100 µL	746	Positive
7	200 µL of Tube No. 6	100 µL	497	Positive
8	200 µL of Tube No. 7	100 µL	331	Positive
9	200 µL of Tube No. 8	100 µL	221	Positive
10	200 µL of Tube No. 9	100 µL	147	Positive
11	200 µL of Tube No. 10	100 µL	98	Positive
12	200 µL of Tube No. 11	100 µL	65	Negative

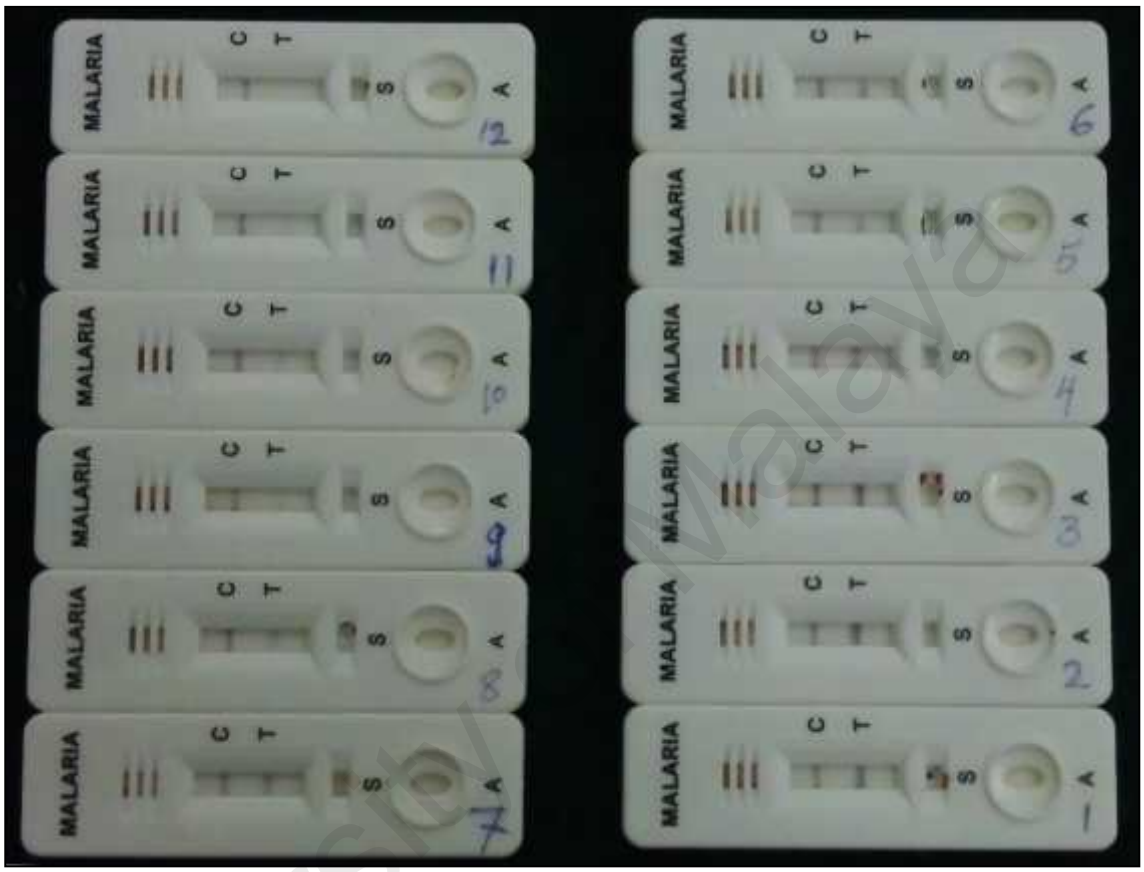


Figure 3.2: Performance of *CareStart*™ malaria HRP2-RDT against serial dilutions of parasite densities

A set of 12 *CareStart*™ malaria HRP2-RDT cartridges, were tested against serial dilutions of *P. falciparum* parasite densities from 5666 parasites/µL (test cartridge No. 1) to 65 parasites/µl (test No. 12): test cartridges 1-8 corresponded to 5666 to 331 parasites/µL showed clear positive results of purple band at the test line (T) in addition to the control line (C). Tests 9-11 (parasitaemia 221-98 parasites/µL) showed faint purple colored band at the test line that means parasite density below 98 showed negative result as no band appeared at the test line.

Table 3.3: Frequency of PfHRP-2 repeat types from *P. falciparum* isolates from Hodeidah and Al-Mahwit, Yemen.

Type	Amino acids repeat	No. of repeats		Mean*	Frequency	
		Min.	Max.		n	%
1	AHHAHHVAD	1	5	3.01	180	100
2	AHHAHHAAD	6	18	9.85	180	100
3	AHHAHHAAY	0	2	1.23	177	98.3
4	AHH	0	2	0.19	28	15.6
5	AHHAHHASD	0	2	0.49	85	47.2
6	AHHATD	2	8	4.11	180	100
7	AHHAAD	3	10	4.97	180	100
8	AHHAAY	0	2	0.52	89	49.4
9	AAY	0	0	0.00	0	0
10	AHHAAAHHATD	1	2	1.25	180	100
11	AHN	0	0	0.00	0	0
12	AHHAAAHHEAATH	1	1	1.00	180	100
13	AHHASD	0	1	0.05	10	5.6
14	AHHAHHATD	0	1	0.03	5	2.8

* Average number of amino acid repeat types in the relevant isolates (n)

3.4 DISCUSSION

Many rapid diagnostic tests (RDTs) are available to detect different malaria-specific antigens. Most RDTs detect *P. falciparum* specific proteins; either histidine rich protein 2 (PfHRP2) or *P. falciparum* lactate dehydrogenase (PfLDH), while others can recognize both *P. falciparum*-specific and pan-specific antigens aldolase (pALD) and pan lactate dehydrogenase (pLDH) (WHO, 2011b). Previous studies have reported genetic variation in PfHRP2 (genetic deletions, frame shift mutations or alterations in protein expression), which can affect the sensitivity of HRP2-based RDTs, while no variability was observed for pALD or pLDH (Baker et al., 2010; Akinyi et al., 2013). The HRP2 of *P. falciparum* is a 2-exons gene connected with an intron, located on chromosome 7 (98671–99734 bp) (NCBI, 2015). The amino acid repeats of pfhrp2 have been characterized into 14 types, based on amino acid motifs being repeated, with Type 2 and Type 7 having been described as possible epitopes targeted by the monoclonal antibodies used to detect hrp2 (Wurtz 2013; Baker et al., 2005; Baker et al., 2010; Lee et al., 2012).

The present study investigated the genetic variation of PfHRP2 among isolates from Yemen, with a possible predicting effect on the performance of the PfHRP2-based RDTs (CareStart® one-step HRP2 RDT; Access Bio Inc., New Jersey, USA) that are used solely for active case detection (ACD) by the national malaria control programme in Yemen (NMCP). A total of 180 isolates were successfully amplified and sequenced for PfHRP2. The sequence lengths of the sequenced isolates varied from 477 to 879 bp (giving proteins of 159 to 293 amino acids). The variations in the number of total amino acids for pfhrp2 for the Yemeni isolates were found to be lower than the global variation (187 to 306 amino acids), as reported for isolates from 19 countries in Africa, South America, the Pacific region and Southeast Asia (Baker et al., 2005). On the other hand, similar variations were reported in Madagascar (145 to 309 amino acids), while a

higher number of variations (157 to 333 amino acids) were reported in isolates collected from six different geographical areas in India (Mariette et al., 2008); Kumar et al., 2012).

Overall, 12 different amino acid repeat types were identified in Yemeni isolates examined by the present study. Of these, six repeat types (types 1, 2, 6, 7, 10 and 12) were found to be present in all isolates, while type 3 was found in 98.3 % of the isolates. These findings are in agreement with isolates from different countries in Africa, Asia and America (Baker et al., 2010). In India, six (types 1, 2, 3, 6, 7 and 11) out of 13 identified types of amino acid repeats were detected in all the examined *P. falciparum* isolates (Kumar et al., 2012). On the other hand, only four types were found in over 98 % of the Senegalese isolates from Dakar (Wurtz et al., 2013; Deme et al., 2014). Our study further showed that repeat types 9 (AAY) and 11 (AHN) were totally absent, a result which has been previously reported in isolates obtained from Senegal, Mali, Uganda and Madagascar (Wurtz et al., 2013; Deme et al., 2014; Mariette et al., 2008).

We found that all of the amplified isolates (100 %) started with a type 1 repeat (AHHAHHVAD), with either a single or multiple of 2 to 5 copies, before ending with a single Type 12 repeat (AHHAAAHHEAATH). Many previous studies in Africa and Asia showed that all pfhrp2 sequences begin with a type 1 repeat and concluded with a type 12 repeat (Deme et al., 2014; Mariette et al., 2008; Baker et al., 2005; Kumar et al., 2012). However, a previous study from Dakar, Senegal on 122 sequenced *P. falciparum* isolates had detected three isolates for which pfhrp2 protein sequences did not begin with a type 1 repeat, and only one isolate found in that study possessed a sequence ending in a type 12 repeat (Wurtz et al., 2013).

The present study also showed that the existence of other repeat types in Yemeni isolates varied from moderate (types 8; 49.4 % and 5; 47.2 %) to low (type 4; 15.6 %). Moreover, other types were found only rarely (types 13; 5.6 % and 14; 2.8 %). Our

findings are consistent with a previous report on Senegalese *P. falciparum* isolates (Wurtz et al., 2013). By contrast, repeat types 11 and 14 were not detected in isolates from Senegal, Mali and Uganda, while type 4 repeats were detected in all of the isolates (Deme et al., 2014). Furthermore, Ugandan isolates were found to be different in that they all contained type 8 (100 %), and lacked type 5 repeats (Deme et al., 2014). Moreover, the number of each motif and the total number of repeats within pfhrp2 vary considerably between countries and within the same country (Mariette et al., 2008; Baker et al., 2005). Yemeni isolates showed high to moderate numbers of the repeat types 2, 7, 6 and 1 within pfhrp2 (means: 9.85, 4.97, 4.11 and 3.01, respectively) which is higher than what it has been reported in Thailand, the Philippines, Madagascar, Papua New Guinea and South America (Mariette et al., 2008; Baker et al., 2005; Rock et al., 1987).

Our findings revealed that the HRP2-based RDT possessed good sensitivity; a low parasitaemia level of 184 asexual stages/µl was strongly detectable by RDT. On the other hand, parasitaemia levels below 100 parasites/ µl were found either weakly detectable (98 parasites/µl) or totally not detected (65 parasites/µl). These results correspond to RDT results on the Yemeni isolates as we found that detection rate increased with parasitaemia level. However, low detection rate (44.4 %) was reported with samples of very low parasitaemia (i.e., < 100 parasites/µl), as well as two samples (positive with microscopy and pfhrp2-PCR) with moderate and high parasitaemia were found negative. These are in agreement with previous studies which revealed that most of the RDT show excellent detection rates for *P. falciparum* at a parasitaemia greater than 500 parasites/µl, with most of the variation reported at relatively low-level parasitaemia (WHO, 2011a; Wurtz et al., 2013; Baker et al., 2010). Moreover, RDT negative results were reported with samples of high-level parasitaemia (Playford & Walker, 2002; Iqbal et al., 2001). This variation could be attributed to either RDT

device-related factors such as poor manufacture and deterioration of the device or parasite-related factors such as the level of parasitaemia, variability in the target epitopes of the parasite antigen, or quantity of parasite antigen produced by the parasite or present in the peripheral blood, or malaria transmission season (WHO, 2011a; Baker et al., 2005; Diarra et al., 2012).

Overall, the present study showed that the sensitivity and specificity of the used HRP2-based RDT were high (90.5 % and 96.1 %, respectively). This was supported by a very good level of agreement between the results of microscopy and RDT tests (Kappa = 0.867). The sensitivity of PfHRP2-based RDT from other reports varied worldwide, ranging from > 90 % (high) to 43.7 % (low) compared to the gold standard microscopy and/or PCR (McMorrow et al., 2008, 2010; Bell et al., 2005; Tahar et al., 2013; Fancony et al., 2013). Within the same context, a previous study conducted at the eastern part of Yemen during a malaria outbreak aimed to evaluate the accuracy of PfHRP2-based RDT among 25 falciparum malaria patients, revealed a sensitivity, specificity and positive predictive value of 74 %, 94 % and 68 %, respectively when compared to the microscopy (Ghouth et al., 2012). However, the small sample size used in the above study, as well as the potential genetic variation in isolates from the eastern part of Yemen collected during an outbreak when compared to the isolates in this study from malaria endemic areas in western Yemen should be taken into consideration.

In the present study, one sample was found positive for falciparum malaria by microscopy and HRP2-RDT while it was negative by PfHRP2-PCR. The DNA quality was confirmed by successful PCR amplification of both falciparum *18S rRNA* and *pfhrp3* genes. Moreover, the HRP2 PCR protocol was tested against falciparum malaria DNA samples with the same and lower parasite densities which all resulted in a successful *hrp2* gene amplification suggesting a *Pfhrp2* gene deletion. Due to *pfhrp3* and *pfhrp2* genes structural homology, *pfhrp3* can cross-react with HRP2-coated

antibodies in the RDT (Wellems & Howard, 1986), and this may explain the false positive PfHRP2-RDT result by our study. On the other hand, *pfhrp2* gene deletion was reported worldwide and more extensively from South America. It was first reported among Peruvian isolates; 41 % of the malaria-microscopically positive isolates have been found negative by RDT, and failed to amplify the PfHRP2 gene by PCR (Gamboa et al., 2010). Later, studies from Brazil and Peru (Akinyi et al., 2013; Houzé et al., 2011; Maltha et al., 2012), Mali in Africa (Koita et al., 2012), and from India in Asia have reported false negative RDT results due to a *pfhrp2* gene deletion (Kumar et al., 2012).

CHAPTER 4: SUSTAINED EFFICACY OF ARTESUNATE-SULFADOXINE-PYRIMETHAMINE AGAINST P. FALCIPARUM IN YEMEN AND A RENEWED CALL FOR AN ADJUNCT SINGLE DOSE PRIMAQUINE TO CLEAR GAMETOCYTES

Wahib M. Atroosh, Hesham M. Al-Mekhlafi, Georges Snounou, Adel Al-Jasari, Hany Sady, Nabil A. Nasr, Yee-Ling Lau, Johari Surin. Sustained efficacy of artesunate-sulfadoxine-pyrimethamine against *P. falciparum* in Yemen and a renewed call for an adjunct single dose primaquine to clear gametocytes. ***Malaria Journal*** **2016;15: 1-11**.

4.1 INTRODUCTION AND LITERATURE REVIEW

The worldwide spread of *Plasmodium falciparum* resistance to anti-malarial drugs led to the development of new strategies to treat malaria and extend the life of novel drugs. Over the last decade, artemisinin-based combination therapy (ACT) has become the cornerstone of malaria treatment policies (White, 2008; WHO, 2015a). This contributed to the global decline in the number of malaria cases (18%) and mortality (48%) between 2000 and 2015 (WHO, 2015a). However, the emergence and spread in Southeast Asia of *P. falciparum* lines with reduced susceptibility to artemisinin (Dondorp et al., 2010; Miotto et al., 2013; WHO, 2014b) raises urgent concern. Mutations in the propeller domain of the parasite's Kelch 13 protein have recently been associated with the delayed clearance that characterizes this resistant phenotype (Ariey et al., 2014). To date, at least 54 single nucleotide polymorphisms (SNPs) have been identified within this domain (WHO, 2014b; Straimer et al., 2015; Tun et al., 2015; Ashley et al., 2014), with C580Y and F446I the most prevalent in Southeast Asia (Tun et al., 2015; Huang et al., 2015; Nyunt et al., 2015; Takala et al., 2015). Numerous novel mutations in the

pfk13 propeller domain have been reported from African *P. falciparum*, but none was associated with artemisinin resistance (Huang et al., 2015; Conrad et al., 2014; Torrentino-Madamet et al., 2014; Kamau et al., 2015; Taylor et al., 2015).

In Yemen, malaria is still a major life-threatening health problem for more than 60% of the population of nearly 25 million people, and in particular in the southwestern corner of the country (WHO, 2014a). The malaria cases are nearly all due to infection by *P. falciparum* (99%), with *Anopheles arabiensis*, *Anopheles sergentii* and *Anopheles azaniae* as the major insect vectors (WHO, 2014a; Al-Eryani et al., 2016). The countries of the Arabian Peninsula are now considered to be malaria-free (Snow et al., 2013), with two exceptions: Yemen where malaria is still prevalent with high rates of mortality and morbidity, and Saudi Arabia where the cases, principally recorded in the southwestern districts bordering Yemen are considered to be mainly imported from the bordering Tehama region of Yemen and from Sudan (Al-Zanbagi, 2014; El Hassan et al., 2015). The decline in chloroquine efficacy in Yemen (Al-Kabsi et al., 2009; Al-Maktari & Bassiouny, 2003; Al-Shamahy et al., 2007; Alkadi et al., 2006) prompted the adoption in 2009 of artesunate in combination with sulfadoxine-pyrimethamine (AS+SP) as the first-line treatment of uncomplicated *P. falciparum* infections, with a combination of artemether with lumefantrine (AL) as a second-line treatment (WHO, 2013). Although clinical efficacy of AS+SP treatment in Yemen has not diminished since its introduction (Al-Kabsi et al., 2009; Adeel et al., 2015), there are indications from the few molecular surveys of *P. falciparum* populations in Yemen that whereas no mutations associated with drug resistance has occurred for *pfdhps*, the prevalence of such mutations in *pfdhfr* is increasing (Mubjer et al., 2011; Al-Hamidhi et al., 2013; Abdul-Ghani et al., 2014; Bamaga et al., 2015b). Finally, it is known that SP does not provide optimal clearance of gametocytes, even when combined with AS, prompting

calls to include monitoring of the sexual stages in drug efficacy studies (Abdul-Ghani et al., 2015).

The present *in vivo* trial aimed to assess the clinical and parasitological efficacy of AS+SP treatment in an area of high endemicity close to the Saudi Arabian border, and to investigate the polymorphisms in the *pfk13* gene propeller domain, *pfdhfr* and *pfdhps* genes of the *P. falciparum* parasites circulating in this area. Furthermore, the impact of the AS+SP treatment on microscopic gametocytaemia was also evaluated throughout the follow-up period.

4.2 MATERIALS AND METHODS

4.2.1 Study area and patients

An active case study among febrile individuals, suspected to have malaria infection, was conducted from March to May 2014 in some malaria-endemic areas in two governorates, Hodeidah and Al-Mahwit located in Tehama region, northwest of Yemen (Figure 3.1). The Hodeidah governorate (14.79° N, 42.97° E) is a coastal area located along the Red Sea in the western part of Yemen, about 226 km from Sana'a, the capital. It is with a total area of 117,145 sq km and a total population of about two million people (NIC, 2014). The Al-Mahwit governorate (16.25° N, 44.717° E) is located about 111 km west of Sana'a, with a total area of 2,858 sq km and a total population of about 600,000 people (NIC, 2014). In Tehama region, the climate is a combination of tropical monsoon with a mean temperature of 37.5ºC in summer and 24ºC in winter. The mean rainfall is 200 mm/year, occasionally during the summer while the weather is dry in winter. Malaria transmission occurs all year around, with a transmission peak from January to March.

Districts and villages with high malaria endemicity were selected for this study based on the malaria incidence records for the year 2013 provided by the National Malaria Control Programme (NMCP), Yemen. These included AdDahi and Bajil Districts in Hodeidah governorate, and Khamis Bani-Saad District in Al-Mahwit governorate. According to the NMCP records for 2013 (Figure 3.1), Hodeidah had the highest number of malaria cases (15,001 – 50,252 cases) followed by Sa'dah, Hajjah and Al-Mahwit governorates (northwest Yemen). However, we could not collect samples from Sa'dah and Hajjah governorates because of the state of civil war during the proposed sampling period. The research team comprised of the Principle Investigator, two medical laboratory experts and a physician who visited each study area and established a station. The heads of the villages were informed about the objectives and the proposed procedures in order to obtain their permission to initiate the study. Afterwards, the village heads asked all residents to present themselves at the station where they were informed of the aims and procedures of the study, and the modalities of their participation. Those who voluntarily agreed to take part in the study were assessed for eligibility. The team also enquired about any individuals who could not attend because of illness or fever and these persons were visited at their home settings. The population in the villages sampled had minimal travel histories.

A total of 622 febrile individuals were examined in ten villages: Halalah, Al-Huamarah, Al-Meshaahra, Al-Rakib, Al-Rufae, Al-Sharjah, Deer Shareef, Kidf Zumailah, Shat Hajal, and Siraj in addition to the city of Bajil. Of these, 86 *P. falciparum*-positive patients were involved in the *in vivo* study.

4.2.2 *Plasmodium falciparum* isolates

A finger-prick blood sample was collected from each participant for rapid diagnostic test (RDT) (CareStart™ Malaria HRP2, Cat. no. G0141, Access Bio, Inc, USA), for preparing thick and thin blood smears, and to obtain blood spots on 3MM Whatman® filter paper (Whatman International Ltd, Maidstone, UK). The filter papers were left to dry in air away from dust and kept in an aluminium foil pouch with desiccant silicon bags at room temperature (25-27ºC) until use. This sample obtained prior to the administration of treatment (for those that had a confirmed malaria diagnosis) and was considered to be the day 0 sample.

The blood smears were Giemsa-stained prior to microscopic examination for the identification of parasite species and stages. The parasitaemia was determined using a modified procedure for counting the asexual stages. Parasites were enumerated against 300 white blood cells (WBC) with a hand tally counter and the number was then multiplied by 25 to obtain the number of parasites per µL of blood (assuming an average of 7,500 WBC/µL blood). The parasitaemia was graded as low (<1,000 parasites/µL blood), moderate (1,000–9,999 parasites/µL blood), or severe (≥10,000 parasites/µL blood). The presence of gametocytes was examined against 500 WBC; though gametocytaemia was treated as qualitative variable in data analysis. Each microscopy-positive slide and a selection of negative slides were examined by two senior specialists and the average parasite count was considered.

4.2.3 *In vivo* test

All the patients who fulfilled the inclusion criteria, including age of six months and above, axillary temperature of ≥ 37.5 °C or history of fever within the last 72 h, microscopically confirmed as uncomplicated *P. falciparum* mono-infection, and ability to swallow oral medication were invited to participate in the *in vivo* trial (Appendix A &

F). Details concerning the study, its benefits and any possible potential risks were fully explained. Only those who willingly agreed to participate and gave informed written and signed consents as well as declared their willingness to comply with the study protocol for the duration of the study and to comply with the study visit schedule were enrolled in the study (Appendix C). Patients who had signs of severe or complicated falciparum malaria or had mixed infection with another *Plasmodium* species (*P. vivax*) or had febrile conditions due to diseases other than malaria or had a history of adverse effects due to any anti-malarial drug or any other medicines were excluded from the *in vivo* study. Moreover, pregnant women and females with positive pregnancy test or females who were unable to or unwilling to do a pregnancy test were also excluded.

Treatment efficacy was evaluated by monitoring the clinical and parasitological parameters over a 28-day follow-up period from the day the first drug dose was administered (day 0). The methods of treatment, schedule of follow-up and analysis of outcomes were as per the WHO guidelines (WHO, 2009). The drug dosage was based on body weight and, according to the national policy of malaria treatment. After treatment administration, patients were observed for at least 30 min to make sure that the medicine was not lost through vomiting or diarrhoea. If vomiting or diarrhoea occurred within 30 min of treatment, a full treatment dose was repeated. Patients then underwent regular clinical and parasitological assessment for another seven specific days after the day of first dose (day 0): days 1, 2, 3, and 7 and then weekly on days 14, 21 and 28. Patients were advised to contact the Principal Investigator at any time during the follow-up period if symptoms returned or in case of appearance of any one of these danger signs: unable to drink or breastfeed (in case of children), severe vomiting, presenting with convulsions, lethargic or unconscious, or difficult breathing, or appearance of jaundice or black urine. Additional clinical and parasitological assessment were obtained in such cases. Patients who missed days 1 and 2 follow-up or

subsequently missed one dose of the treatment were withdrawn from the study. After day 3, patients who were lost to follow-up on day 7 but were present on days 6 or 8 (likewise days 13/15, days 20/22, days 27/29 for day 14, day 21 and day 28, respectively), were still included in the study. However, absence from the follow-up after day 3 for more than one day led to withdrawal from the study. Treatment outcomes were classified as early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), or adequate clinical and parasitological response (ACPR), according to the WHO guidelines (WHO, 2009). [Appendix G & K]

4.2.4 Treatment regimen

ACT in the form of AS+SP is the first-line treatment in Yemen for uncomplicated falciparum malaria. AS+SP used in this study was provided by WHO through NMCP: 50 mg artesunate tablets in a strip of 12 tablets (Artesunate, Batch No. AS 120901, Guilin Pharmaceutical Co Ltd, China) and sulfadoxine 500 mg/pyrimethamine 25 mg tablets in a pack of 1,000 tablets (Batch No. SP IH0132, Micro Labs Ltd, The Netherlands). The drug was administered at a daily dose of artesunate 4 mg per kg of body weight (BW) for three successive days (days 0, 1 and 2) plus a single dose of 25/1.25 mg per kg BW of SP, respectively, on the first day (day 0). Additionally, NMCP provided the study with the second-line treatment AL should AS+SP therapeutic failure occur, and parenteral quinine therapy for those who might vomit the treatment twice and/or could not take the medication orally, for pregnant women during first trimester, for patients with severe or complicated malaria and in case of AL therapeutic failure (Appendix I).

4.2.5 DNA extraction

Two to three discs (6-mm diameter) of the blood spots dried on the filter paper were cut using a flamed-sterile puncher, and were then used for DNA extraction using a Qiagen blood and tissue kit (QIAGEN, DNeasy® Blood & Tissue Kit, Cat. no. 69506, Germany) according to manufacturer's instruction. DNA was eluted using 100 µL of the elution buffer AE (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0) and kept at -20^{o}C until use.

4.2.6 Species-specific PCR identification of malaria parasites

All malaria-positive samples from patients recruited to the *in vivo* study were subjected to PCR confirmation, targeting the small sub-unit ribosomal RNA genes (Singh et al., 1999) prior to any further molecular analyses. Only samples that were positive for *P. falciparum* were considered for molecular characterization of anti-malarial drug resistance. Species other than *P. falciparum* were not detected in any of the admission samples.

4.2.7 *Msp1*, *msp2* and *glurp* genotyping

Recurrent cases were distinguished from new infections (PCR-correction) according to the protocols described previously (Snounou & Beck, 1998; WHO, 2008) for the amplification and analysis of selected polymorphic domains of the merozoite surface protein-1 (*msp1*), merozoite surface protein-2 (*msp2*) and glutamate rich protein (*glurp*) genes.

Amplification of the three markers was achieved using nested PCR (Snounou et al., 1999). Amplicons were visualized in 2.5% of agarose gel stained with Sybr® safe DNA gel stain (Invitrogen, USA) using UV documenting system (Bio-Rad, Hercules, CA, USA). The allelic variants observed were then grouped into different size bins of

25 bp for *msp1* and *msp2*, and of 50 bp for *glurp*. Details of primers sequences and PCR conditions for amplifying *msp1*, *msp2* and *glurp* are in Table 4.1.

For those cases where parasites were observed during the follow-up period, *msp1* and *msp2* allelic variants were compared between the samples obtained before and after treatment (i.e. the base line day 0 sample, and the one collected at the recurrent episode) by capillary electrophoresis in order to increase sensitivity and resolution. Forward oligonucleotide primers of secondary PCR for *msp1* (M1-KF, M1-MF and M1-RF) and for *msp2* (M2-FCF and ICF) were labelled with 6-FAM fluorescent dye. Amplicons were analysed by the automated ABI 3730XL Genetic Analyzer and then interpreted using GeneMapper® analysis software version 4.0 (Applied Biosystems, USA).

4.2.8 Gene mutation of molecular markers of anti-malarial drug resistance

Selected domains of the parasite genes associated with drug resistance were amplified by conventional or nested PCR using genomic DNA purified from the *P. falciparum* isolates, and the mutations identified following direct sequencing or through restriction fragment length polymorphism (RFLP) analysis.

4.2.8.1 *Pfk13* propeller domain: A single run PCR using forward K13-F (5'-GTTGGTGGAGCTATTTTTGAAACATCTAG-3') and reverse K13-R (5'-GCCAAGCTGCCATTCATTTGTATC-3') primers that were designed based on *P. falciparum* 3D7 kelch gene (PF13_0238, Gene ID: 814205), flanking 1062 bp amplicon that corresponds to nucleotides 1094-2127 (codons 364-709). The PCR products were then visualized by 1% agarose gel stained with Sybr® safe DNA gel stain under UV, then gel-purified, sequenced in both directions, and the resulting sequences aligned using BioEdit Sequence Alignment Editor Software (version 7.1.9) and compared to *P. falciparum* 3D7 Kelch 13 gene (Gene ID: 814205). All positions were evaluated for the

presence of mutations. Details of the primers sequences and PCR conditions for detecting mutations in K13 gene are in Table 4.2.

4.2.8.2 *pfdhfr* and *pfdhps*: *Pfdhfr* was analysed for mutations at six codons (A16V, C50R, N51I, C59R, S108N, and I164L) and *pfdhps* at seven codons (S436A/F, A437G, K540E, A581G, A613T/S, I640F, and H645P). Amplification of *pfdhfr* was achieved by a modified nested PCR protocol (Tinto et al., 2007) followed by purification and sequencing of the 700 bp amplicons instead of restriction enzymes (RE) digestion. The primary reaction was performed using Amp1 (5' TTTATATTTTCTCCTTTTTA-3') and Amp2 (5'- CATTTTATTATTCGTTTTCT-3') oligonucleotides pair, and the secondary amplification reaction using SP1 (5'- ATGATGGAACAAGTCTGCGAC-3') and SP2 (5'- ACATTTTATTATTCGTTTTC-3') (Isozumi et al., 2015). The PCR protocol for *pfdhps* amplification was slightly modified: the upstream primers for both primary and secondary PCR were changed so as to yield a longer amplicon (1,005 bp). PCR amplification was performed using PS1-F (5'-GAATTTTTATCCATTCCTCATG-3') + O2 (5'-TTCCTCATGTAATTCATCTGA-3') and PSA-F (5'- GTATACAACACACAGATATAG-3') + O2 primers pairs for the primary and secondary amplification reactions, respectively (Tinto et al., 2007). All PCR products were visualized in 1.5% agarose gels and the PCR products were purified, sequenced and then aligned using BioEdit Sequence Alignment Editor Software compared to *dhfr* (ID: 9221804) and *dhps* (ID: 2655294) genes sequences of 3D7 *P. falciparum*. (Table 4.2).

4.2.9 Data analysis

Data were double entered into Microsoft Office Excel 2007 spreadsheets and cross-checked for accuracy before being exported to IBM SPSS statistical package version 20 (IBM Corp, NY, USA) for data analysis. For descriptive analysis, frequency and proportion were used to present the distribution categorical variables. All quantitative variables were examined for normality by Shapiro-Wilk test before analysis. Statistical associations between point mutations and explanatory variables, including age, gender, sites, and parasitaemia, were assessed using the Chi-square test or Fisher's Exact test where applicable. Per-protocol and Kaplan-Meier survival analysis was used to evaluate the treatment outcome and to plot the decline in gametocytaemic cases after treatment among individuals who were gametocyte-positive at enrolment. Patients were censored from the per-protocol analysis of treatment outcome if they were lost to follow-up, identified with re-infection (after PCR-correction) or decided to withdraw from the study, but they were considered in the Kaplan-Meier survival analysis until the day of withdrawal. A *P* value of <0.05 was considered statistically significant.

4.2.10 Ethical statement

The study protocol was approved by the Medical Ethics Committee of the University of Malaya Medical Centre, Kuala Lumpur (Ref. 974.19), and by the Ministry of Health and the NMCP in Yemen (Appendix E). At the villages, the residents were informed about the aims and procedures of the study. Moreover, the participants were also informed that they could withdraw from the study at any point of time without citing reasons for doing so. Afterwards, written and signed or thumb-printed informed consents were taken from adult participant or from the parents on behalf of their children before enrolment, as approved by the mentioned Ethics Committees (Appendix C). The consent request form was translated into Arabic language and was read entirely

to the patients or their parents/guardians before they were asked to sign the document (Appendix C). Furthermore, permission for pregnancy testing was sought from married female participants aged 18 years and above. RDT-positive individuals were treated according to the national malaria treatment policy, Ministry of Health and Population, Yemen (as stated in Methods). Any patient who decided not to participate or to continue in the *in vivo* study was also treated and followed-up until total clearance of parasitaemia by day 28.

Table 4.1: Oligonucleotide sequences and cycling conditions for genotyping *P. falciparum* msp1, msp2 and glurp genes

	Gene-Family	Primer name	Primer sequence	Thermal conditions
Primary PCR	MSP-1	M1-OF M1-OR	5'-CTAGAAGCTTTAGAAGATGCAGTATTG-3' 5'-CTTAAATAGTATTCTAATTCAAGTGGATCA-3'	1 cycle (95 ^{O}C/5 min, 58 ^{O}C/2 min, 72 ^{O}C/2 min) 25 cycles (94 ^{O}C/1 min, 58 ^{O}C/2 min, 72 ^{O}C/ 2min) Extension at 72 ^{O}C/2 min Annealing at 58 ^{O}C/2 min Final extension at 72 ^{O}C/5 min
	MSP-2	M2-OF M2-OR	5'-ATGAAGGTAATTAAAACATTGTCTATTATA-3' 5'-CTTTGTTACCATCGGTACATTCTT-3'	
	Glurp	G-OF G-OR	5'-TGAATTTGAAGATGTTCACACTGAAC-3' 5'-GTGGAATTGCTTTTTCTTCAACACTAA-3'	
Secondary PCR	MSP1-K1	M1-KF_FAM M1-KR	/56-FAM/AAATGAAGAAGAAATTACTACAAAAGGTGC 5'-GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3'	1 cycle (95 ^{O}C/5 min, 61 ^{O}C/2 min, 72 ^{O}C/2 min) 25 cycles (94 ^{O}C/1 min, 61 ^{O}C/2min, 72 ^{O}C/2min) Extension at 72 ^{O}C/2 min Annealing at 61 ^{O}C/2 min Final extension at 72 ^{O}C/5 min
	MSP1-MAD20	M1-MF_FAM M1-MR	/56-FAM/AAATGAAGGAACAAGTGGAACAGCTGTTAC 5'-ATCTGAAGGATTTGTACGTCTTGAATTACC-3'	
	MSP1-RO33	M1-RF_FAM M1-RR	/56-FAM/TAAAGGATGGAGCAAATACTCAAGTTGTTG 5'-CATCTGAAGGATTTGCAGCACCTGGAGATC-3'	
	MSP2-FC27	M2-FCF_FAM M2-FCR	/56-FAM/AATACTAAGAGTGTAGGTGCARATGCTCCA 5'-TTTTATTTGGTGCATTGCCAGAACTTGAAC-3'	
	MSP2-IC	M2-ICF_FAM M2-ICR	/56-FAM/AGAAGTATGGCAGAAAGTAAkCCTYCTACT 5'-GATTGTAATTCGGGGGATTCAGTTTGTTCG-3'	
	Glurp	G-NF G-OR	5'-TGTTCACACTGAACAATTAGATTTAGATCA-3' 5'-GTGGAATTGCTTTTTCTTCAACACTAA-3'	

Msp1: merozoite surface protein 1, *Msp2*: merozoite surface protein 2, *Glurp*: glutamate rich protein.

Table 4.2: Oligonucleotide sequences and cycling conditions for genotyping *P. falciparum k13*, *pfdhfr* and *pfdhps* genes

Gene	Primer	Sequence	Size (bp)	Thermal conditions
Kelch 13	K13-F	GTTGGTGGAGCTATTTTTGAAACATCTAG	1062	94 ^{O}C/5 min 40 cycles (94 ^{O}C/30 sec, 60 ^{O}C/90 sec, 72 ^{O}C/90 sec) 72 ^{O}C/10 min
	K13-R	GCCAAGCTGCCATTCATTTGTATC		
Pfdhfr	Amp1	TTTATATTTTCTCCTTTTTA	718	94 ^{O}C/5 min 30 cycles (94 ^{O}C/30 sec, 49 ^{O}C/60 sec, 72 ^{O}C/60 sec) 72 ^{O}C/5 min
	Amp2	CATTTTATTATTCGTTTTCT		
	SP1	ATGATGGAACAAGTCTGCGAC	700	94 ^{O}C/5 min 25 cycles (94 ^{O}C/30 sec, 49 ^{O}C/60 sec, 72 ^{O}C/60 sec) 72 ^{O}C/5 min
	SP2	ACATTTTATTATTCGTTTTC		
Pfdhps	PS1-F	GAATTTTTATCCATTCCTCATG	1028	94 ^{O}C/5 min 30 cycles (94 ^{O}C/60 sec, 56 ^{O}C/2 min, 72 ^{O}C/60 sec) 72 ^{O}C/5 min
	O2	TTCCTCATGTAATTCATCTGA		
	PSA-F	GTATACAACACACAGATATAG	1005	94 ^{O}C/5 min 25 cycles (94 ^{O}C/60 sec, 56 ^{O}C/2 min, 72 ^{O}C/60 sec) 72 ^{O}C/5 min
	O2	TTCCTCATGTAATTCATCTGA		

Pfdhfr: Plasmodium falciparum dihydrofolate reductase, Pfdhps: Plasmodium falciparum dihydropteroate synthase, bp: base pare.

4.3 RESULTS

Of the 622 individuals screened, 188 (30.2%) and 189 (30.4%) individuals were found positive for malaria by using the CareStart^{TM} HRP2-RDT and microscopy, respectively. However, only 89 met the inclusion criteria and consented to be enrolled in the study. All were given AS+SP and followed up for 28 days. Two patients were lost to follow-up from day 14 onwards and one patient refused to continue from day 2, leaving 86 patients (40 males and 46 females; aged between eight months and 65 years, mean age of 12.4 years) who were successfully followed up until day 28. Admission asexual parasite levels varied from 561 to 55,555 and with mean parasitaemia of 8,199 parasites/μL blood. The general characteristics of the 86 participants are shown in Table 4.3.

Overall, asexual parasite clearance was rapid as no patient was found parasitaemic on days 3 and 7. By day 1, 51 patients (59.3%) were cleared of parasites and only four (4.7%) patients still had microscopically detectable parasitaemia on day 2 which subsequently cleared on day 3. Asexual parasites with fever reappeared (recrudescent/re-infection cases) on day 14 in two patients, on day 21 in another, and finally on day 28 in two further patients (Table 4.4). Interestingly, these five patients were from Khamis Bani-Saad District of Al-Mahweet governorate, from the following villages: two from Deer Shareef, one from Al-Meshaahra, one from Shat Hajal, and one from Al-Rufae. Table 4.6 shows the parasitological and clinical outcomes reported among the patients treated with AS+SP.

After Kaplan-Meier analysis, therapeutic outcome of AS+SP was 94.2% (before PCR-correction) with 81 cases showing adequate clinical and parasitological response (Figure 4.1. a). After PCR-correction, three cases were classified as late clinical failure (recrudescences) as the genotypes of the parasites from the pre-treatment samples (i.e.

samples collected on day 0 before the first dose of treatment was administered) and those on the day of parasite reappearance were similar. The remaining two cases were classified as new infections (Table 4.4 & 4.5). Thus, the PCR-corrected cure rate increased to 96.5% (Figure 4.1. b). All reported treatment failures were observed in the age groups under the age of 15 (four below five years of age and one aged between five to fifteen). Overall, the mean age of the five patients who were classified as LCF was significantly lower than patients who were classified as ACPR (3.3 years vs. 12.9 years; $P = 0.006$). Moreover, almost similar mean baseline parasitaemia was reported between the LCF and the ACPR cases (8,375 vs. 8,188 parasites/µL; $P = 0.612$).

Table 4.3: General characteristics of the participants at enrolment (n = 86)

Variable	Frequency	%
Governorate		
Hodeidah	28	32.6
Al-Mahwit	58	67.4
Gender		
Male	40	46.5
Female	46	53.5
Age group (years)		
< 5	20	23.3
5-15	50	58.1
>15	16	18.6
History of fever	86	100.0
Weight (kg)	20.0 (17.5) [b]	-
Haemoglobin level (g/dl)	10.2 (1.9) [a]	-
Parasitaemia group (asexual parasites/µL)		
Parasitaemia on day 0	5247 (9648) [b]	-
≤ 999	13	15.1
1,000-9,999	50	58.1
≥ 10,000	23	26.7
Type of house		
Wooden	44	51.2
Cement	27	31.4
Cement & wood	15	17.4
Source of drinking water		
Piped-water	22	25.6
Well	35	40.7
Stream/river	29	33.7
Having electricity supply	69	80.2

Table 4.3, continued

Having vehicles		
Don't have	72	83.7
Motorcycle	9	10.5
Car/truck	4	4.7
Motorcycle & car	1	1.2
Having radio	35	40.7
Having TV	30	34.9
History of IRS (in the last 12 months)		
No	59	68.6
Yes	21	24.4
Don't remember	6	7.0
Mosquito bed nets		
Having bed nets	45	52.3
Using bed nets	28	32.6
Source of bed nets (government)	45	100.0
What do you do first when you have fever?		
Go to clinic/hospital	47	54.7
self -treatment	18	20.9
Do nothing	21	24.4

IRS indoor residual spraying

[a] Mean (SD)

[b] Median (interquartile range)

Table 4.4: Parasitaemia clearance and re-appearance of the five recrudescent/re-infection cases

CASE NUMBER	DAY	*MSP1*		*MSP2*			*GLURP*	OUTCOME
		K1	MAD20	RO33	FC27	IC		
25	0	180	-	-	300	-	1000	Recrudescence
	21	180	-	-	300	-	1000	
85	0	-	-	150	450	-	900	New infection
	14	200	-	-	450	500	1000	
255	0	180	-	-	-	500	1000	Recrudescence
	28	180	-	-	-	500	1000	
257	0	180	-	150	300	600	900	Recrudescence
	14	180	-	150	300	600	900	
383	0	230	200	-	350	-	900	New infection
	28	-	230	-	280	-	900	

MSP1: merozoite surface protein 1, MSP2: merozoite surface protein 2, *GLURP*: glutamate rich protein.

Table 4.5: Genotyping data for the five patients with renewed clinical activity during follow-up (using capillary electrophoresis fragment analysis).

Case Number	Sample File Name	Marker	Size (bp)
25/K1-0	1st_BASE_241189_K1-25_0.fsa	FAM-1	185.64
25/K1-21	1st_BASE_241190_K1-25_21.fsa	FAM-1	185.72
255/K1-0	1st_BASE_241191_K1-255_0.fsa	FAM-1	185.82
255/K1-28	1st_BASE_241192_K1-255_28.fsa	FAM-1	185.83
257/K1-0	1st_BASE_241193_K1-257_0.fsa	FAM-1	185.59
257/K1-14	1st_BASE_241194_K1-257_14.fsa	FAM-1	185.54
257/RO33-0	1st_BASE_241197_RO33-257_0.fsa	FAM-2	151.16
257/RO33-14	1st_BASE_241198_RO33-257_14.fsa	FAM-2	151.22
25/FC27-0	1st_BASE_241205_FC27-25_0.fsa	FAM-3	324.6
25/FC27-21	1st_BASE_241206_FC27-25_21.fsa	FAM-3	324.6
257/FC27-0	1st_BASE_241207_FC27-257_0.fsa	FAM-3	324.52
257/FC27-14	1st_BASE_241208_FC27-257_14.fsa	FAM-3	324.52
255/IC-0	1st_BASE_241213_IC-255_0.fsa	FAM-4	~ 555
255/IC-28	1st_BASE_241214_IC-255_28.fsa	FAM-4	~ 555
257/IC-0	1st_BASE_241215_IC-257_0.fsa	FAM-5	~ 650
257/IC-14	1st_BASE_241216_IC-257_14.fsa	FAM-5	~ 650
CONTROL 1	1st_BASE_1_Control.fsa	FAM	200.00
CONTROL 2	1st_BASE_1_Control.fsa	FAM	300.00

Table 4.6: Summary of parasitological and clinical outcomes among patients treated with AS + SP after 28 days of follow-up

Classification	Without PCR correction		PCR-corrected	
	Number	**Proportion (95% CI)**	**Number**	**Proportion (95% CI)**
EFT	0	0 (0)	0	0 (0)
LCT	5	5.8 (0.019, 0.130)	3	3.5 (0.007, 0.099)
LPF	0	0 (0)	0	0 (0)
ACPR	81	94.2 (0.870, 0.981)	83	96.5 (0.901, 0.993)
Total analysis	86			
WTH	1			
LFU	2			
Total	89			

CI Confidence interval, *ACPR* Adequate clinical and parasitological response, *ETF* Early treatment failure, *LCF* Late clinical failure, *LPF* Late parasitological failure, *LFU* Loss to follow-up, *WTH* Withdrawn.

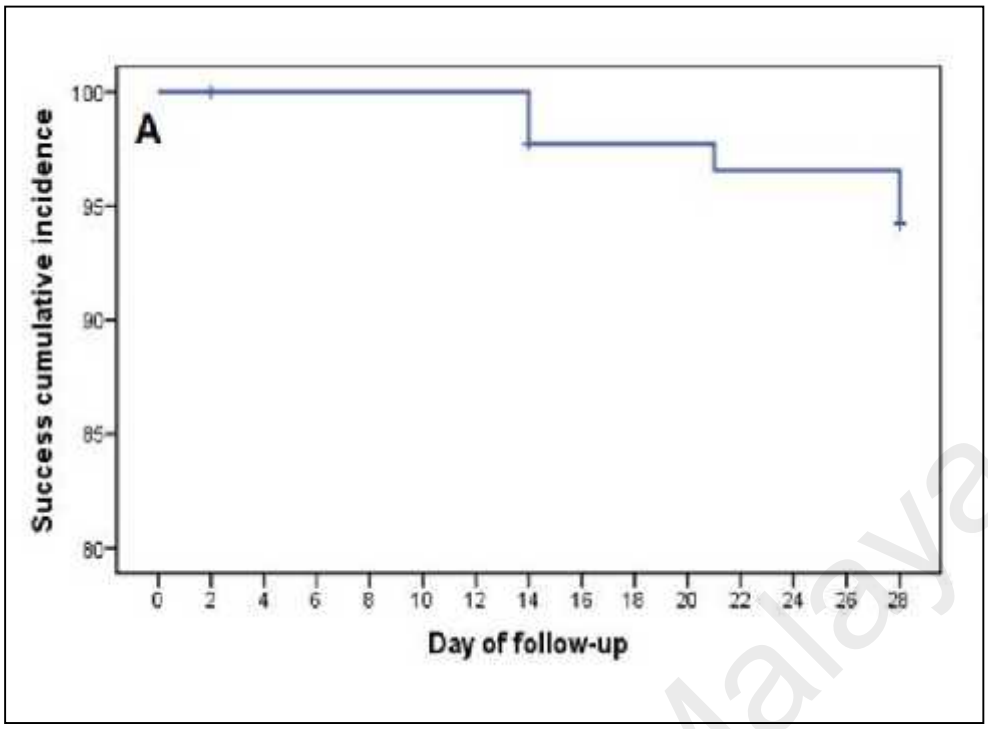


Figure 4.1. a: Kaplan-Meier curves showing treatment success cumulative proportion for the population under study for AS + SP up to day 28 of follow-up (PCR-uncorrected).

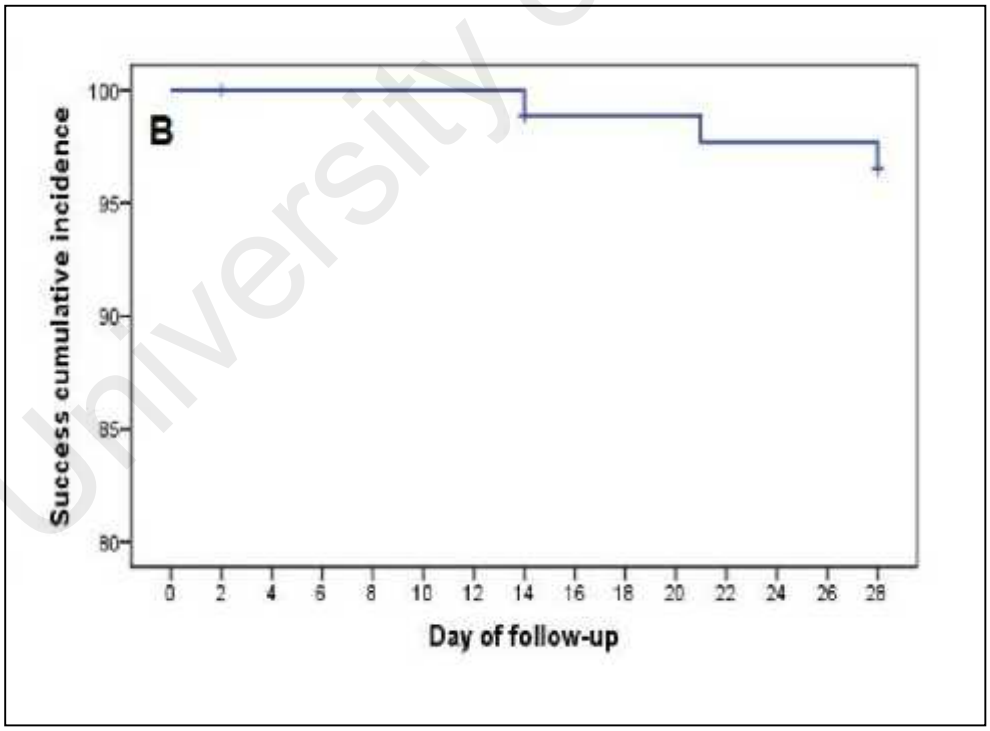


Figure 4.1. b: Kaplan-Meier curves showing treatment success cumulative proportion for the population under study for AS + SP up to day 28 of follow-up (PCR-corrected).

4.3.1 Molecular markers of drug resistance

4.3.1.1 *Pfk13*-propeller: Sequences were obtained from all 86 (100%) isolates collected in this study. None of the study isolates analysed for mutations of the *pfK13* propeller domain (including the three recrudescent isolates) carried any mutations at the 39 codons previously published to be associated with artemisinin resistance (Ariey et al., 2014; Huang et al., 2015; Isozumi et al., 2015).

4.3.1.2 *Pfdhfr* and *pfdhps*: For *pfdhfr* gene, sequences were obtained from 81 (94.2%) of the study isolates. Double mutations at S108N and N51I were observed in 53 isolates (65.4%), and all the other codons (16, 50, 59, and 164) were found to be wild type. Thus, the ACICNI *pfdhfr* haplotype was predominant (65.4%) over the wild-type ACNCSI haplotype. For *pfdhps,* sequences were obtained from 76 isolates (88.4%), and no mutations were observed for codons 436, 437, 540, 581, and 613, though mutations were in two other codons: 640 (n=6) and 645 (n=4). The distribution of the *pfdhfr* double-mutated haplotype frequency was found significantly associated with the districts and sites from which the participants were recruited: in 87.5 and 80.4% of those from Ad-Dahee and Khamis Bani-Saad, respectively, while patients from Bajil districts harboured parasites that were mostly wild type (5.9% with mutated haplotypes). Parasites obtained from patients from the villages of Al-Humarah (AdDahi) and Shat Hajal (Khamis Bani-Saad) all carried the double mutation. No association was reported between the frequency of *pfdhfr* mutations and other variables such as sex and age, admission parasite density, or gametocytaemia.

4.3.2 Gametocytaemia

Gametocytes were observed in 69 of the 189 persons found infected with *P. falciparum* during the initial survey, and a similar proportion of the patients recruited (35/86) were also gametocytaemic at the initiation of AS+SP treatment. Most of these patients still harboured gametocytes by day 7 post-treatment, and 14% were still gametocytaemic at the end of the trail follow-up period (Figure 4.2).

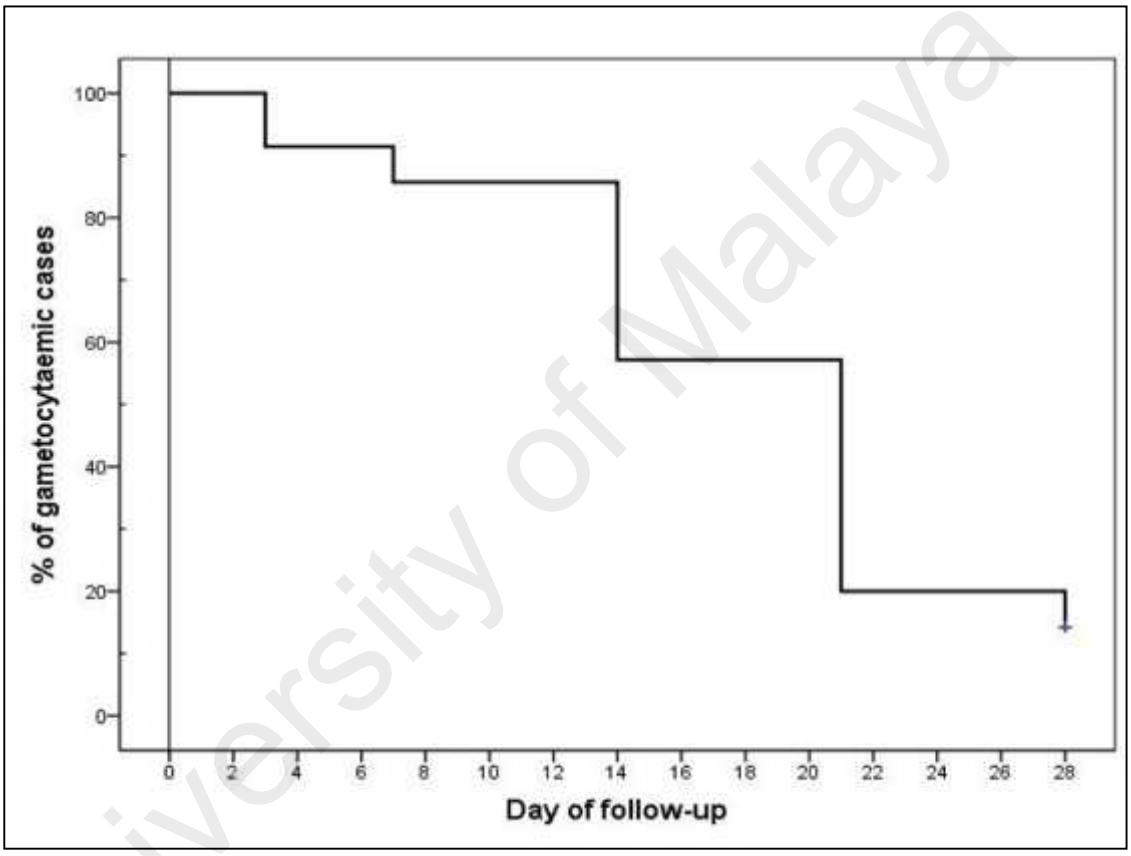


Figure 4.2: Kaplan-Meier curves showing time to disappearance of microscopic gametocytaemia in gametocytaemic individuals at enrolment and following AS+SP treatment (n = 35)

4.4 DISCUSSION

ACT is now the recommended first-line treatment in nearly all the malaria-endemic countries of the world (all Caribbean and Central American countries, except Panama, still rely on chloroquine + primaquine). High levels of resistance to SP have generally precluded the selection of SP as the ACT companion drug, and the AS+SP combination has been uniquely adopted in countries of WHO's Eastern Mediterranean Region (except for Djibouti) and India where it is supplemented by primaquine, but it is administered alone in Azerbaijan, Somalia, Sudan, and Yemen. Increased frequencies of mutations associated with SP failure have been recorded in Sudan (Gadalla et al., 2013**)**, and in particular in Somalia where unacceptable levels of treatment failure (22%) were recorded in one area (Warsame et al., 2015**)**.

The study presented here indicates that the efficacy of AS+SP treatment is still high in Yemen (>95%), which concords with the only other *in vivo* efficacy trial that was recently conducted in the same country (Adeel et al., 2015). In both studies, asexual parasitaemia was cleared by day 3 post-treatment, and only a few PCR-confirmed cases of true recrudescence were observed. In neither study were drug levels in those where treatment failed measured, and drug absorption or metabolism as a basis for failure could not be formally excluded. It is noteworthy that the three true recrudescences detected in the current study originated from the district with the highest percentage of malaria infection in surveyed residents (Khamis Bani-Saad district in Al-Mahwit governorate) and where all the isolates had the *pfdhfr* double mutation. Given high cure rate and the absence of resistance-associated mutations in the *pfdhps* gene and the rarity of triple mutations in the *pfdhfr* gene in Yemen (Bamaga et al., 2015b), a recommendation to abandon SP as the partner drug is not warranted in Yemen. Nonetheless, constant monitoring of AS+SP efficacy should be a priority, not least because SP tablets are available in pharmacies as well as ordinary stores in rural areas,

where they are kept at home and used for self-medication, a practice that could contribute to a rapid emergence of SP resistance. Previous studies revealed that CQ, quinine and SP are the most available anti-malaria drugs in private drug stores in Yemen. They are prescribed for uncomplicated falciparum malaria, and when it is financially possible they are used for self-administration, particularly in rural areas (Ghouth, 2013; Bashrahil et al., 2010).

In neighbouring and other Eastern Mediterranean countries, a similar profile of *pfdhfr* and *pfdhps* mutations has been reported in the Jazan region in southwest Saudi Arabia that shares borders with our study area (Bin Dajem et al., 2012), and in Afghanistan where the *pfdhfr* double mutation (C59R, S108N) predominates (Awab et al., 2013). Despite the high frequency of mutations in both *pfdhfr* and *pfdhps* genes reported in Pakistan, the triple *pfdhfr* mutant was not observed, though 52% (88/170) of the isolates studied harboured the *pfdhps* A437G mutation with the double mutated (C59R+S108N) *pfdhfr* (Khattak et al., 2013). Studies from south-eastern Iran showed an initial steady reduction in the prevalence of *pfdhfr* + *pfdhps* triple mutations (*pfdhfr* C59R+S108N and *pfdhps* A437G) during 2008-2010 following the adoption of AS+SP in 2007 (Afsharpad et al., 2012), but a significant increase in their prevalence was later reported in the same area during 2012-2014 (Rouhani et al., 2015). Ultimately, SP remains a suitable partner drug to AS for the treatment of uncomplicated falciparum malaria, and regular monitoring for the increase in resistance-associated mutations is warranted.

This first investigation of the *pfk13* propeller domain in *P. falciparum* from Yemen indicated that the parasites are still free of the mutations associated with artemisinin resistance in Asia. This might be due to the relatively short exposure of the parasites to artemisinin as well as the use of SP as the partner drug. Indeed, although ACT was introduced in 2009, its distribution at district level was gradual and its

widespread use is slowed by continuing availability and prescription of chloroquine in both urban and rural areas (Bamaga et al., 2014; Ghouth, 2013; Bashrahil et al., 2010). Nonetheless, the potential for rapid selection and spread of mutants should not be underestimated and *pfk13* should be regularly monitored in Yemen.

In the context of sustainable malaria control, the use of SP for treatment suffers from a major disadvantage, namely a poor efficacy in eliminating the sexual stages. Thus, when SP was assessed in *in vivo* trials alone or in combination with artemisinins, the rapid clearance of asexual parasites was not observed for the gametocytes that often persisted for many days, sometimes throughout the follow-up period, in the circulation and remained infectious to mosquitoes. It is likely that the prevalence of post-treatment gametocytaemia was underestimated when microscopy alone was used to detect the gametocytes. Indeed, numerous studies showed that molecularly detectable sub-microscopic gametocytes are common following treatment and could be sufficient to sustain transmission (Babiker et al., 1999; Bousema et al., 2006; Drakeley et al., 2006; Mens et al., 2008; Okell et al., 2009; Karl et al., 2011). In Yemen, this is exacerbated by a delay in seeking treatment (Al-Taiar et al., 2009), probably due to limited financial resources. In the current study, approximately half of the persons surveyed admitted that they self-medicated or did not seek treatment when fever developed (Table 4.3), and this is reflected in the high proportion of screened patients (69/189, 36.5%) that were gametocytaemic at the time of admission. A pattern similar to the one observed in this study was obtained for the sexual stages in the other *in vivo* trial of AS+SP efficacy conducted in Yemen (Adeel et al., 2015). Given that artemisinin has limited activity against mature gametocytes (Targett et al., 2001; Bousema & Drakeley, 2011), the use of AS+SP in Yemen might provide a selective advantage for the transmission of SP-resistant parasites (Sutherland et al., 2002; White et al., 2012).

The data presented here reinforce the suggestion that the fate of the sexual stages should be considered as an additional factor to measure in standardized protocols of *in vivo* anti-malarial efficacy studies when the objectives of the malaria control program also aim to reduce transmission (Abdul-Ghani et al., 2015). Furthermore, it will be now judicious to recommend the addition of primaquine to the standard AS+SP first-line treatment in Somalia, Sudan and Yemen. Concerns over primaquine dose-dependent haemolytic effects in persons with glucose-6-phosphate dehydrogenase deficiency (G6PD) (Shekalaghe et al., 2010) are allayed by recent WHO guidelines that indicate that a single primaquine dose (0.25 mg base/kg) concomitantly administered on the first day of ACT treatment, is both effective in blocking transmission and unlikely to cause serious toxicity in individuals with any of the G6PD-deficiency variants, obviating the need for systematic and onerous G6PD testing (White et al., 2012; White, 2013; WHO, 2015b).

CHAPTER 5: DIFFERENT PATTERNS OF *PFCRT* AND *PFMDR1* POLYMORPHISM IN *PLASMODIUM FALCIPARUM* ISOLATES FROM TEHAMA REGION, YEMEN

Wahib M. Atroosh, Hesham M. Al-Mekhlafi, Adel Al-Jasari, Salwa S. Dawaki, Fatin N. Elyana, Mona A. Al-Areeqi, Hany Sady, Nabil A. Nasr, Awatif M. Abdulsalam, Lahvanya R. Subramaniam, Meram Azzani, Init Ithoi, Yee Ling Lau and Johari Surin. Different patterns of *pfcrt* and *pfmdr1* polymorphism in *Plasmodium falciparum* isolates from Tehama region, Yemen. ***Peer Journal*** **2016; 4: e2191**

5.1 INTRODUCTION AND LITERATURE REVIEW

Chloroquine (CQ), a safe and cheap antimalarial, has been the drug of choice for treating uncomplicated falciparum malaria over the past few decades. However, its effectiveness has been greatly hampered by the emergence of resistant *Plasmodium falciparum* strains that emerged along the Thai–Cambodian border early in 1957 and then spread to other foci in Asia, and also to South America, Papua New Guinea, and Africa (Moore & Lanier, 1961; Harinasuta et al., 1965; Grimmond et al., 1976; Campbell et al., 1979; Fogh et al., 1979). The emergence and spread of *P. falciparum* strains that are resistant to CQ and other antimalarials has necessitated a switch to new drugs to ensure maximum effectiveness and prevent the development of ongoing parasite resistance. It has been suggested that a combination therapy rather than monotherapies is highly efficient for falciparum malaria case management and malaria control.

Currently, artemisinin combination therapy (ACT) is the cornerstone of malaria treatment policies and control worldwide (Nosten & White, 2007; White, 2008; WHO, 2015a). Interestingly, the replacement of CQ with an ACT in areas where falciparum isolates were CQ-resistant resulted in the re-emergence of CQ-sensitive strains a few years after the cessation of CQ use (Liu et al., 1995; Kublin et al., 2002; Kublin et al., 2003; Mita et al., 2004).

Chloroquine resistance is determined by the major point mutation at codon 76 of the *P. falciparum* CQ resistance transporter (*pfcrt*) gene, through the substitution of lysine amino acid (K) by threonine (T) (Fidock et al., 2000), which is highly correlated with increased clinical CQ tolerance and treatment failure (Djimdé et al., 2001; Wellems & Plowe, 2001; Wongsrichanalai et al., 2002; White, 2004). However, the accumulation of mutations along the *pfcrt* gene, especially at codons A220S, Q271E, N326S, I356T, and R371, in addition to the leader *pfcrt* K76T point mutation was found significantly associated with CQ resistance (Djimdé et al., 2001). Also, point mutations in the *P. falciparum* multidrug resistance 1 (*pfmdr1*) gene have been reported to modulate sensitivity and resistance to multiple antimalarials (Basco et al., 1995; Reed et al., 2000; Sisowath et al., 2005).

In Yemen, malaria is still a major public health problem that threatens more than 60% of the population, with *P. falciparum* causing 99% of the malaria cases (WHO, 2015a). The emergence in Yemen of parasite resistance to CQ as well as to other antimalarials prompted the adoption of ACT in 2009, which includes artesunate plus sulfadoxine-pyrimethamine (AS + SP) and artemether plus lumefantrine (AL) as the first line and second line treatment, respectively, for uncomplicated falciparum malaria infections. However, CQ was retained as the drug of choice for vivax malaria.

Some *in vivo* and *in vitro* studies have been conducted in different malaria-endemic districts of Yemen in order to evaluate the efficacy of CQ against *P. falciparum* isolates. These studies found that CQ resistance varied from 16.1% in the Taiz governorate (Alkadi et al., 2006) to more than 40% in the Lahj (Mubjer et al., 2011) and Hodeidah governorates (Al-Maktari et al., 2003; Al-Shamahy et al., 2007). However, despite these reports and the introduction of a new malaria treatment policy, CQ continues to be available in drug markets and private drug stores in Yemen. It is still prescribed for falciparum malaria in some private health facilities, and is also used by individuals for self-administration, especially in remote malarious areas (Bashrahil et al., 2010; Ghouth, 2013).

Some molecular studies have reported a high prevalence of *pfcrt* 76T mutations (ranging from 88% to 100%) in different districts of Yemen (Al-Mekhlafi et al., 2011; Mubjer et al., 2011; Abdul-Ghani et al., 2013; Al-Hamidhi et al., 2013). Nevertheless, there is still a paucity of information about the full genetic profile of *pfcrt* and *pfmdr1* in the country. Yet, such genetic markers are useful tools for monitoring parasite susceptibility to various antimalarials. In addition, they help researchers to monitor changes in parasite genotypes after a change in treatment policy. Therefore, the present study aimed to investigate the susceptibility of *P. falciparum* to CQ (and some other antimalarials) 5 years after the adoption of an ACT by conducting a molecular analysis of the related mutations in the *pfcrt* and *pfmdr1* genes in isolates from the Tehama region in northwestern Yemen. Furthermore, because there is a diverse market for antimalarials in this region, improving our knowledge about drug pressure would be useful in targeting interventions aimed at reducing inappropriate drug use in the region.

5.2 MATERIALS AND METHODS

5.2.1 Study area and subjects

An active case survey that targeted individuals with fever (as a group suspected to have malaria) was conducted from March to May 2014 in two malaria-endemic districts of the Hodeidah governorate and one in the Al-Mahwit governorate in the Tehama region in northwestern Yemen (Figure 3.1). The Hodeidah governorate (14.79° N, 42.97° E) is located about 226 kilometres from Sana'a, the capital of Yemen. Hodeidah is a coastal area along the Red Sea with a total area of 117,145 km^2 and a total population of 2.16 million (NIC, 2014). The Al-Mahwit governorate (16.25° N, 44.717° E) is located between Hodeidah and Sana'a (about 111 km west of Sana'a), covers a total area of 2,858 km^2, and has a total population of 597,000 (NIC, 2014). The climate in the Tehama region is a combination of tropical monsoons with occasional rain in summer and dry weather in winter with a mean rainfall of 200 mm/year. The mean temperature is 37.5 ºC in summer and 24 ºC in winter with humidity ranging between 70% and 90%. Malaria transmission is perennial, but there is a high malaria transmission peak from January to March. Transmission is known to be more prevalent in Hodeidah.

Villages in the districts with the highest malaria endemicity in the region were chosen based on the national malaria records for the period 2010–2013 that were provided by the National Malaria Control Programme (NMCP), Yemen. Based on these records, samples were collected from 10 villages in the AdDahi and Bajil districts in Hodeidah and the Khamis Bani Saad district in Al-Mahwit. The villages in question were Halalah, Al-Huamarah, Al-Meshaahra, Al-Rakib, Al-Rufae, Al-Sharjah, Deer Shareef, Kidf Zumailah, Shat Hajal, and Siraj. Samples were also collected from the city of Bajil.

The present study employed an *in vivo* efficacy trial that was designed to investigate the effectiveness of the AS + SP therapy in treating uncomplicated malaria and that used the World Health Organization (WHO) 28-day follow-up protocol. Initially, 622 individuals with fever in the 11mentioned sites were examined. From this number, 86 individuals with falciparum malaria monoinfection, who voluntarily agreed to take part in the *in vivo* study, were recruited based on the inclusion criteria in the WHO guidelines (WHO, 2009). All 86 were considered for the investigation of different molecular markers related to resistance to antimalarial drugs, including the *pfcrt* and *pfmdr1* genes.

5.2.2 *Plasmodium falciparum* isolates

A finger-prick blood sample was collected from each participant for use in the RDT test (*CareStart*^{TM} Malaria HRP2-RDT) and for preparing thick and thin blood films and a filter paper blood spot. Blood films were stained with 5% of buffer-diluted Giemsa stain for 30 minutes and examined microscopically for malaria parasites. Filter papers were left to dry in air away from dust and then kept in an aluminium pouch together with desiccated silicon bags until used.

Parasite species and stages were recorded and parasitaemia (parasite density) was determined by counting only the asexual stages against 300 white blood cells (WBCs) and then multiplying by 25, assuming the average of the total WBC count of the individuals was equal to 7500 cells per µl of blood (Billo et al., 2013). The level of parasitaemia was graded as low (<1000 parasites/µL of blood), moderate (1000–9999 parasites/µL of blood), or severe (≥10,000 parasites/µL of blood).

5.2.3 DNA extraction

Two to three discs (6 mm diameter) of 3MM Whatman's filter paper blood spots (cut by a flamed-sterile puncher) were used for DNA extraction, which was performed using a Qiagen blood and tissue kit (QIAGEN, DNeasy® Blood & Tissue Kit, Cat. no. 69506, Germany) according to the manufacturer's instructions. The DNA was eluted using 100 µL of AE (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0) elution buffer (included in the kit) and kept at -20 ºC until used.

5.2.4 Detection of gene mutations in *pfcrt* and *pfmdr1*

All extracted DNA samples of *P. falciparum* were subjected to mutation analysis using polymerase chain reaction (PCR) amplification followed by restricted fragment length polymorphism (RFLP) to investigate the mutations in CQ resistance transporter (*pfcrt*) and multidrug resistance1 (*pfmdr1*) genes.

Amplification of *pfcrt* was performed using the protocol designed by Djimdé et al. (2001) with the aim of detecting mutations at codons 72-76, 220, 271, 326, 356, and 371 of the *pfcrt* gene and at codons 86 and 1246 of the *pfmdr1* gene. The other codon mutations of *pfmdr1* (184, 1034, and 1042) were analysed using the PCR-RFLP protocol in Duraisingh et al. (2000). However, the PCR amplification and restriction enzyme (RE) digestion in the protocol was modified for codon 184 by designing a forward primer MDR184-F (5'- ATAATAATCCTGGATCTAAATTAAGA-3') to replace A4 to amplify an amplicon of 155 bp instead of 560 bp and by using Swa1 endonuclease which cuts once into 123 bp and 32 bp for the mutated allele but not the wild.

Restriction enzyme digestion was done in 20 µL of reaction mixture that consisted of 6–8 µL of PCR product, 1X of the specific buffer (included in the RE kit),

and 1 unit of the specific RE (New England Biolabs Inc., UK), then was incubated for 15–60 minutes (based on the RE) according to the manufacturer's instructions, and then visualised in 2.5%–4.0% of TAE buffered agarose gel (based on the size of the cleaves) stained with Sybr® safe DNA gel stain **(**Invitrogen, USA) using a Bio-Rad Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA, USA). Oligonucleotide primers and restriction enzymes for detecting mutations in *pfcrt* and *pfmdr1* are in Table 5.1.

Genomic DNA of *P. falciparum* strains 3D7 (MRA-102G) and HB3 (MRA-155G) were used as positive controls for the wild types of *pfcrt* and *pfmdr1*, while the Dd2 strain (MRA-150G) was used as positive control for the mutated types. All the strains were provided by the Malaria Research and Reference Reagents Resources Center (MR4, ATCCW, Manassas VA, USA).

5.2.5 Ethical consideration

The study protocol was approved by the Medical Ethics Committee of the University of Malaya Medical Centre, Kuala Lumpur (Ref. 974.19), and by the Ministry of Health and Population, in conjunction with the National Malaria Control Programme in Yemen (Appendix E).

Table 5.1: Detection of point mutation of *Pfcrt* and *Pfmdr1* genes using PCR-RFLP

Gene	PCR	Codon	Primer	Sequence	Size (bp)	Thermal conditions	RE	Target	Cleaves size (bp)
Pfcrt	Primary	72, 74, 75 & 76	76-A	GCGCGCGCATGGCTCACGTTTAGGTGGAG	206	94 ºC /5 min 25 cycles: (94 ºC/30 sec, 50 ºC/90 sec, 65 ºC/90 sec) 65 ºC/10 min	----		
			76-B	GGGCCCGGCGGATGTTACAAAACTATAGTTACC					
	Secondary	72	72MS-F1	TTTATATTTTAAGTATTATTTATTTAAGTGGA	93		FokI	Wild	55 + 38
			76D2-R	CAAAACTATAGTTACCAATTTTG					
		74 & 75	745MS-F	TAAGTATTATTTATTTAAGTGTATGTGTCAT	84		74: NlaIII 75: BspHI	Wild	53 + 31 **(If 75 Wild)**
			76D2-R	CAAAACTATAGTTACCAATTTTG				74 Wild	
		76	76D1	TGTGCTCATGTGTTTAAACTT	145		ApoI	Wild	98 + 43
			76D2	CAAAACTATAGTTACCAATTTTG					
	Primary	200s	CRT-2A	CCCAAGAATAAACATGCGAAAC	706	As above	----		
			CRT-2B	ACAATTATCTCGGAGCAGTT					
	Secondary	220	CRT220-a	TATTTATTTATTTATATATTTTGTTTTCTT**G**C**G**ATTAAGG	154	As above	BglI	Wild	110 + 40
			CRT220-b	ACAATTATCTCGGAGCAGTT					
		271	CRT271-a	GGCACATTTCATTTTATTTTATTTTTTCTTTCCTAATTAAT **GAA**TACGTT	124		XmnI	Mutant	50
			CRT271-b	GGCTATGGTATCCTTTTTCC					
	Primary	300s	CRT3A	CCTTGGCATTGTTTTCCT	533		----		
			CRT3B	CCAAAGTTACGAAATCTAATAATCTTGG					
	Secondary	326	CRT326-a	CCTTTTTATTCTTACATAGCTGGTTATTGAATTATCAC	68		Mse1	Wild	24
			CRT326-b	TGGCATTGTTTTCCTTCT					
		356	CRT356-a	ATATATATGGCTAAGAATTTAAAGTAATAAGCAGTTGCT	100		AlwNI	Mutant	40
			CRT356-b	AATTATCGACAAATTTTCTACC					
		371	CRT371-a	TATTATTTTTACTTTTTAATTTTATAGGGTGATGTCTTAA	80		AflII	Wild	40
			CRT371-b	AAGTTACGAAATCTAATAATCTTGGTTC					

Continued, Table 5.1.

Pfmdr1	Primary	86	MDR-A	GCGCGCGTTGAACAAAAAGAGTACCGCTG	450	As above	----		
			MDR-B	GGGCCCTCGTACCAATTCCTGAACTCAC					
	Secondary		MDR-D1	TTTACCGTTTAAATGTTTACCTGC	291	As above	AflIII	Mutant	126+165
			MDR-D2	CCATCTTGATAAAAAACACTTCTT					
	Primary	1246	1246-A	GGGGGATGACAAATTTTCAAGATTA	295	As above	----		
			1246-B	GGGGGACTAACACGTTTAACATCTT					
	Secondary		1246-D1	AATGTAAATGAATTTTCAAACC	202	As above	BglII	Wild	111 + 90
			1246-D2	CATCTTCTCTTCCAAATTTGATA					
	Primary	184	A1	TGTTGAAAGATGGGTAAAGAGCAGAAAGAG	657	94 ºC /5 min 25 cycles: (94 ºC/30 sec, 45 ºC/60 sec, 72 ºC/60 sec) 72 ºC/5 min	----		
			A3	TACTTTCTTATTACATATGACACCACAAACA					
	Secondary		A2	GTCAAACGTGCATTTTTTATTAATGACCATTTA	155		SwaI	Mutant	123 + 32
			MDR184-F	GATAATAATCCTGGATCTAAATTAAGA					
	Primary	1034 & 1042	O1	AGAAGATTATTTCTGTAATTTGATACAAAAAGC	877	As above	----		
			O2	ATGATTCGATAAATTCATCTATAGCAGCAA					
	Secondary		1034-F	AGAATTATTGTAAATGCAGCTTTATGGGGACTC	233	As above	DdeI	Wild	Wild: 2 sites cut114 + 56 Mutant: 1site 172 + 59
			1042-R	AATGGATAATATTTCTCAAATGATAACTTAGCA			AseI	Wild	

PCR: polymerase chain reaction, RFLP: restriction enzyme length polymorphism, RE: restriction enzyme, CRT: chloroquine resistance transporter, MDR: multidrug resistance, bp: base pair.

5.3 RESULTS

A total of 86 malaria-positive individuals participated in the present study; 40 (46.5%) were males and 46 (53.5%) were females. The ages of participants ranged from 8 months to 65 years, with a mean age of 12.4 years. The age group of 5–15 years represented 58.1% of the study participants. Asexual parasite density (parasitaemia) varied from 561 to 55,555 parasites/μL, with a mean parasitaemia of 8,199 parasites/μL.

The *pfcrt* gene mutations were screened as molecular markers of CQ, and possibly of other antimalarials such as amodiaquine and lumefantrine. All isolates were successfully amplified (100%). Mutation at *pfcrt* 76T was found at a virtual fixation level in almost all districts (97.7%), followed by 88.4% for position 75E. The prevalence of mutations at other codons of *pfcrt* was found to vary from moderate as in codons 74I (79.1%), 220S (69.8%), 271E (69.8%) and 371I (53.5%) to low as in 326S (36%) and 72S (10.5%). None of the isolates considered in the present study carried the mutated type at codon 356 of *pfcrt* (Table 5.2.)

Eight haplotypes of *pfcrt* 72-76 amino acids were found to be present in the study area. The CVIET classical, old-world African/Southeast Asian haplotype was the most prevalent among all *pfcrt* haplotypes (77.9%), followed by SVMNT (9.3%) and CVMET (7%). Only one isolate (1.2%) was found to carry each of the other mutated haplotypes, namely CVMEK, CVINT, SVMET and CVMNT, as well as the CVMNK wild haplotype.

Among all 10 types of codon mutation in *pfcrt*, sextuple mutations (i.e., mutations at six codons) had the highest presence among the isolates (33.7%), followed by quadruple mutations (four codons) with 23.3%, quintuple (five codons) with 14.4%,

septuple (seven codons) with 12.2%, and triple (three codons) with 8.1%. The frequency of double and single mutations was very low; 3.5% and 1.2%, respectively.

As regards to the results of the mutation analysis of the *pfmdr1* gene, *P. falciparum* field isolates from the Tehama region showed at least a single point mutation in the *pfmdr1* gene (Table 5.3). Mutation at position 184 of *pfmdr1* was found at the fixation level (100%), together with a low prevalence of mutation for codons 1034 and 86 of *pfmdr1* of 20.9% and 16.3%, respectively. No mutation was detected for codons 1042 and 1246.

The NFSND single-mutated haplotype of phenyl alanine amino acid at position 184 was predominant (64%), followed by NFCND (19.8%), and YFSND (15.1%), with an overall presence of 34.9% for all double-mutated haplotypes. The YFCND triple-mutated haplotype was found in only one isolate (1.2%) carrying the mutated amino acids tyrosine (codon 86), phenyl alanine (184), and cysteine (1034).

Interestingly, falciparum malaria in the study area, which consisted of districts known to have the highest malaria endemicity in the country, showed unexpected variation in *pfcrt* and *pfmdr1* gene mutations. Isolates from the AdDahi district in Hodeidah and the Khamis Bani Saad district in Al-Mahwit were almost similar in terms of carrying the majority of mutated alleles for most of the codons, and consequently carrying the mutated haplotypes. In contrast, the malaria isolates from the Bajil district in Hodeidah were found to be mostly of the wild type and consequently carried less mutated haplotypes.

Isolates from the study area showed a high prevalence of CQ resistant haplotypes, with a predominance of CVIET classical old-world African/Southeast Asian haplotypes in the Khamis Bani Saad district of Al-Mahwit (93.1%) and the AdDahi district in Hodeidah (88.9%) compared to only 26.3% for isolates from the Bajil district

of Hodeidah. In the same context, mutation at the *pfcrt* 72 codon was only reported in isolates from the Bajil district (47.4%), whereas it was totally absent from the other two districts. Accordingly, the SVMNT haplotype was found exclusively in 42.1% of isolates from Bajil (Table 5.2).

Table 5.2: Frequency distribution of *pfcrt* mutations and haplotypes for *P. falciparum* isolates from different districts of Tehama, Yemen

Marker		AdDahi		Bajil		Khamis Bani Saad		Total	
		n	%	n	%	N	%	n	%
Crt 72	wild	9	100	10	52.6	58	100	77	89.5
	mutated	0	0	9	47.4	0	0	9	10.5
Crt 74	wild	1	11.1	14	73.7	3	5.2	18	20.9
	mutated	8	88.9	5	26.3	55	94.8	68	79.1
Crt 75	wild	0	0	8	42.1	2	3.4	10	11.6
	mutated	9	100	11	57.9	56	96.6	76	88.4
Crt 76	wild	0	0	0	0	2	3.4	2	2.3
	mutated	9	100	19	100	56	96.6	84	97.7
Crt 220	wild	4	44.4	4	21.1	18	31	26	30.2
	mutated	5	55.6	15	78.9	40	69	60	69.8
Crt 271	wild	0	0	18	94.7	8	13.8	26	30.2
	mutated	9	100	1	5.3	50	86.2	60	69.8
Crt 326	wild	5	55.6	2	10.5	48	82.8	55	64
	mutated	4	44.4	17	89.5	10	17.2	31	36
Crt 371	wild	3	33.3	18	94.7	19	32.8	40	46.5
	mutated	6	66.7	1	5.3	39	67.2	46	53.5
Haplotypes	CV**IET**	8	88.9	5	26.3	54	93.1	67	77.9
	SVMN**T**	0	0	8	42.1	0	0	8	9.3
	CVM**ET**	1	11.1	5	26.3	0	0	6	7
	CV**I**N**T**	0	0	0	0	1	1.2	1	1.2
	CVM**E**K	0	0	0	0	1	1.2	1	1.2
	CVMNK	0	0	0	0	1	1.2	1	1.2
	CVMN**T**	0	0	0	0	1	1.2	1	1.2
	SVM**ET**	0	0	1	1.2	0	0	1	1.2

Similarly, mutations at *pfmdr1* showed a geographic variation in terms of *pfmdr1* allele and haplotype frequencies. That is to say, in addition to the fixation level of mutation at *pfmdr1* 184, mutations at 86 and 1034 of *pfmdr1* were found only in the AdDahi and Khamis Bani Saad districts, whereas they were not present in isolates from the Bajil district. The triple-mutated haplotype of 86, 1084 and 1034 (YFCND) was found in one isolate from Khamis Bani Saad. (Table 5.3)

Table 5.3: Frequency distribution of *pfmdr1* mutations and haplotypes for *P. falciparum* isolates from different districts of Tehama, Yemen

Marker		AdDahi		Bajil		Khamis Bani Saad		Total	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Mdr1 86	wild	5	55.6	19	100	48	82.8	72	83.7
	mutated	4	44.4	0	0	10	17.2	14	16.3
Mdr1 1034	wild	8	88.9	19	100	41	70.7	68	79.1
	mutated	1	11.1	0	0	17	29.3	18	20.9
Haplotypes	N**F**SND	4	44.4	19	100	32	55.2	55	64
	N**FC**ND	1	11.1	0	0	16	27.6	17	19.8
	YFSND	4	44.4	0	0	9	15.5	13	15.1
	YFCND	0	0	0	0	1	1.7	1	1.2

5.4 DISCUSSION

The present study provides information on the *pfcrt* and *pfmdr1* genetic profile of *P. falciparum* isolates from the districts with the highest malaria endemicity in the Tehama region of Yemen. The isolates were found to mostly carry mutated alleles, especially for *pfcrt* codons 74I, 75E, 76T, 220S, 271E, and 371I, while no mutations were detected at codon 356 of *pfcrt*. This is consistent with a previous study conducted in Yemen that reported a high prevalence of mutated alleles for codons 74I and 75E (89%) and 76T (93%) (Al-Hamidhi et al., 2013). The present study found that the overall prevalence of *pfcrt* 76T was 74%–100%, which is similar to the figure previously reported for isolates from other parts of Yemen (Al-Mekhlafi et al., 2011; Mubjer et al., 2011; Bamaga et al., 2015a).

The high level of mutation at position 76 of *pfcrt* (96.6%–100%) appears to indicate that, 5 years on from changing the malaria treatment policy to one that is ACT based and the official cessation of CQ used for *P. falciparum*, the re-emergence of a CQ-sensitive strain of *P. falciparum* has not yet occurred in the study area. One possible explanation for the virtual fixation of the *pfcrt* 76 mutation in the study area could be the continued availability of CQ. It is still being prescribed to falciparum malaria patients in Yemen particularly in private health facilities and is also being used as a self-administered treatment (Bashrahil et al., 2010; Ghouth, 2013; Bamaga et al., 2014). Moreover, CQ has not yet been totally withdrawn from governmental drug stores as it is still the drug of choice for treating vivax malaria infections.

The findings of the present study support those of previous research conducted elsewhere in Yemen that have reported 79%–100% mutated alleles for *pfcrt* 76T (Al-Mekhlafi et al., 2011; Mubjer et al., 2011; Abdul-Ghani et al., 2013; Al-Hamidhi et al., 2013). The finding in previous studies such as these that there was a decrease in the

prevalence of the *pfcrt* 76T mutated allele and a re-emergence of CQ-sensitive strains some years after abandoning CQ was controversial, particularly as many studies in Africa documented an increase in the re-emergence of the wild type of *pfcrt* 76 after complete CQ withdrawal (Laufer et al., 2006; Mohammed et al., 2013; Mbogo et al., 2014; Mekonnen et al., 2014). In contrast, South American isolates of *P. falciparum* have been reported to retain their mutated types despite the cessation of CQ use (Vieira et al., 2004; Griffing et al., 2010; Adhin et al., 2013). Similarly, studies conducted in Ethiopia have found a fixation of *pfcrt* 76T mutations after cessation of CQ use for more than a decade (Mula et al., 2011; Golassa et al., 2014).

The results of the present study also revealed that the CVIET classical old-world, African/ Southeast Asian mutated haplotype was predominant (77.9%) among all *pfcrt* haplotypes in the study area and that seven other haplotypes were also present, including 9.3% (eight cases) of the SVMNT new-world South American mutated haplotype (only in the Bajil district) and 7.0% (six cases) of CVMET. This finding is in agreement with the only two other available studies based in Yemen that attempted to examine the mutations along the *pfcrt* gene (Al-Hamidhi et al., 2013; Mubjer et al., 2011). For instance, CVIET was reported for the majority of the isolates from different parts of Yemen (Dhamar 100%, Taiz 88%, and Hodeidah 70.6%), with a presence of only 4% of the SVMNT haplotype in isolates from the Hodeidah governorate (Al-Hamidhi et al., 2013). Similarly, CVIET was the only haplotype reported for isolates from the Al-Musaimeer malaria-endemic district in Lahj governorate (Mubjer et al., 2011).

Interestingly, the present study found the SVMNT haplotype exclusively in 42.1% of isolates from the Bajil district in Hodeidah. This finding might be due to a ‘parasite response’ to a particular drug pressure other than CQ. However, data on the use of a unique antimalarial in this particular area were not available, so it is not

possible to relate the occurrence of the SVMNT haplotype to the increased use of any of the antimalarials. A previous study conducted in Tanzania reported the unusual existence of the SVMNT haplotype in Africa and attributed the finding, without supporting evidence, to the increased drug pressure of amodiaquine (AQ) use in the area under study (Alifrangis et al., 2006).

Moreover, malaria transmission in the Bajil district, an area with the SVMNT haplotype, was found to be lower than that in the AdDahi and Khamis Bani Saad districts. This is in line with the study on Tanzania, which reported a higher existence of the SVMNT mutated haplotype in areas with low malaria transmission than in areas with high transmission (Alifrangis et al., 2006). As mentioned above, it has been suggested that the SVMNT haplotype occurs as a response to the intense pressure of AQ, and *P. falciparum* isolates with SVMNT are supposedly less susceptible to AQ monotherapy and combination therapy. Interestingly, the NMCP in Yemen conducted six *in vivo* clinical trials for CQ and AQ drug efficacy from 2002 to 2004; four studies looked at CQ efficacy and found that there was 30%–57% treatment failure, while two studies monitored the efficacy of AQ monotherapy and artesunate-amodiaquine (AST-AQ) combination therapy and found 44.3% and 18.5% treatment failure rate, respectively (unpublished data).

The existence of SVMNT in Bajil rather than in the other two districts raises an interesting question about the source of the evolution of this CQ resistant haplotype in the country. Typically, SVMNT is associated with new-world South American or Papua New Guinea isolates, and importation of this haplotype from South America or Papua New Guinea, or even from the Philippines or India in Asia, was logically unaccepted based on the population characteristics of the study area. Hence, the apparent independent evolution of SVMNT in Bajil isolates will need further study to confirm the finding presented herein. However, the results of a study conducted in Papua New

Guinea suggest that there is a recombination of de novo point mutation that transforms the old-world classical African CVIET haplotype into a new-world South American SVMNT one (Mehlotra et al., 2001).

As regards to the *pfmdr1* gene, the present study found that mutations varied among the isolates from the three districts, from fixation (100%) for 184F to 20.9% for 1034C and 16.3% for 86Y, while no mutation was detected for the 1042 and 1246 codons. These results are consistent with those of a previous study in Hodeidah that reported a high prevalence (99%) of 184F and a relatively low existence (20%) of the 86Y mutated allele (Al-Hamidhi et al., 2013). Likewise, a recent study conducted in Hadramout in the southeastern part of Yemen, which has low malaria transmission, reported a low frequency (16.7%) of *pfmdr1* 86 mutated allele (Bamaga et al., 2015a). However, a high prevalence (70%) for mutations of 1034C and 1042D has been reported for the Taiz, Dhamar, and Hodeidah governorates (Al-Hamidhi et al., 2013).

It has been found that polymorphism in the *pfmdr1* gene is related to an increase in parasite tolerance/resistance to some antimalarials (Gamo, 2014). Nevertheless, the findings regarding the association of mutations in the *pfmdr1* codons with antimalarial drug resistance are mixed. For instance, 86Y has been linked to CQ resistance, while mutations at positions 184, 1034, 1042, and 1246 were found to be mostly related to resistance to AQ, mefloquine, halofantrine, and quinine (Reed et al., 2000; Danquah et al., 2010; Gamo, 2014). Moreover, the occurrence of *pfmdr1* N86 and 184F has been found to increase with the use of lumefantrine (Sisowath et al., 2007; Nwakanma et al., 2008; Malmberg et al., 2013). In the same vein, another study reported an increase of *pfmdr1* N86 and 184F, together with the wild alleles of D1246, and concluded that this was linked to the extensive and prolonged use of AQ in combination with artesunate (Fröberg et al., 2012). In other studies, *P. falciparum* isolates carrying *pfmdr1* haplotypes of mutated 86Y and Y184 wild alleles were found to be associated with the

usage of CQ or AQ, which continued to change over time with changes in the antimalarials used. For instance, mutated 86Y and Y184 wild alleles changed to a haplotype carrying wild N86 and 184F mutated alleles years after the implementation of ACT, and the selection of parasites to one of artemisinin's derivatives (Humphreys et al., 2007; Dlamini et al., 2010; Mungthin et al., 2010; Thomsen et al., 2011). Overall, the findings of the present study provide a broad view on the polymorphism of *pfcrt* and *pfmdr1* genes in areas with the highest level of malarial transmission in Yemen, 5 years after official cessation of CQ.

The present study showed no significant association of mutations in the *pfcrt* and *pfmdr1* codons with the AS+SP reported outcomes. This could be explained by the very high rate of 76T mutated alleles, the high efficacy of the ACT drug (with the very small number of treatment failure), and the low frequency of point mutations reported for the *pfdhfr* and *pfdhps* genes in the isolates studied (these findings were not reported in the published article). It has been suggested that polymorphism *pfmdr1* gene at codons 86, 184, and 1246 could serve as markers of changes in parasite susceptibility to other drugs, including ACT combinations (Humphreys *et al.*, 2007;). A recent study has reported a possible association between the capacity of *P. falciparum* to develop resistance to ACT *in vitro* based on the mutations in *Pfmdr1*, however, the *in-vivo* assay of the study did not detect any polymorphisms for the *Pfk13* gene (Njokah *et al.*, 2016).

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSION

Malaria is one of the most important parasitic diseases and health problem worldwide, and most of the victims reside in sub-Saharan Africa. Half of the world's population is at risk of malaria with more than 200 million cases and half a million deaths reported annually (WHO, 2015a). Malaria transmission occurs in around 90 countries and these are located mostly in Africa, Asia and South America. Children below 5 years and pregnant women are the two most affected groups. The disease is caused by a unicellular protozoan parasite belonging to the genus *Plasmodium* that requires a female *Anopheles* mosquito to continue its life cycle. Several species of the *Plasmodium* parasite are responsible for producing malaria in mammals, reptiles and birds. Five main species have been identified as causing malaria in humans: *P. falciparum*, *P. vivax, P. malariae*, *P. ovale* and *P. knowlesi*. In addition, *P. cynomolgi* was recently reported as a causative human malaria parasite in Southeast Asia (Ta et al., 2014). However, of these species, *P. falciparum* is the most important in terms of its capability to produce the more severe form of the disease, cause a high mortality rate and develop resistance to different antimalarials.

Yemen is the only country in the Arabian Peninsula with a high rate of malaria transmission, and this threatens the malaria control efforts of neighbouring countries. *Plasmodium falciparum* is responsible for 99% of malaria cases in Yemen with a few scattered cases of vivax and malariae malaria infection.

Up to the 2000s, malaria diagnosis in Yemen was achieved only by malaria microscopy. Also, the infrastructure for malaria diagnosis was optimal only in principal cities and there was high variation in the outcomes of microscopy. Moreover, peripheral

areas where malaria is endemic had the lowest level of microscopy performance due to either a lack of well-trained personnel, irregular supply of logistics, lack of electricity or a combination thereof. Therefore, the NMCP of Yemen started using a RDT in 2009 as an alternative tool for malaria diagnosis in remote endemic areas. It also updated its malaria treatment policy the same year to substitute CQ with ACT as a response to the increase in and spread of CQ-resistant strains of *P. falciparum*. However, the RDT and ACT have not been evaluated enough since their year of implementation (2009). Therefore, the present study aimed to evaluate both by focusing on two parameters; malaria diagnosis and malaria treatment. It is important to mention that this study is also the first that attempts to use molecular analysis for evaluating both the performance of the hrp2-based RDT and the efficacy of ACT in Yemen through the genetic analysis of *Pfhrp2* and *P. falciparum kelch13* genes, respectively.

The *CareStart*™ one-step HRP2-based RDT showed high sensitivity and specificity in detecting malaria parasites from isolates collected specifically from endemic areas in the Hodeidah and Al-Mahwait governorates of Tehama region. The CareStart™ one-step HRP2-based RDT showed high performance especially for those cases with parasitaemia of >100 parasites/µL. The findings reveal that the high genetic variation of the *pfhrp2* has no impact on the performance of the hrp2-based RDT. The findings of the present study provide insights into the genetic diversity of *pfhrp2* in *P. falciparum* isolates from Yemen, revealing a high *pfhrp2* polymorphism.

The data from the present *in-vivo* study indicates that AS+SP is still efficacious for the treatment of uncomplicated falciparum malaria with a cure rate of 96.5% (PCR corrected). Molecular analysis of the genes related to SP resistance shows an existence of double mutant *pfdhfr* genotypes that were found in 65.4% of the isolates while almost no mutation was reported in the *pfdhps* gene. *Pfk13* gene related to AS resistance for the Yemen isolates was found all to be of wild type as no mutation detected along the

amplified *k13* gene of *P. falciparum* isolates. This data shows the high effectiveness of artemisinin against *P. falciparum* isolates from Yemen with a consideration of increasing the chance of resistance to the ACT combination therapy in the future due to increasing in the mutations in *pfdhfr* for SP partner drug resistance.

Although the drug combination (AS+SP) was found to be highly effective in eliminating the asexual forms of the parasite and in curing the clinical symptoms of malaria, it showed poor efficacy against the sexual stages of the parasite (gametocytaemia). The persistence of gametocytes in patients' blood throughout the follow-up period (28 days) is a major problem in terms of continued malaria transmission. Furthermore, the persistence of post-treatment gametocytaemia in Yemen might provide a selective advantage for the transmission of parasites that are resistant to SP and that will later become resistant to artemisinin.

The present study also aimed to provide a genetic profile of *pfcrt* and *pfmdr1* as molecular markers of the *P. falciparum* parasite's resistance to antimalarials, especially CQ. The high prevalence of *pfcrt* mutations and mutated haplotypes suggests a high CQ resistance in *P. falciparum* isolates. This reflects the continuation of CQ pressure in the study area due to the availability of CQ in many of the private pharmacies and drugstores where it is usually sold to the population when requested.

6.2 RECOMMENDATIONS

The present study aimed to evaluate the government's new policies of rapid malaria diagnosis using the CareStart hrp2-based RDT and malaria treatment using AS+SP combination therapy in the peripheral endemic areas of Yemen. The following are the most important recommendations by the present study: -

1. A population-based national study that includes other endemic areas throughout Yemen is strongly recommended in order to genetically analyse the *P. falciparum* isolates based on the pfhrp2 gene. Such study should be undertaken to accurately determine the magnitude of the false negative results among the malaria population in Yemen (parasites that cannot be detected employing the currently used pfhrp2-based RDT) either because of the low parasitaemia or due to partial or total hrp2 gene deletion.
2. The increase in the mutations in *pfdhfr* among *P. falciparum* Yemen isolates requires continuous monitoring via *in vivo*, *in vitro* and molecular examination of SP efficacy not only the Tehama region, but also other malaria-endemic areas. This is because SP resistance, if it occurs, will hamper the future efficacy of artemisinin in Yemen and the whole region.
3. Persistent gametocytaemia after AS+SP treatment suggests that primaquine should also be administered in order to reduce malaria transmission in a sustainable way. This is all the more important in Yemen, where disruption of the health infrastructure and displacement of the population due to the current political situation are likely to lead to an exacerbation of malaria and to threaten the surrounding countries in the Arabian Peninsula.
4. The high prevalence of mutations in the *pfcrt* gene, especially *pfcrt* 76T, reflects a high CQ resistance in *P. falciparum* isolates in the study area and suggests that there is continuous use of CQ in the treatment of malaria. It is therefore

recommended that the availability of CQ in private hospitals, clinics, and drugstores should be controlled to ensure its complete withdrawal. Moreover, treatment of vivax malaria using CQ should only be available in governmental health facilities to prevent the random, uncontrolled use of CQ.

6.3 LIMITATIONS OF THE STUDY

The present study was conducted from March to May 2014 in areas with high rates of malaria transmission and poverty in the Hodeidah and Al-Mahwait governorates of Tehama region, Yemen. The study was designed to investigate AS+SP efficacy through parallel *in vivo* and *in vitro* assays. However, Yemen at that time was in the throes of a political crisis and experiencing growing instability, which later led to a civil war in some areas of the country including parts of Tehama, and at the time of writing this crisis is still ongoing. During our research, electricity in the city of Bajil, where a laboratory is available in the Bajil Malaria Unit, was rarely available for more than 4 to 5 hours per day, and when it was available the supply was intermittent and irregular. Sometimes when it came on, it did not last for more than 1 hour at best. Another problem encountered during our study was the lack of access to a regular supply of fuel for the transportation required for visiting patients for follow-up and to generate electricity for our tests, an issue that was especially important to address in the hot climate of Yemen. Sometimes we bought fuel from the black market at a cost that was three times higher than its normal price, but even using this option there was no real guarantee of a continuous supply of fuel. Thus, the study was limited in terms of the comprehensiveness of our *in vivo* evaluation of AS+SP, which we eventually had to abandon along with our *in vitro* assessment due to the above-mentioned conditions.

REFERENCE

Aaby, P., Rodrigues, A., Kofoed, P.-E., & Benn, C. S. (2015). RTS,S/AS01 malaria vaccine and child mortality. *Lancet, 386*: 1735-1736.

Abdul-Ghani, R., Basco, L. K. , Beier J. C., & Mahdy M. A. K. (2015). Inclusion of gametocyte parameters in anti-malarial drug efficacy studies: filling a neglected gap needed for malaria elimination. *Malaria Journal, 14*: 413.

Abdul-Ghani, R., Farag, H. F., & Allam, A. F. (2013). Sulfadoxine-pyrimethamine resistance in *Plasmodium falciparum*: A zoomed image at the molecular level within a geographic context. *Acta Tropica, 125*: 163-190.

Abdul-Ghani, R., Farag, H. F., Allam, A. F., & Shawky, S. M. (2014). Prevailing *Plasmodium falciparum* dihydrofolate reductase 108-asparagine in Hodeidah, Yemen: A questionable sulfadoxine–pyrimethamine partner within the artemisinin-based combination therapy. *Acta Tropica, 132*: 39-44.

Adagu, I. S., Warhurst, D. C., Ogala, W. N., Abdu-Aguye, I., Audu, L. I., Bamgbola, F. O., & Ovwigho, U. B. (1995). Antimalarial drug response of *Plasmodium falciparum* from Zaria, Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 89*: 422-425.

Adeel, A. A., Saeed, N. A., Aljasari, A., Almohager, A. M., Galab, M. H., & AlMahdi, A. (2015). High efficacy of two artemisinin-based combinations: artesunate+sulfadoxine-pyrimethamine and artemether-lumefantrine for falciparum malaria in Yemen. *Malaria Journal, 14*: 449.

Adhin, M. R., Labadie-Bracho, M., & Bretas, G. (2013). Molecular surveillance as monitoring tool for drug-resistant *Plasmodium falciparum* in Suriname. *The American Journal of Tropical Medicine and Hygiene, 89*: 311–316.

Afsharpad, M., Zakeri, S., Pirahmadi, S. & Djadid, N. D. (2012). Molecular monitoring of *Plasmodium falciparum* resistance to antimalarial drugs after adoption of sulfadoxine-pyrimethamine plus artesunate as the first line treatment in Iran. *Acta Tropica* 121:13-8.

Akinyi, S., Hayden, T., Gamboa, D., Torres, K., Bendezu, J., Abdallah, J. F., Griffing, S. M., Quezada, W. M., Arrospide, N., De Oliveira, A. M., Lucas, C., Magill, A. J., Bacon, D. J., Barnwell, J. W., & Udhayakumar, V. (2013). Multiple genetic origins of histidine-rich protein 2 gene deletion in *Plasmodium falciparum* parasites from Peru. *Scientific Report, 3*: 2797.

Alifrangis, M., Dalgaard, M. B., Lusingu, J. P., Vestergaard, L. S., Staalsoe, T., Jensen, A. T. R., Enevold A., Rønn A. M., Khalil I. F., Warhurst D. C., Lemnge M. M., Theander T. G., & Bygbjerg I. C. (2006). Occurrence of the Southeast Asian/South American SVMNT haplotype of the chloroquine-resistance transporter gene in *Plasmodium falciparum* in Tanzania. *The Journal of Infectious Diseases, 193*: 1738-1741.

Alkadi, H., Al-Maktari, M., & Nooman, M. (2006). Chloroquine-resistant *Plasmodium falciparum* local strain in Taiz Governorate, Republic of Yemen. *Chemotherapy, 52*: 166-170.

Alker, A. P., Lim, P., Sem, R., Shah, N. K., Yi, P., Bouth, D. M., Tsuyuoka, R., Maguire, J. D., Fandeur, T., Ariey, F., Wongsrichanalai, C., & Meshnick, S. R. (2007). *Pfmdr1* and *in vivo* resistance to artesunate-mefloquine in falciparum malaria on the Cambodian–Thai border. *The American Journal of Tropical Medicine and Hygiene, 76*: 641-647.

Al-Eryani, S. M. A., Kelly-Hope, L., Harbach, R. E., Briscoe, A. G., Barnish, G., Azazy, A., & McCall, P. J. (2016). Entomological aspects and the role of human behaviour in malaria transmission in a highland region of the Republic of Yemen. *Malaria Journal, 15*: 130.

Al-Hamidhi, S., Mahdy, M. A., Al-Hashami, Z., Al-Farsi, H., Al-Mekhlafi, A. M., & Idris, M. A. (2013). Genetic diversity of *Plasmodium falciparum* and distribution of drug resistance haplotypes in Yemen. *Malaria Journal, 12*: 244.

Al-Kabsi, A. M., Al-Shamahy, H. A., Al-Harazy, A. H., & Harmal, N. S. (2009). The therapeutic efficacy of sulfadoxine/pyrimethamine against *Plasmodium falciparum* in Yemen. *Medical Principles and Practice, 18*: 62-6.

Al-Maktari, M., & Bassiouny, H. (2003). Malaria status in Al-Hodeidah Governorate, Republic of Yemen. Part II: Human factors causing the persistence of chloroquine resistant *P. falciparum* local strain. *Journal of the Egyptian Society of Parasitology, 33*: 829-39.

Al-Maktari, M. T., Bassiouny, H. K., Al-Hamd, Z. S., Assabri, A. M., El-Massry, A. G., & Shatat, H. Z. (2003). Malaria status in Al-Hodeidah Governorate, Yemen: malariometric parasitic survey & chloroquine resistance *P. falciparum* local strain. *Journal of the Egyptian Society of Parasitology, 33*: 361-372.

Al-Mekhlafi, A. M., Mahdy, M. A., Al-Mekhlafi, H. M., Azazy, A. A., & Fong, M. Y. (2011). High frequency of *Plasmodium falciparum* chloroquine resistance marker (pfcrt T76 mutation) in Yemen: an urgent need to re-examine malaria drug policy. *Parasites & Vectors, 4*: 94.

Al-Shamahy, H., Al-Harazy, A., Harmal, N., & Al-Kabsi, A. (2007). The prevalence and degree of resistance of *Plasmodium falciparum* to first-line antimalarial drugs: an *in vitro* study from a malaria endemic region in Yemen. *Annals of Saudi Medicine, 27*: 432-6.

Al-Taiar, A., Chandler, C., Al-Eryani, S., & Whitty, C. J. (2009). Knowledge and practices for preventing severe malaria in Yemen: the importance of gender in planning policy. *Health Policy Planning, 24*: 428-37.

Al-Zanbagi, N. A. (2014). Review of malaria in Saudi Arabia, current status and future prospects. *Journal of International Academic Research for Multidisciplinary, 2*: 1-15.

Ansell, J., Hamilton, K. A., Pinder, M., Walraven, G. E., & Lindsay, S. W. (2002). Short-range attractiveness of pregnant women to *Anopheles gambiae* mosquitoes. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 96*: 113-6.

Arasu, G. (1992). Risk behavior in malaria transmission in Malaysia. *Southeast Asian Journal of Tropical Medicine and Public Health, 23*: 51-56.

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A. C., Khim, N., Kim, S., Duru, V., Bouchier, C., Ma, L., Lim, P., Leang, R., Duong, S., Sreng, S., Suon, S., Chuor, C. M., Bout, D. M., Menard, S., Rogers, W. O., Genton, B., Fandeur, T., Miotto, O., Ringwald, P., Le Bras, J., Berry, A., Barale, J.-C., Fairhurst, R. M., Benoit-Vical, F., Mercereau-Puijalon, O., & Menard, D. (2014). A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature, 505*: 50-5.

Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J. M., Mao, S., Sam, B., Sopha, C., Chuor, C. M., Nguon, C., Sovannaroth, S., Pukrittayakamee, S., Jittamala, P., Chotivanich, K., Chutasmit, K., Suchatsoonthorn, C., Runcharoen, R., Hien, T. T., Thuy-Nhien, N. T., Thanh, N. V., Phu, N. H., Htut, Y., Han, K.-T., Aye, K. H., Mokuolu, O. A., Olaosebikan, R. R., Folaranmi, O. O., Mayxay, M., Khanthavong, M., Hongvanthong, B., Newton, P. N., Onyamboko, M. A., Fanello, C. I., Tshefu, A. K., Mishra, N., Valecha, N., Phyo, A. P., Nosten, F., Yi, P., Tripura, R., Borrmann, S., Bashraheil, M., Peshu, J., Faiz, M. A., Ghose, A., Hossain, M. A., Samad, R., Rahman, M. R., Hasan, M. M., Islam, A., Miotto, O., Amato, R., MacInnis, B., Stalker, J., Kwiatkowski, D. P., Bozdech, Z., Jeeyapant, A., Cheah, P. Y., Sakulthaew, T., Chalk, J., Intharabut, B., Silamut, K., Lee, S. J., Vihokhern, B., Kunasol, C., Imwong, M., Tarning, J., Taylor, W. J., Yeung, S., Woodrow, C. J., Flegg, J. A., Das, D., Smith, J., Venkatesan, M., Plowe, C. V., Stepniewska, K., Guerin, P. J., Dondorp, A. M., Day, N. P., & White, N. J. (2014). Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *The New Englaand Journal of Medicine, 371*: 411-23.

Avila P. E., Kirchgatter K., Brunialti K. C., Oliveira A. M., Siciliano R. F., & Di Santi S. M. (2002). Evaluation of a rapid dipstick test, Malar-CheckÔ, for the diagnosis of *Plasmodium falciparum* malaria in Brazil. *Revista do Instituto de Medicina Tropical de São Paulo, 44*: 293-6.

Awab, G., Pukrittayakamee, S., Jamornthanyawat, N., Yamin, F., Dondorp, A., Day, N., White, N., Woodrow, C., & Imwong, M. (2013). Prevalence of antifolate resistance mutations in *Plasmodium falciparum* isolates in Afghanistan. *Malaria Journal, 12*: 96.

Babiker, H. A., Abdel-Wahab, A., Ahmed, S., Suleiman, S., Ranford-Cartwright, L., Carter, R., & Walliker, D. (1999). Detection of low level *Plasmodium falciparum* gametocytes using reverse transcriptase polymerase chain reaction. *Molecular and Biochemical Parasitology, 99*: 143-8.

Baker, J., Ho, M., Pelecanos, A., Gatton, M., Chen, N., Abdullah, S., Albertini, A., Ariey, F., Barnwell, J., Bell, D., Cunningham, J., Djalle, D., Echeverry, D., Gamboa, D., Hii, J., Kyaw, M., Luchavez, J., Membi, C., Menard, D., Murillo, C., Nhem, S., Ogutu, B., Onyor, P., Oyibo, W., Wang, S., McCarthy, J., & Cheng, Q. (2010). Global sequence variation in the histidine-rich proteins 2 and 3 of *Plasmodium falciparum*: implications for the performance of malaria rapid diagnostic tests. *Malaria Journal, 9*: 129.

Baker, J., McCarthy, J., Gatton, M., Kyle, D., Belizario, V., Luchavez, J., Bell, D., & Cheng, Q. (2005). Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests. *Journal of Infectious Diseases, 192*: 870-7.

Balls, M. J., Bødker, R., Thomas, C. J., Kisinza, W., Msangeni, H. A., & Lindsay, S. W. (2004). Effect of topography on the risk of malaria infection in the Usambara mountains, Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 98*: 400-408.

Bamaga, O., Mahdy, M., & Lim, Y. (2015a). Survey of chloroquine-resistant mutations in the *Plasmodium falciparum* pfcrt and pfmdr-1 genes in Hadhramout, Yemen. *Acta Tropica, 149*: 59-63.

Bamaga, O., Mahdy, M. & Lim, Y. (2015b). Frequencies distribution of dihydrofolate reductase and dihydropteroate synthetase mutant alleles associated with sulfadoxine-pyrimethamine resistance in *Plasmodium falciparum* population from Hadhramout Governorate, Yemen. *Malaria Journal*, 14:516.

Bamaga, O., Mahdy, M., Mahmud, R., & Lim, Y. (2014). Malaria in Hadhramout, a southeast governorate of Yemen: prevalence, risk factors, knowledge, attitude and practices (KAPs). *Parasites & Vectors, 7*: 351.

Basco, L. K., Bras, J. L., Rhoades, Z., & Wilson, C. M. (1995). Analysis of pfmdr1 and drug susceptibility in fresh isolates of *Plasmodium falciparum* from Sub-Saharan Africa. *Molecular and Biochemical Parasitology, 74*: 157-166.

Bashrahil K. A., Bingouth A. S., & Baruzaig A. S. (2010). Antimalarial drugs: availability and mode of prescribing in Mukalla, Yemen. *East Mediterranean Health Journal, 16*: 146-50.

Bastiaens G. J. H., Bousema T., & Leslie T. (2014). Scale-up of malaria rapid diagnostic tests and artemisinin- based combination therapy: challenges and perspectives in sub-Saharan Africa. *PLoS Medicine, 11*: e1001590.

Bell D., Wilson D., & Martin L. (2005). False-positive results of a *Plasmodium falciparum* histidine-rich protein 2–detecting malaria rapid diagnostic test due to high sensitivity in a community with fluctuating low parasite density. *The American Journal of Tropical Medicine and Hygiene, 73*: 199-203.

Berga, D. K. (1999). Assignment report to Yemen on: Monitoring therapeutic efficacy of chloroquine in the republic of Yemen. Ministry of Public Health. Yemen, Sana'a 21-Dec-1998-18 Jan. 1999.

Bhattacharya PR, B. S., Kabilan L. (1997). Alleles of the *Plasmodium falciparum* pfmdr1 gene appear not to be associated with chloroquine resistance in India. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 91*: 91.

Billo M. A., Diakité M., Dolo A., Diallo M., Poudiougou B., Diawara S. I., Johnson E. S., Rice J. C., Krogstad D. J., & Doumbo O. K. (2013). Inter-observer agreement according to malaria parasite density. *Malaria Journal, 12*: 335-335.

Bin Dajem S. M., Al-Farsi H. M., Al-Hashami Z. S., Al-Sheikh A. A., Al-Qahtani A., & Babiker H. A. (2012). Distribution of drug resistance genotypes in *Plasmodium*

falciparum in an area of limited parasite diversity in Saudi Arabia. *The American Journal of Tropical Medicine and Hygiene, 86*: 782-8.

Bousema, J. T., Schneider, P., Gouagna, L. C., Drakeley, C. J., Tostmann, A., Houben, R., Githure, J. I., Ord, R., Sutherland, C. J., Omar, S. A., & Sauerwein, R. W. (2006). Moderate effect of artemisinin-based combination therapy on transmission of *Plasmodium falciparum*. *Journal of Infectious Diseases, 193*: 1151-9.

Bousema T., & Drakeley C. (2011). Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. *Clinical Microbiology Reviews, 24*: 377-410.

Bradley, D. J., Newbold, C. I., & Warrell D. A. (1987) *Malaria. In: Oxford Textbook of Medicine*. Eds: Weatherall D. J., Ledingham, J. G. G. & Warrell, D. A. Oxford: Oxford University Press.

Brier, B. (2004). Infectious diseases in ancient Egypt. *Infectious Disease Clinics of North America, 18*: 17-27.

Brossi, A., Venugopalan, B., Dominguez Gerpe, L., Yeh, H. J., FlippenAnderson, J. L., Buchs, P., Luo, X. D., Milhous, W., & Peters, W., (1988). Arteether, a new antimalarial drug: synthesis and antimalarial properties. *Journal of Medicinal Chemistry, 31*:645–50.

Bruce-Chwatt, L. J. (1988). *History of malaria from prehistory to eradication*. In: Wernsdorfer, W. H., McGregor, I. (eds) Malaria: Principles and practice of microbiology. 1st ed. Edinburgh, United Kingdom: Churchill Livingstone, pp 1-59.

Bruce-Chwatt, L., & Gockel, C. (1960). A study of the bloodfeeding patterns of Anopheles mosquitoes through precipitin tests. *Bulletin of the World Health Organization, 22*:685-720.

Butraporn, P., Sornmani, S., & Hungsapruek, T. (1986). Social, behavioral, housing factors and their interactive effects associated with malaria occurrence in East Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health, 17*: 386-392.

Campbell, C. C., Chin, W., Collins, W. E., Teutsch, S. M., & Moss, D. M. (1979). Chloroquine-resistant *Plasmodium falciparum* from East Africa: cultivation and drug sensitivity of the Tanzanian I/CDC strain from an American tourist. *Lancet, 2*: 1151-1154.

Casares, S., Brumeanu, T.-D., & Richie, T. L. (2010). The RTS,S malaria vaccine. *Vaccine, 28*: 4880-4894.

Chaiyaroj, S. C., Buranakiti, A., Angkasekwinai, P., Looressuwan, S., & Cowman, A. F. (1999). Analysis of mefloquine resistance and amplification of pfmdr1 in multidrug-resistant *Plasmodium falciparum* isolates from Thailand. *Am J Trop Med Hyg, 61*: 780-783.

Cheng, Q., Gatton, M., Barnwell, J., Chiodini, P., McCarthy, J., Bell, D., & Cunningham, J. (2014). *Plasmodium falciparum* parasites lacking histidine-rich protein 2 and 3: a review and recommendations for accurate reporting. *Malaria Journal, 13*: 283.

Cheruiyot, J., Ingasia, L. A., Omondi, A. A., Juma, D. W., Opot, B. H., Ndegwa, J. M., Mativo, J., Cheruiyot, A. C., Yeda, R., Okudo, C., Muiruri, P., Bidii, N. S., Chebon, L. J., Angienda, P. O., Eyase, F. L., Johnson, J. D., Bulimo, W. D., Andagalu, B., Akala, H. M., & Kamau, E. (2014). Polymorphisms in pfmdr1, pfcrt, and pfnhe1 genes are associated with reduced *in vitro* activities of quinine in *Plasmodium falciparum* isolates from Western Kenya. *Antimicrobial Agents and Chemotherapy, 58*: 3737-3743.

Chin, W., Contacos, P. G., Coatney, G. R., & Kimball, H. R. (1965). A naturally acquired quotidian-type malaria in man transferable to monkeys. *Science, 149*: 865-865.

Cojean, S., Noel, A., Garnier, D., Hubert, V., Le Bras, J., & Durand, R. (2006). Lack of association between putative transporter gene polymorphisms in *Plasmodium falciparum* and chloroquine resistance in imported malaria isolates from Africa. *Malaria Journal, 5*: 24.

Conrad, M. D., Bigira, V., Kapisi, J., Muhindo, M., Kamya, M. R., Havlir, D. V., Dorsey, G., & Rosenthal, P. J. (2014). Polymorphisms in K13 and falcipain-2 associated with artemisinin resistance are not prevalent in *Plasmodium falciparum* isolated from Ugandan children. *PLoS One, 9*: e105690.

Cowman, A. F., Karcz, S., Galatis, D., Culvenor, J. G. (1991). A P-glycoprotein homologue of *Plasmodium falciparum* is localized on the digestive vacuole. *The Journal of Cell Biology, 113*:1033–1042.

Cox, F. E. (2010). History of the discovery of the malaria parasites and their vectors. *Parasites & Vectors, 3*: 1-9.

Cox-Singh J., Davis T. M., Lee K. S., Shamsul S. S., Matusop A., Ratnam S., Rahman H. A., Conway D. J., & Singh, B. (2008). *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life-threatening. *Clinical Infectious Diseases, 46*: 165–171.

Craig, M. H., Snow, R. W., & le Sueur, D. (1999). A Climate-based distribution model of malaria transmission in sub-Saharan Africa. *Parasitology Today, 15*: 105-111.

Dal-Bianco M. P., Köster K. B., Kombila U. D., Kun J. F., Grobusch M. P., Ngoma G. M., Matsiegui P. B., Supan C., Salazar C. L., Missinou M. A., Issifou S., Lell B., & Kremsner P. (2007). High prevalence of asymptomatic *Plasmodium falciparum* infection in Gabonese adults. *The American Journal of Tropical Medicine and Hygiene, 7*: 939-942.

Danquah I., Coulibaly B., Meissner P., Petruschke I., Müller O., & Mockenhaupt F. P. (2010). Selection of pfmdr1 and pfcrt alleles in amodiaquine treatment failure in north-western Burkina Faso. *Acta Tropica, 114*: 63-66.

Deme, A., Park, D., Bei, A., Sarr, O., Badiane, A., Gueye, P. E. H., Ahouidi, A., Ndir, O., Mboup, S., Wirth, D., Ndiaye, D., & Volkman, S. (2014). Analysis of pfhrp2 genetic diversity in Senegal and implications for use of rapid diagnostic tests. *Malaria Journal, 13*: 34.

Diarra, A., Nébié, I., Tiono, A., Sanon, S., Soulama, I., Ouédraogo, A., Gansané, A., Yaro, J. B., Ouédraogo, E., Traoré, A. S., & Sirima, S. B. (2012). Seasonal

performance of a malaria rapid diagnosis test at community health clinics in a malaria-hyperendemic region of Burkina Faso. *Parasites & Vectors, 5*: 103.

Djimdé, A. A., Fofana, B., Sagara, I., Sidibe, B., Toure, S., Dembele, D., Dama, S., Ouologuem, D., Dicko, A., & Doumbo, O. K. (2008). Efficacy, safety, and selection of molecular markers of drug resistance by two ACTs in Mali. *The American Journal of Tropical Medicine and Hygiene, 78*: 455-461.

Djimdé A., Doumbo O. K., Cortese J. F., Kayentao K., Doumbo S., Diourté Y., Coulibaly D., Dicko A., Su X. Z., Nomura T., Fidock D. A., Wellems T. E., & Plowe C. V. (2001). A molecular marker for chloroquine-resistant falciparum malaria. *The New England Journal of Medicine, 344*: 257-263.

Dlamini S. V, Beshir K., & Sutherland C. J. (2010). Markers of anti-malarial drug resistance in *Plasmodium falciparum* isolates from Swaziland: identification of pfmdr1-86F in natural parasite isolates. *Malaria Journal, 9*: 1-9.

Do Manh, C., Beebe, N., Thi Van, V. N., Le Quang, T., Lein, C., Van Nguyen, D., Xuan, T., Le Ngoc, A., & Cooper, R. (2010). Vectors and malaria transmission in deforested, rural communities in north-central Vietnam. *Malaria Journal, 9*: 259.

Dondorp, A. M., Nosten, F., Yi, P., Das, D., Phyo, A. P., Tarning, J., Lwin, K. M., Ariey, F., Hanpithakpong, W., Lee, S. J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Herdman, T., An, S. S., Yeung, S., Singhasivanon, P., Day, N. P. J., Lindegardh, N., Socheat, D., & White, N. J. (2009). Artemisinin resistance in *Plasmodium falciparum* malaria. *The New England Journal of Medicine, 361*: 455-467.

Dondorp, A. M., Yeung, S., White, L., Nguon, C., Day, N. P. J., Socheat, D., & von Seidlein, L. (2010). Artemisinin resistance: current status and scenarios for containment. *Nature Reviews Microbiology, 8*: 272-280.

Drakeley C., Sutherland C., Bousema J. T., Sauerwein R. W., & Targett G. A. (2006). The epidemiology of *Plasmodium falciparum* gametocytes: weapons of mass dispersion. *Trends in Parasitology, 22*: 424-30.

Duraisingh M. T., Jones P., Sambou I., von Seidlein L., Pinder M., & Warhurst D. C. (2000). The tyrosine-86 allele of the pfmdr1 gene of *Plasmodium falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. *Molecular and Biochemical Parasitology, 108*: 13-23.

Ekvall, H., Z, P., & Bjorkman, A. (1998). Chloroquine treatment for uncomplicated childhood malaria in an area with drug resistance: early treatment failure aggravates anaemia. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 92*: 556-560.

El Hassan, I. M., Sahly, A., Alzahrani, M. H., Alhakeem, R. F., Alhelal, M., Alhogail, A., Alsheikh, A. A. H., Assiri, A. M., ElGamri, T. B., Faragalla, I. A., Al-Atas, M., Akeel, M. A., Bani, I., Ageely, H. M., BinSaeed, A. A., Kyalo, D., Noor, A. M., & Snow, R. W. (2015). Progress toward malaria elimination in Jazan Province, Kingdom of Saudi Arabia: 2000-2014. *Malaria Journal, 14*: 444.

Eyles, D. E., Hoo, C. C., Warren, M., & Sandosham, A. A. (1963). *Plasmodium falciparum* resistant to chloroquine in cambodia. *The American Journal of Tropical Medicine and Hygiene, 12*: 840-843.

Fancony C., Sebastiao Y., Pires J., Gamboa D., & Nery S. (2013). Performance of microscopy and RDTs in the context of a malaria prevalence survey in Angola: A comparison using PCR as the gold standard. *Malaria Journal, 12*: 284.

Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekunov, S. M., Ferdig, M. T., Ursos, L. M. B., Sidhu, A. b. S., Naudé, B., Deitsch, K. W., Su, X.-z., Wootton, J. C., Roepe, P. D., & Wellems, T. E. (2000). Mutations in the *P. falciparum* digestive vacuole transmembrane protein pfcrt and evidence for their role in chloroquine resistance. *Molecular Cell, 6*: 861–871.

Fogh S., Jepsen S., & Effersoe, P. (1979). Chloroquine-resistant *Plasmodium falciparum* malaria in Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 73*: 228-229.

Fogh, S., Jepsen, S., & Mataya, R. H. (1984). R-III chloroquine-resistant *Plasmodium falciparum* malaria from northern Malawi. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 78*: 282.

Foote, S. J., Kyle, D. E., Martin, R. K., Oduola, A. M. J., Forsyth, K., Kemp, D. J., & Cowman, A. F. (1990). Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature, 345*: 255-258.

Fröberg G., Jörnhagen L., Morris U., Shakely D., Msellem M. I., Gil J. P., Björkman A., & Mårtensson A. (2012). Decreased prevalence of *Plasmodium falciparum* resistance markers to amodiaquine despite its wide scale use as ACT partner drug in Zanzibar. *Malaria Journal, 11*: 321-321.

Fungladda, W., & Sornmani, S. (1986). Health behavior, treatment-seeking patterns, and cost of treatment for patients visiting malaria clinics in Western Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health, 17*: 379-385.

Fungladda, W., Sornmani, S., Klongkamnuankarn, K., & Hungsapruek, T. (1987). Sociodemographic and behavioral factors associated with hospital malaria patients in Kanchanaburi. *Thailand Journal of Tropical Medicine and Hygiene, 90*: 233-237.

Gadalla N. B., Abdallah T. M., Atwal S., Sutherland C. J., & Adam I. (2013). Selection of pfdhfr/pfdhps alleles and declining artesunate/sulphadoxine-pyrimethamine efficacy against *Plasmodium falciparum* 8 years after deployment in eastern Sudan. *Malaria Journal, 12*: 255.

Gamboa, D., Ho, M.-F., Bendezu, J., Torres, K., Chiodini, P. L., Barnwell, J. W., Incardona, S., Perkins, M., Bell, D., McCarthy, J., & Cheng, Q. (2010). A large proportion of *P. falciparum* isolates in the Amazon region of Peru lack pfhrp2 and pfhrp3: implications for malaria rapid diagnostic tests. *PLoS One, 5*: e8091.

Gamo F-J. (2014). Antimalarial drug resistance: new treatments options for Plasmodium. *Drug Discovery Today: Technologies, 11*: 81-88.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A.,

Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M.-S., Nene, V., Shallom, S. J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M. A., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W., Fraser, C. M., & Barrell, B. (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature, 419*: 498-511.

Girard, M., Reed, Z., Friede, M., & Kieny, M. (2007). A review of human vaccine research and development: Malaria. *Vaccine, 25*: 1567-1580.

Ghebreyesus, T. A., Haile, M., Witten, K. H., Getachew, A., Yohannes, A. M., Yohannes, M., Teklehaimanot, H. D., Lindsay, S. W., & Byass, P. (1999). Incidence of malaria among children living near dams in northern Ethiopia: community based incidence survey. *British Medical Journal, 319*, 663–666.

Ghouth A., Nasseb F., & Al-Kaldy K. (2012). The accuracy of the first response histidine-rich protein2 rapid diagnostic test compared with malaria microscopy for guiding field treatment in an outbreak of falciparum malaria. *Tropical Parasitology, 2*: 35-7.

Ghouth A. S. B. (2013). Availability and prescription practice of anti-malaria drugs in the private health sector in Yemen. *The Journal of Infection in Developing Countries, 7*: 404-12.

Golassa L., Enweji N., Erko B., Aseffa A., & Swedberg G. (2014). High prevalence of pfcrt-CVIET haplotype in isolates from asymptomatic and symptomatic patients in south-central Oromia, Ethiopia. *Malaria Journal, 13*: 1-8.

Gonzalez, A. (2000). Malaria Epidemic in Guajira, December 1, 1999 to February 1, 2000. *Infect Quinc Epidemiol Nac, 5*: 17-18.

Good, M. F., Xu, H., Wykes, M., & Engwerda, C. R. (2005). Development and regulation of cell-mediated immune responses to the blood stages of malaria: implications for vaccine research. *Annual Review of Immunology, 23*: 69-99.

Griffing S., Syphard L., Sridaran S., McCollum A. M., Mixson-Hayden T., Vinayak S., Villegas L., Barnwell J. W., Escalante A. A., & Udhayakumar V. (2010). Pfmdr1 amplification and fixation of pfcrt chloroquine resistance alleles in *Plasmodium falciparum* in Venezuela. *Antimicrobial Agents and Chemotherapy, 54*: 1572–1579.

Grimmond, T. R., Donovan, K. O., & Riley, I. D. (1976). Chloroquine resistant malaria in Papua New Guinea. Papua New Guinea. *Papua New Guinea Medical Journal*, 19: 184-185.

Harinasuta, T., Suntharasamai, P., & Viravan, C. (1965). Chloroquine resistant falciparum malaria in Thailand. *Lancet, 2*: 657-660.

Harrison, G. (1978). *Mosquitoes, malaria and man: A history of the hostilities since 1880*. New York: Dutton.

Hawass, Z., Gad, Y. Z., Ismail, S., Khairat, R., Fathalla, D., Hasan, N., Ahmed, A., Elleithy, H., Ball, M., Gaballah, F., Wasef, S., Fateen, M., Amer, H., Gostner, P.,

Selim, A., Zink, A., & Pusch, C. M. (2010). Ancestry and pathology in king Tutankhamun's family. *Journal of American Medicine Association, 303*: 638-647.

Higgins, C. F. (1992). ABC transporters: from microorganisms to man. *Annual Review of Cell and Developmental Biology, 8*: 67–113.

Himeidan, Y. E., Elbashir, M. I., & Adam, I. (2004). Attractiveness of pregnant women to the malaria vector, *Anopheles arabiensis*, in Sudan. *Annals of Tropical Medicine and Parasitology, 98*: 631-633.

Hermsen, C. C., Verhage, D. F., Telgt, D. S. C., Teelen, K., Bousema, J. T., Roestenberg, M., Bolad, A., Berzins, K., Corradin, G., Leroy, O., Theisen, M., & Sauerwein, R. W. (2007). Glutamate-rich protein (GLURP) induces antibodies that inhibit *in vitro* growth of *Plasmodium falciparum* in a phase 1 malaria vaccine trial. *Vaccine, 25*: 2930-2940.

Holder, A. A. (2009). Malaria vaccines: Where Next?. *PLoS Pathogens, 5*: e1000638.

Holder, A. A., Guevara Patiño, J. A., Uthaipibull, C., Syed, S. E., Ling, I. T., Scott-Finnigan, T., & Blackman, M. J. (1999). Merozoite surface protein 1, immune evasion, and vaccines against asexual blood stage malaria. *Parassitologia, 41*: 409-414.

Holmgren, G., Gil, J. P., Ferreira, P. M., Veiga, M. I., Obonyo, C. O., & Björkman, A. (2006). Amodiaquine resistant *Plasmodium falciparum* malaria in vivo is associated with selection of pfcrt 76T and pfmdr1 86Y. *Infection, Genetics and Evolution, 6*: 309-314.

Houzé S., Hubert V., Le Pessec G., Le Bras J., & Clain J. (2011). Combined deletions of pfhrp2 and pfhrp3 genes results in *Plasmodium falciparum* malaria false negative rapid diagnostic test. *Journal of Clinical Microbiology, 49*: 2694–6.

Huang, B., Deng, C., Yang, T., Xue, L., Wang, Q., Huang, S., Su, X.-z., Liu, Y., Zheng, S., Guan, Y., Xu, Q., Zhou, J., Yuan, J., Bacar, A., Abdallah, K., Attoumane, R., Mliva, A., Zhong, Y., Lu, F., & Song, J. (2015). Polymorphisms of the artemisinin resistant marker (K13) in *Plasmodium falciparum* parasite populations of Grande Comore Island 10 years after artemisinin combination therapy. *Parasites & Vectors, 8*: 634.

Humphreys G. S., Merinopoulos I., Ahmed J., Whitty C. J., Mutabingwa T. K., Sutherland C. J., & Hallett R. L. (2007). Amodiaquine and artemether-lumefantrine select distinct alleles of the *Plasmodium falciparum* mdr1 gene in Tanzanian children treated for uncomplicated malaria. *Antimicrobial Agents and Chemotherapy, 51*: 991-997.

Imwong, M., Tanomsing, N., Pukrittayakamee, S., Day, N. P. J., White, N. J., & Snounou, G. (2009). Spurious amplification of a *Plasmodium vivax* small-subunit RNA gene by use of primers currently used to detect *P. knowlesi*. *Journal of Clinical Microbiology, 47*: 4173-4175.

Iqbal J., Hira P. R., Sher A., & Al Enezi A. A. (2001). Diagnosis of imported malaria by Plasmodium lactate dehydrogenase (pLDH) and histidine-rich protein 2 (PfHRP-2)-based immunocapture assays. *The American Journal of Tropical Medicine and Hygiene, 64*: 20-3.

Isozumi R., Uemura H., Kimata I., Ichinose Y., Logedi J., Omar A. H., & Kaneko A. (2015). Novel mutations in K13 propeller gene of artemisinin-resistant *Plasmodium falciparum*. *Emerging Infectious Diseases, 21*: 490-2.

Jarcho, S. (1984). Laveran's discovery in the retrospect of a century. *Bulletin of the History of Medicine, 58*: 215-224.

Jongwutiwes, S., Putaporntip, C., Iwasaki, T., Sata, T., & Kanbara, H. (2004). Naturally acquired *Plasmodium knowlesi* malaria in human, Thailand. *Emerging Infectious Diseases, 10*: 2211-2213.

Kamau, E., Campino, S., Amenga-Etego, L., Drury, E., Ishengoma, D., Johnson, K., Mumba, D., Kekre, M., William, Y., Mead, D., Marielle, B.-A., Apinjoh, T., Golassa, L., Randrianarivelojosia, M., Andagalu, B., Maiga-Ascofare, O., Amambua-Ngwa, A., Tindana, P., Ghansah, A., MacInnis, B., Kwiatkowski, D., & Djimde, A. (2015). K13-propeller polymorphisms in *Plasmodium falciparum* parasites from sub-Saharan Africa. *Journal of Infectious Diseases, 211*: 1352-1355.

Kantele, A., & Jokiranta, T. S. (2011). Review of cases with the emerging fifth human malaria parasite, *Plasmodium knowlesi*. *Clinical Infectious Diseases, 52*: 1356-1362.

Karl S., Gurarie D., Zimmerman P. A., King C. H., St Pierre T. G., & Davis T. M. (2011). A submicroscopic gametocyte reservoir can sustain malaria transmission. *PLoS One, 6*: e20805.

Khattak, A. A., Venkatesan, M., Jacob, C. G., Artimovich, E. M., Nadeem, M. F., Nighat, F., Hombhanje, F., Mita, T., Malik, S. A., & Plowe, C. V. (2013). A comprehensive survey of polymorphisms conferring anti-malarial resistance in *Plasmodium falciparum* across Pakistan. *Malaria Journal, 12*: 300.

Kiszewski, A. E., & Teklehaimanot, A. (2004). A review of the clinical and epidemiologic burdens of epidemic malaria. *The American Journal of Tropical Medicine and Hygiene, 71*: 128-135.

Kocken, C. H. M., Withers-Martinez, C., Dubbeld, M. A., van der Wel, A., Hackett, F., Blackman, M. J., & Thomas, A. W. (2002). High-level expression of the malaria blood-stage vaccine candidate *Plasmodium falciparum* apical membrane antigen 1 and induction of antibodies that inhibit erythrocyte invasion. *Infection and Immunity, 70*: 4471-4476.

Koita, O., Doumbo, O., Ouattara, A., Tall, L., Konare, A., Diakite, M., Diallo, M., Sagara, I., Masinde, G., Doumbo, S., Dolo, A., Tounkara, A., Traore, I., & Krogstad, D. (2012). False-negative rapid diagnostic tests for malaria and deletion of the histidine-rich repeat region of the hrp2 gene. *The American Journal of Tropical Medicine and Hygiene, 86*: 194-8.

Krungkrai, S. R., & Yuthavong, Y. (1987). The antimalarial action on *Plasmodium falciparum* of qinghaosu and artesunate in combination with agents which modulate oxidant stress. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 81*: 710–4.

Krungkrai, J., & Krungkrai, S. R. (2016). Antimalarial qinghaosu/artemisinin: The therapy worthy of a Nobel Prize. *Asian Pacific Journal of Tropical Biomedicine, 6*: 371-375.

Kublin, J. G., Cortese, J. F., Njunju, E. M., G. Mukadam, R. A., Wirima, J. J., Kazembe, P. N., Djimdé, A. A., Kouriba, B., Taylor, T. E., & Plowe, C. V. (2003). Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *J Infectious Diseases, 187*: 1870-1875.

Kublin J. G., Dzinjalamala F. K., Kamwendo D. D., Malkin E. M., Cortese J. F., & Martino L. M. (2002). Molecular markers for failure of sulfadoxine–pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. *International Journal of Infectious Diseases, 185*: 380-388.

Kumar, N., Singh, J., Pande, V., Mishra, N., Srivastava, B., Kapoor, R., Valecha, N., & Anvikar, A. (2012). Genetic variation in histidine rich proteins among Indian *Plasmodium falciparum* population: possible cause of variable sensitivity of malaria rapid diagnostic tests. *Malaria Journal, 11*: 298.

Laufer M. K., Thesing P. C., Eddington N. D., Masonga R., Dzinjalamala F. K., Takala S. L., Taylor T. E., & Plowe C. V. (2006). Return of chloroquine antimalarial efficacy in Malawi. *The New England Journal of Medicine, 355*: 1959-1966.

Laveran, C. L. A., Kean, B. H., Mott, K. E., & Russell, A. J. (1982). A newly discovered parasite in the blood of patients suffering from malaria. Parasitic etiology of Attacks of malaria. *Reviews of infectious diseases, 4*: 908-911.

Lee, N., Gatton, M., Pelecanos, A., Bubb, M., Gonzalez, I., Bell, D., Cheng, Q., & McCarthy, J. (2012). Identification of optimal epitopes for *Plasmodium falciparum* rapid diagnostic tests that target histidine-rich proteins 2 and 3. *Journal of Clinical Microbiology, 50*: 1397-405.

Levander, O. A., Ager Jr, A. L., Morris, V. C., & May, R. G. (1989). Qinghaosu, dietary vitamin E, selenium, and cod-liver oil: effect on the susceptibility of mice to the malarial parasite *Plasmodium yoelii*. *The American Journal of Clinical Nutrition, 50*: 346–52.

Liljander, A. (2010). Malaria: Multiclonal infections and protective immunity. (PhD), Karolinska institute, Stockholm, Sweden. Retrieved from https://openarchive.ki.se/xmlui/handle/10616/38651

Lindsay, S., Ansell, J., Selman, C., Cox, V., Hamilton, K., & Walraven, G. (2000). Effect of pregnancy on exposure to malaria mosquitoes. *Lancet.3;355*:1972.

Ling, I. T., Ogun, S. A., & Holder, A. A. (1994). Immunization against malaria with a recombinant protein. *Parasite Immunology, 16*(2): 63-67.

Liu D. Q., Liu R. J., Ren D. X., Gao D. Q., Zhang C. Y., Qui C. P., Cai X. Z., Ling C. F., Song A. H., & Tang X. (1995). Changes in the resistance of *Plasmodium falciparum* to chloroquine in Hainan, China. *Bulletin of the World Health Organization, 73*: 483-486.

Looareesuwan, S., Viravan, C., Webster, H. K., Kyle, D. E., Hutchinson, D. B. & Canfield, C. J. (1996). Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand. *The American Journal of Tropical Medicine and Hygiene, 54*: 62-66.

Lusingu J. P., Vestergaard L. S., Mmbando B. P., Drakeley C. J., Jones C, Akida J, Savaeli Z. X., Kitua A. Y., Lemnge M. M., Theander T. G. (2004). Malaria morbidity and immunity among residents of villages with different *Plasmodium falciparum* transmission intensity in North-Eastern Tanzania. *Malaria Journal, 3*:26.

Luxemburger, C., Ricci, F., Nosten, F., Raimond, D., Sather, S. & White, N. (1997). The epidemiology of severe malaria in an area of low transmission in Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 91*: 256-262.

Macdonald, G. (1957). *The epidemiology and control of malaria*. London, United Kingdom: Oxford University Press.

Malmberg M., Ferreira P. E., Tarning J., Ursing J., Ngasala B., Björkman A., Mårtensson A., & Gil J. P. (2013). *Plasmodium falciparum* drug resistance phenotype as assessed by patient antimalarial drug levels and its association with pfmdr1 polymorphisms. *Journal of Infectious Diseases, 207*: 842-847.

Maltha, J., Gamboa, D., Bendezu, J., Sanchez, L., Cnops, L., Gillet, P., & Jacobs, J. (2012). Rapid diagnostic tests for malaria diagnosis in the Peruvian Amazon: Impact of pfhrp2 gene deletions and cross-reactions. *PLoS One, 7*: 43094.

Mamser A. (1989). Report on a visit to the Yemen Arab Republic from 14–2 to 13-3-1989. Geneva, EMRO: World Health Organization. pp. 1–5. (WHO/RA/MAL/005).

Mariette N., Barnadas C., Bouchier C., Tichit M., & Menard D. (2008). Country-wide assessment of the genetic polymorphism in *Plasmodium falciparum* and *Plasmodium vivax* antigens detected with rapid diagnostic tests for malaria. *Malaria Journal, 7*: 219.

Mbogo G. W., Nankoberanyi S., Tukwasibwe S., Baliraine F. N., Nsobya S. L., Conrad M. D., & Rosenthal P. J. (2014). Temporal changes in prevalence of molecular markers mediating antimalarial drug resistance in a high malaria transmission setting in Uganda. *The American Journal of Tropical Medicine and Hygiene, 91*: 54–61.

McMorrow M. L., Masanja M. I., Abdulla S. M. K., Kahigwa E., & Kachur S. P. (2008). Challenges in routine implementation and quality control of rapid diagnostic tests for malaria–Rufiji district, Tanzania. *The American Journal of Tropical Medicine and Hygiene, 79*: 385-90.

Meek, S. (1988). Epidemiology of malaria in displaced Khmers on the Thailand-Kampuchean border. *Southeast Asian Journal of Tropical Medicine and Public Health, 19*: 243-252.

Mehlotra R. K., Fujioka H., Roepe P. D., Janneh O., Ursos L. M., Jacobs-Lorena V., McNamara D. T., Bockarie M. J., Kazura J. W., Kyle D. E., Fidock D. A., & Zimmerman P. A. (2001). Evolution of a unique *Plasmodium falciparum* chloroquine-resistance phenotype in association with pfcrt polymorphism in Papua New Guinea and South America. *Proceedings of the National Academy of Sciences of the United States of America, 98*: 12689–12694.

Mekonnen S. K., Aseffa A., Berhe N., Teklehaymanot T., Clouse R. M., Gebru T., & Velavan T. P. (2014). Return of chloroquine-sensitive *Plasmodium falciparum* parasites and emergence of chloroquine-resistant *Plasmodium vivax* in Ethiopia. *Malaria Journal, 13*: 244.

Mens P. F., Sawa P., van Amsterdam S. M., Versteeg I., Omar S. A., Schallig H. D., & Kager P. A. (2008). A randomized trial to monitor the efficacy and effectiveness by QT-NASBA of artemether-lumefantrine versus dihydroartemisinin-piperaquine for treatment and transmission control of uncomplicated *Plasmodium falciparum* malaria in western Kenya. *Malaria Journal, 7*: 237.

Meshnick, S. R., Taylor, T. E., & Kamchonwongpaisan, S. (1996). Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiological Reviews, 60*: 301–315.

Meshnick, S. R., Thomas, A., Ranz, A., Xu, C. M., & Pan, H. Z. (1991). Artemisinin (qinghaosu): the role of intracellular hemin in its mechanism of antimalarial action. *Molecular and Biochemical Parasitology, 49*:181-9.

Meshnick, S. R., Tsang, T. W., Lin, F. B., Pan, H. Z., Chang, C. N., Kuypers, F., Chiu, D., & Lubin, B. (1989). Activated oxygen mediates the antimalarial activity of qinghaosu. *Progress in clinical and biological research, 313*: 95–104.

Metselaar, D. & Van Thiel, P. (1959). Classification of malaria. *Tropical and Geographical Medicine, 11*: 157-161.

Miller R. L., Ikram S., Armelagos G. J., Walker R., Harer W. B., Shiff C. J., Baggett D., Carrigan M., & Maret S. M. (1994a). Diagnosis of *Plasmodium falciparum* infections in mummies using the rapid manual ParaSight-F test. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 88*: 31-32.

Miller, L., Good, M., & Milon, G. (1994b). Malaria pathogenesis. *Science, 264*: 1878-1883.

Minakawa, N., Omukunda, E., Zhou, G., Githeko, A., & Yan, G. (2006). Malaria vector productivity in relation to the highland environment in Kenya. *The American Journal of Tropical Medicine and Hygiene, 75*: 448-453.

Miotto, O., Almagro-Garcia, J., Manske, M., MacInnis, B., Campino, S., Rockett, K. A., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J. M., Duong, S., Nguon, C., Chuor, C. M., Saunders, D., Se, Y., Lon, C., Fukuda, M. M., Amenga-Etego, L., Hodgson, A. V. O., Asoala, V., Imwong, M., Takala-Harrison, S., Nosten, F., Su, X.-z., Ringwald, P., Ariey, F., Dolecek, C., Hien, T. T., Boni, M. F., Thai, C. Q., Amambua-Ngwa, A., Conway, D. J., Djimde, A. A., Doumbo, O. K., Zongo, I., Ouedraogo, J.-B., Alcock, D., Drury, E., Auburn, S., Koch, O., Sanders, M., Hubbart, C., Maslen, G., Ruano-Rubio, V., Jyothi, D., Miles, A., O'Brien, J., Gamble, C., Oyola, S. O., Rayner, J. C., Newbold, C. I., Berriman, M., Spencer, C. C. A., McVean, G., Day, N. P., White, N. J., Bethell, D., Dondorp, A. M., Plowe, C. V., Fairhurst, R. M., & Kwiatkowski, D. P. (2013). Multiple populations of artemisinin-resistant *Plasmodium falciparum* in Cambodia. *Nature Genetics, 45*: 648-55.

Mita T., Kaneko A., Lum J. K., Zungu I. L., Tsukahara T., Eto H., Kobayakawa T., Björkman A., & Tanabe K. (2004). Expansion of wild type allele rather than back mutation in pfcrt explains the recent recovery of chloroquine sensitivity of *Plasmodium falciparum* in Malawi. *Molecular and Biochemical Parasitology, 135*: 159-163.

Mita, T., Tanabe, K., Kita, K. (2009). Spread and evolution of *Plasmodium falciparum* drug resistance. *Parasitology International, 58*:201-9.

Mohammed A., Ndaro A., Kalinga A., Manjurano A., Mosha J. F., Mosha D. F., & Kavishe R. A. (2013). Trends in chloroquine resistance marker, Pfcrt-K76T mutation ten years after chloroquine withdrawal in Tanzania. *Malaria Journal, 12*: 415.

MOHP. (2014). National Health Statistics Report: Sana'a: Ministry of Health and Population, Yemen.

Montgomery, C. R., & Eyles, D. E. (1963). Chloroquine resistant falciparum malaria in Malaya. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 57*: 409-416.

Moore, D. V., & Lanier, J. E. (1961). Observations on two *Plasmodium falciparum* infections with an abnormal response to chloroquine. *The American Journal of Tropical Medicine and Hygiene, 10*: 5-9.

Mubjer R., Adeel A., Chance M., & Hassan A. (2011). Molecular markers of anti-malarial drug resistance in Lahj Governorate, Yemen: baseline data and implications. *Malaria Journal, 10*: 245.

Mula P., Fernández-Martínez A., de Lucio A., Ramos J. M., Reyes F., González V., Benito A., & Berzosa P. (2011). Detection of high levels of mutations involved in anti-malarial drug resistance in *Plasmodium falciparum* and *Plasmodium vivax* at a rural hospital in southern Ethiopia. *Malaria Journal, 10*: 214.

Mungthin M., Suwandittakul N., Chaijaroenkul W., Rungsrihirunrat K., Harnyuttanakorn P., Seugorn A., & Na Bangchang K. (2010). The patterns of mutation and amplification of *Plasmodium falciparum* pfcrt and pfmdr1 genes in Thailand during the year 1988 to 2003. *Parasitology Research, 107*: 539-545.

Murray C. K., & Bennett J. W. (2009). Rapid diagnosis of malaria. *Interdiscip Perspect Infect Dis,* 415953.

National Centre of Biotechnology Information. http://www.ncbi.nlm.nih.gov/gene/9221889. Access date: 18 Mar 2015.

National Information Centre, Yemen. (2014). http://www.yemen-nic.info/sectors/popul. Access date 16 Jan 2015.

NMCP. (2009) Updating National Malaria Treatment Policy, Yemen. Sana'a. National Malaria Control Program, Ministry of Health and Population, Yemen.

NMCP. (2014). Malaria Integrated Vector Control. Sana'a: National Malaria Control Program, Yemen.

Njokah, M. J., Kang'ethe, J. N., Kinyua, J., Kariuki, D. & Kimani, F. T. (2016). *In vitro* selection of *Plasmodium falciparum* pfcrt and pfmdr1 variants by artemisinin. *Malaria Journal, 15*(1): 381.

Nosten, F., Kuile, F. t., Chongsuphajaisiddhi, T., Luxemburger, C., Webster, H. K., Edstein, M., Phaipun, L., Thew, K. L., & White, N. J. (1991). Mefloquine-resistant falciparum malaria on the Thai-Burmese border. *Lancet, 337*: 1140-1143.

Nosten F., & White N. J. (2007). Artemisinin-based combination treatment of falciparum malaria. *The American Journal of Tropical Medicine and Hygiene, 77*: 181-192.

Nunn, J. F., & Tapp, E. (2000). Tropical diseases in ancient Egypt. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 94*: 147-153.

Nwakanma D., Kheir A., Sowa M., Dunyo S., Jawara M., Pinder M., Milligan P., Walliker D., & Babiker H. A. (2008). High gametocyte complexity and mosquito infectivity of *Plasmodium falciparum* in the Gambia. *International Journal for Parasitology, 38*: 219-227.

Nyunt, M. H., Hlaing, T., Oo, H. W., Tin-Oo, L.-L. K., Phway, H. P., Wang, B., Zaw, N. N., Han, S. S., Tun, T., San, K. K., Kyaw, M. P., & Han, E.-T. (2015). Molecular assessment of artemisinin resistance markers, polymorphisms in the k13 propeller, and a multidrug-resistance gene in the eastern and western border areas of Myanmar. *Clinical Infectious Diseases, 60*: 1208-15.

Oemijati, S. (1992). Risk behavior in malaria transmission in Indonesia. *Southeast Asian Pacific Journal of Tropical Medicine, 23*: 47-50.

Okell L. C., Ghani A. C., Lyons E., & Drakeley C. J. (2009). Submicroscopic infection in *Plasmodium falciparum*-endemic populations: a systematic review and meta-analysis. *Journal of Infectious Disease, 200*: 1509-17.

Okoko, B. J., Enwere G., Ota M. O. (2003). The epidemiology and consequences of maternal malaria: a review of immunological basis. *Acta Tropica, 87*:193-205.

Olotu, A., Lusingu, J., Leach, A., Lievens, M., Vekemans, J., Msham, S., Lang, T., Gould, J., Dubois, M.-C., Jongert, E., Vansadia, P., Carter, T., Njuguna, P., Awuondo, K. O., Malabeja, A., Abdul, O., Gesase, S., Mturi, N., Drakeley, C. J., Savarese, B., Villafana, T., Lapierre, D., Ballou, W. R., Cohen, J., Lemnge, M. M., Peshu, N., Marsh, K., Riley, E. M., von Seidlein, L., & Bejon, P. (2011). Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5–17 months in Kenya and Tanzania: a randomised controlled trial. *The Lancet Infectious Diseases, 11*: 102-109.

Onori, E. (1984). The problem of *Plasmodium falciparum* drug resistance in Africa south of the Sahara. *Bulletin of the World Health Organization, 62*: 55-62.

Overbosch, D., van den Wall Bake AW, P. C. Stuiver, and H. J. van der Kaay. (1984). Chloroquine-resistant falciparum malaria from Malawi. *Tropical and Geographical Medicine, 36*: 71-72.

Owusu-Agyei, S., Smith, T., Beck, H. P., Amenga-Etego, L., & Felger, I. (2002). Molecular epidemiology of *Plasmodium falciparum* infections among asymptomatic inhabitants of a holoendemic malarious area in northern Ghana. *Tropical Medicine & International Health, 7*: 421-428.

Paaijmans, K. P., Read, A. F., & Thomas, M. B. (2009). Understanding the link between malaria risk and climate. *Proceedings of the National Academy of Sciences of the United States of America, 106*: 13844-13849.

Pålsson, K., Jaenson, T. G., Dias, F., Laugen, A. T., & Bjorkman, A. (2004). Endophilic Anopheles mosquitoes in Guinea Bissau, west Africa, in relation to human housing conditions. *Journal of Medical Entomology*. 41:746–752.

Partnership RBM. (2008). The Global Malaria Action Plan for a Malaria Free World. Geneva: Roll Back Malaria Partnership.

Pasvol G. C. B., Carlsson, J. & Snounou G., (1995). The Pathogenesis of severe falciparum malaria. *Bailliere's Clinical Infious Diseases, 2*: 249-270.

Payne, D. (1987). Spread of chloroquine resistance in *Plasmodium falciparum*. *Parasitology Today, 3*: 241-246.

Peters, W., (1987). *Chemotherapy and drug resistance in malaria* (2nd ed.). New York: Academic Press, Inc.

Peterson, D. S., Walliker, D., Wellems, T. E. (1988). Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. *Proceedings of the National Academy of Sciences of the United States of America, 85*:9114-8.

Picot, S., Olliaro, P., de Monbrison, F., Bienvenu, A. L., Price, R. N., & Ringwald, P. (2009). A systematic review and meta-analysis of evidence for correlation between molecular markers of parasite resistance and treatment outcome in falciparum malaria. *Malaria Journal*, 8: 89-10.

Pinsent, A., Read, J. M., Griffin, J. T., Smith, V., Gething, P. W., Ghani, A. C., Pasvol, G., & Hollingsworth, T. D. (2014). Risk factors for UK *Plasmodium falciparum* cases. *Malaria Journal, 13*: 1-8.

Playford E. G., & Walker J. (2002). Evaluation of the ICT malaria P.f/P.v and the OptiMal rapid diagnostic tests for malaria in febrile returned travellers. *Journal of Clinical Microbiology, 40*: 4166-71.

Plowe, C. V., Cortese, J. F., Djimde, A., Nwanyanwu, O. C., Watkins, W. M., Winstanley, P. A., Franco, J. G. E., Mollinedo, R. E., Avila, J. C., Cespedes, J. L., Carter, D., & Doumbo, O. K. (1997). Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. *Journal of Infectious Diseases, 176*: 1590-1596.

Price, R. N., Cassar, C., Brockman, A., Duraisingh, M., van Vugt, M., White, N. J., Nosten, F., & Krishna, S. (1999). The pfmdr1 gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. *Antimicrobial Agents and Chemotherapy, 43*: 2943-2949.

Reed M. B., Saliba K. J., Caruana S. R., Kirk K., & Cowman A. F. (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature, 403*: 906-909.

Reyburn, H., Mbatia, R., Drakeley, C., Bruce, J., Carneiro, I., Olomi, R., Cox, J., Nkya, W. M., Lemnge, M., Greenwood, B. M., & Riley, E. M. (2005). Association of transmission intensity and age with clinical manifestations and case fatality of severe

Plasmodium falciparum malaria. *Journal of American Medicine Association,* 293:1461-1470.

Richard, C. (2001). Transmission blocking malaria vaccines. *Vaccine, 19*: 2309-2314.

Rieckmann, K., Campbell, G., Sax, L., & Mrema, J. (1978). Drug sensitivity of *Plasmodium falciparum*. An in-vitro microtechnique. *Lancet, 1*: 22-23.

RBM., (2002). Epidemiology of Malaria. Retrieved from: Roll Back Malaria: http://chsr.aua.am/malaria/eng/epidemiology.php. Accessed date: 1 Dec 2014

Rock, E. P., Marsh, K., Saul, A. J., Wellems, T. E., Taylor, D. W., Maloy, W. L., & Howard, R. J. (1987). Comparative analysis of the *Plasmodium falciparum* histidine-rich proteins HRP-I, HRP-II and HRP-III in malaria parasites of diverse origin. *Parasitology, 95*: 209-27.

Rosenthal, P. J. & Meshnick, S. R. (1996) Hemoglobin catabolism and iron utilization by malaria parasites. *Molecular and Biochemical Parasitology, 83*:131-9.

Rouhani M., Zakeri S., Pirahmadi S., Raeisi A., & Djadid N. D. (2015). High prevalence of *pfdhfr-pfdhps* triple mutations associated with anti-malarial drugs resistance in *Plasmodium falciparum* isolates seven years after the adoption of sulfadoxine-pyrimethamine in combination with artesunate as first-line treatment in Iran. *Infection, Genetics and Evolution, 31*: 183-9.

RTS,S Clinical Trials Partnership. (2015). Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. *Lancet, 386*: 31-45.

Rutgers, T., Gordon, D., Gathoye, A. M., Hollingdale, M., Hockmeyer, W., Rosenberg, M., & Wilde, M. D. (1988). Hepatitis B surface antigen as carrier matrix for the repetitive epitope of the circumsporozoite protein of *Plasmodium Falciparum*. *Nature Biotechnology, 6*: 1065-1070.

Sabatinelli, G., Ejov, M., & Joergensen, P. (2000). Malaria in the WHO European Region (1971-1999). *Euro Surveillance, 6*: 61-65.

Satoskar, A. R. (2009). *Medical parasitology*. Austin, Tex., United States: Landes Bioscience.

Schofield, L., Michael, C., Hewitt, K. E., Siomos, M. A. & Seeberger, P. H. (2002). Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. *Nature, 418*:785-9**.**

Senok, A. C., Nelson, E. A., Li, K., & Oppenheimer, S. J. (1997). Thalassaemia trait, red blood cell age and oxidant stress: effects on *Plasmodium falciparum* growth and sensitivity to artemisinin. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 91*: 585–9.

Shekalaghe, S. A., ter Braak, R., Daou, M., Kavishe, R., van den Bijllaardt, W., van den Bosch, S., Koenderink, J. B., Luty, A. J. F., Whitty, C. J. M., Drakeley, C., Sauerwein, R. W., & Bousema, T. (2010). Haemolysis after a single dose of primaquine co-administered with an artemisinin is not restricted to glucose-6-

phosphate dehydrogenase (G6PD A- variant) deficient individuals in Tanzania. *Antimicrobial Agents and Chemotherapy, 54*: 1762-68.

Sherman I. W. (1998). *A brief history of malaria and discovery of the parasite's life cycle*. In: Sherman I. W., editor. ed. Malaria: Parasite Biology, Pathogenesis and Protection. Washington, DC: ASM.

Sidhu, A. B. S., Valderramos, S. G., & Fidock, D. A. (2005). Pfmdr1 mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. *Molecular Microbiology, 57*: 913-926.

Singh, B., Bobogare, A., Cox-Singh, J., Snounou, G., Abdullah, M., & Rahman, H. (1999). A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *The American Journal of Tropical Medicine and Hygiene, 60*: 687 - 692.

Singh B., Sung L. K., Matusop A., Radhakrishnan A., Shamsul S. S. G., Cox-Singh J., & Thomas A. (2004). A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet, 363*: 1017-1024.

Singhanetra-Renard, A. (1986). Population movement, socioeconomic behavior and the transmission of malaria in Northern Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health, 17*: 396-405.

Singhanetra-Renard, A. (1993). Malaria and mobility in Thailand. *Social Science & Medicine, 37*: 1147-1154.

Sinton J. A. & Mulligan H. W. (1933). A critical review of the literature relating to the identification of the malarial parasites recorded from monkeys of the families Cercopithecidae and Colobidae. *Records of the Malaria Survey of India*, *3*:381–443.

Sirawaraporn, W., Sathitkul, T., Sirawaraporn, R., Yuthavong, Y., & Santi, D. V. (1997). Antifolate-resistant mutants of *Plasmodium falciparum* dihydrofolate reductase. *Proceedings of the National Academy of Sciences of the United States of America*, *94*:1124-9.

Sisowath, C., Ferreira, P. E., Bustamante, L. Y., Dahlström, S., Mårtensson, A., Björkman, A., Krishna, S., & Gil, J. P. (2007). The role of pfmdr1 in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa. *Tropical Medicine & International Health, 12*: 736-742.

Sisowath, C., Strömberg, J., Mårtensson, A., Msellem, M., Obondo, C., Björkman, A., & Gil, J. P. (2005). In vivo selection of *Plasmodium falciparum* pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). *Journal of Infectious Diseases, 191*: 1014-1017.

Slatter, M. J., Pettengell, K., & Taylor, R. K. (1983). Chloroquine-resistant malaria. *South African Medical Journal, 63*: 838.

Snounou G., & Beck H. P. (1998). The use of PCR genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. *Parasitology Today, 14*: 462-467.

Snounou G., Zhu X., Siripoon N., Jarra W., Thaithong S., Brown K., & Viriyakosol S. (1999). Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 93*: 369-74.

Snow, R. W., Amratia, P., Zamani, G., Mundia, C. W., Noor, A. M., Memish, Z. A., Al Zahrani, M. H., Al Jasari, A., Fikri, M., & Atta, H. (2013). The malaria transition on the Arabian Peninsula: Progress toward a malaria-free region between 1960–2010. *Advances in parasitology, 82*: 205-251.

Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. Y., & Hay, S. I. (2005). The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature, 434*: 214-217.

Sornmani, S., Butraporn, P., Fungladda, W., Okanurak, K., & Dissapongsa, S. (1983). Migration and disease problems: A study of patterns of migration in an endemic area of malaria in Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health, 14*: 64-68.

Starzengruber, P., Fuehrer, H.-P., Ley, B., Thriemer, K., Swoboda, P., Habler, V. E., Jung, M., Graninger, W., Khan, W. A., Haque, R., & Noedl, H. (2014). High prevalence of asymptomatic malaria in south-eastern Bangladesh. *Malaria Journal, 13*: 1-10.

Straimer, J., Gnädig, N. F., Witkowski, B., Amaratunga, C., Duru, V., Ramadani, A. P., Dacheux, M., Khim, N., Zhang, L., Lam, S., Gregory, P. D., Urnov, F. D., Mercereau-Puijalon, O., Benoit-Vical, F., Fairhurst, R. M., Ménard, D., & Fidock, D. A. (2015). K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. *Science, 347*: 428-31.

Stepniewska, K., Taylor, W. R. J., Mayxay, M., Price, R., Smithuis, F., Guthmann, J.-P., Barnes, K., Myint, H. Y., Adjuik, M., Olliaro, P., Pukrittayakamee, S., Looareesuwan, S., Hien, T. T., Farrar, J., Nosten, F., Day, N. P. J., & White, N. J. (2004). *In vivo* assessment of drug efficacy against *Plasmodium falciparum* malaria: Duration of follow-up. *Antimicrobial Agents and Chemotherapy, 48*: 4271-4280.

Sutherland, C. J., Alloueche, A., Curtis, J., Drakeley, C. J., Ord, R., Duraisingh, M., Greenwood, B. M., Pinder, M., Warhurst, D., & Targett, G. A. T. (2002). Gambian children successfully treated with chloroquine can harbor and transmit *Plasmodium falciparum* gametocytes carrying resistance genes. *The American Journal of Tropical Medicine and Hygiene, 67*: 578-85.

Swales, C. A., Chiodini, P. L., & Bannister, B. A. (2007). New guidelines on malaria prevention: a summary. *Journal of Infection, 54*: 107-110.

Tachibana, S.-I., Sullivan, S. A., Kawai, S., Nakamura, S., Kim, H. R., Goto, N., Arisue, N., Palacpac, N. M. Q., Honma, H., Yagi, M., Tougan, T., Katakai, Y., Kaneko, O., Mita, T., Kita, K., Yasutomi, Y., Sutton, P. L., Shakhbatyan, R., Horii, T., Yasunaga, T., Barnwell, J. W., Escalante, A. A., Carlton, J. M., & Tanabe, K. (2012). Plasmodium cynomolgi genome sequences provide insight into *Plasmodium vivax* and the monkey malaria clade. *Nature Genetics, 44*: 1051-1055.

Tahar R., Sayang C., Ngane Foumane V., Soula G., Moyou-Somo R., Delmont J., & Basco L. K. (2013). Field evaluation of rapid diagnostic tests for malaria in Yaounde, Cameroon. *Acta Tropica, 125*: 214-9.

Takala-Harrison, S., Jacob, C. G., Arze, C., Cummings, M. P., Silva, J. C., Dondorp, A. M., Fukuda, M. M., Hien, T. T., Mayxay, M., Noedl, H., Nosten, F., Kyaw, M. P., Nhien, N. T. T., Imwong, M., Bethell, D., Se, Y., Lon, C., Tyner, S. D., Saunders, D. L., Ariey, F., Mercereau-Puijalon, O., Menard, D., Newton, P. N., Khanthavong, M., Hongvanthong, B., Starzengruber, P., Fuehrer, H.-P., Swoboda, P., Khan, W. A., Phyo, A. P., Nyunt, M. M., Nyunt, M. H., Brown, T. S., Adams, M., Pepin, C. S., Bailey, J., Tan, J. C., Ferdig, M. T., Clark, T. G., Miotto, O., MacInnis, B., Kwiatkowski, D. P., White, N. J., Ringwald, P., & Plowe, C. V. (2015). Independent emergence of artemisinin resistance mutations among *Plasmodium falciparum* in Southeast Asia. *Journal of Infectious Diseases, 211*: 670-9.

Targett, G., Drakeley, C., Jawara, M., von Seidlein, L., Coleman, R., Deen, J., Pinder, M., Doherty, T., Sutherland, C., Walraven, G., & Milligan, P. (2001). Artesunate reduces but does not prevent post-treatment transmission of *Plasmodium falciparum* to *Anopheles gambiae*. *Journal of Infectious Diseases, 183*: 1254-9.

Ta, T. H., Hisam, S., Lanza, M., Jiram, A. I., Ismail, N., & Rubio, J. M. (2014). First case of a naturally acquired human infection with *Plasmodium cynomolgi*. *Malaria Journal, 13*: 1-7.

Taylor-Robinson, A. W. (2000). Vaccination against malaria: targets, strategies and potentiation of immunity to blood stage parasites. *Frontiers in Bioscience, **1***: 16-29.

Taylor, S. M., Parobek, C. M., DeConti, D. K., Kayentao, K., Coulibaly, S. O., Greenwood, B. M., Tagbor, H., Williams, J., Bojang, K., Njie, F., Desai, M., Kariuki, S., Gutman, J., Mathanga, D. P., Mårtensson, A., Ngasala, B., Conrad, M. D., Rosenthal, P. J., Tshefu, A. K., Moormann, A. M., Vulule, J. M., Doumbo, O. K., ter Kuile, F. O., Meshnick, S. R., Bailey, J. A., & Juliano, J. J. (2015). Absence of putative artemisinin resistance mutations among *Plasmodium falciparum* in Sub-Saharan Africa: a molecular epidemiologic study. *Journal of Infectious Diseases, 211*: 680-8.

TDR. (2003). Drugs against parasitic diseases: R & D methodologies and issues. Discoveries and drug development. Geneva, Switzerland: World Health Organization.

The malERA Consultative Group on Vaccines (2011), A research agenda for malaria eradication: vaccines. *PLOS Medicine 8*: e1000398.

Thomsen T. T., Ishengoma D. S., Mmbando B. P., Lusingu J. P., Vestergaard L. S., Theander T.G., Lemnge M. M., Bygbjerg I. C., & Alifrangis, M. (2011). Prevalence of single nucleotide polymorphisms in the *Plasmodium falciparum*multidrug resistance gene (Pfmdr-1) in Korogwe District in Tanzania before and after introduction of artemisinin-based combination therapy. *The American Journal of Tropical Medicine and Hygiene, 85*: 979-983.

Tinto H., Ouédraogo J. B., Zongo I., van Overmeir C., van Marck E., Guiguemdé T. R., & D'Alessandro U. (2007). Sulfadoxine–pyrimethamine efficacy and selection of *Plasmodium falciparum* dhfr mutations in Burkina Faso before its introduction as

intermittent preventive treatment for pregnant women. *The American Journal of Tropical Medicine and Hygiene, 76*: 608-13.

Torrentino-Madamet, M., Fall, B., Benoit, N., Camara, C., Amalvict, R., Fall, M., Dionne, P., Ba Fall, K., Nakoulima, A., Diatta, B., Dieme, Y., Menard, D., Wade, B., & Pradines, B. (2014). Limited polymorphisms in k13 gene in *Plasmodium falciparum* isolates from Dakar, Senegal in 2012-2013. *Malaria Journal, 13*: 472.

Toshihiro Mita, Akira Kaneko, J. Koji LUM, Bwijo Bwijo, Miho Takechi, Innocent L. Zungu, Takahiro Tsukahara, Kazuyuki Tanabe, Takatoshi Kobayakawa, & Anders Bjorkman. (2003). Recovery of chloroquine sensitivity and low prevalence of *Plasmodium falciparum* Chloroquine resistance transporter gene mutation K76T following the discontinuance of chloroquine use in Malawi. *The American Journal of Tropical Medicine and Hygiene, 68*: 413-415.

Trape, J.-F., Lefebvre-Zante, E., Legros, F., Ndiaye, G., Bouganali, H., Druilhe, P., & Salem, G. (1992). Vector density gradients and the epidemiology of urban malaria in Dakar, Senegal. *The American Journal of Tropical Medicine and Hygiene, 47*: 181-189.

Triglia, T., Menting, J. G. T., Wilson, C., & Cowman, A. F. (1997). Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences of the United States of America, 94*, 13944–13949.

Tun, K. M., Imwong, M., Lwin, K. M., Win, A. A., Hlaing, T. M., Hlaing, T., Lin, K., Kyaw, M. P., Plewes, K., Faiz, M. A., Dhorda, M., Cheah, P. Y., Pukrittayakamee, S., Ashley, E. A., Anderson, T. J. C., Nair, S., McDew-White, M., Flegg, J. A., Grist, E. P. M., Guerin, P., Maude, R. J., Smithuis, F., Dondorp, A. M., Day, N. P. J., Nosten, F., White, N. J., & Woodrow, C. J. (2015). Spread of artemisinin-resistant *Plasmodium falciparum* in Myanmar: a cross-sectional survey of the K13 molecular marker. *The Lancet Infectious Diseases, 15*: 415-21.

Vafa, M., Troye-Blomberg, M., Anchang, J., Garcia, A., & Migot-Nabias, F. (2008). Multiplicity of *Plasmodium falciparum* infection in asymptomatic children in Senegal: relation to transmission, age and erythrocyte variants. *Malaria Journal, 7*: 17.

Verdrager, J. (1995). Localized permanent epidemics: the genesis of chloroquine resistance in *Plasmodium falciparum*. *Southeast Asian Journal of Tropical Medicine and Public Health, 26*: 23-28.

Vestergaard, L. S., & Ringwald, P. (2007). Responding to the challenge of antimalarial drug resistance by routine monitoring to update national malaria treatment policies. *The American Journal of Tropical Medicine and Hygiene, 77*: 153-159.

Vieira, P. P., Urbano Ferreira, M., Alecrim, M. G., Alecrim, W. D., da Silva, L. H. P., Sihuincha, M. M., & Zalis, M. G. (2004). Pfcrt polymorphism and the spread of chloroquine resistance in *Plasmodium falciparum* populations across the Amazon Basin. *Journal of Infectious Diseases, 190*: 417–424.

Warsame, M., Hassan, A. M., Barrette, A., Jibril, A. M., Elmi, H. H., Arale, A. M., Mohammady, H. E., Nada, R. A., Amran, J. G. H., Muse, A., Yusuf, F. E., & Omar, A. S. (2015). Treatment of uncomplicated malaria with artesunate plus sulfadoxine-

pyrimethamine is failing in Somalia: evidence from therapeutic efficacy studies and Pfdhfr and Pfdhps mutant alleles. *Tropical Medicine & International Health, 20*: 510-7.

Wellems, T. E., & Howard, R. J., (1986). Homologous genes encode two distinct histidine-rich proteins in a cloned isolate of *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences of the United States of America, 83*: 6065-9.

Wellems, T. E., Panton, L. J., Gluzman, I. Y., do Rosario, V. E., Gwadz, R. W., Walker-Jonah, A., & Krogstad, D. J. (1990). Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross. *Nature, 345*: 253-255.

Wellems T. E., & Plowe C. V. (2001). Chloroquine-resistant malaria. *Journal of Infectious Diseases, 184*: 770-776.

Wernsdorfer, W. H., & Payne, D. (1991). The dynamics of drug resistance in *Plasmodium falciparum*. *Pharmacology & Therapeutics, 50*: 95-121.

White, N., (1999). Antimalarial drug resistance and combination chemotherapy. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*. *354*:739-749.

White N. J. (2004). Antimalarial drug resistance. *Journal of Clinical Investigation, 113*: 1084-1092.

White N. J. (2008). Qinghaosu (artemisinin): The price of success. *Science, 18*: 330-334.

White N. J. (2013). Primaquine to prevent transmission of falciparum malaria. *The Lancet Infectious Diseases, 13*: 175-81.

White, N. J., & Pongtavornpinyo, W. (2003). The de novo selection of drug-resistant malaria parasites. *Proceedings: Biological Sciences, 270*: 545–554.

White N., Qiao L., Qi G., & Luzzatto L. (2012). Rationale for recommending a lower dose of primaquine as a *Plasmodium falciparum* gametocytocide in populations where G6PD deficiency is common. *Malaria Journal, 11*: 418.

WHO. (1993). A Global Malaria Control Strategy. Geneva: World Health Organization.

WHO. (2001). Monitoring antimalarial drug resistance. Report of a WHO consultation. Geneve: World Health Organization. http://apps.who.int/iris/bitstream/10665/67590/1/WHO_CDS_CSR_EPH_2002.17.pdf

WHO. (2008). Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations. Geneva: World Health Organization. http://www.who.int/malaria/publications/atoz/9789241596305/en/. Accessed 31 Dec 2015.

WHO. (2009). Methods for Surveillance of antimalarial drug efficacy. Geneva: World Health Organization. http://www.who.int/malaria/publications/atoz/9789241597531/en/. Accessed 29 Dec 2015.

WHO. (2010a). World Malaria Report 2010. Geneve: World Health Organization.

WHO. (2010b). Global report on antimalarial drug efficacy and drug resistance: 2000–2010. Geneve: World Health Organization.

WHO. (2011a). Eliminating malaria: learning from the past, looking ahead *Progress & impact series*. Geneva: World Health Organization.

WHO. (2011b). Malaria rapid diagnostic test performance. Results of WHO product testing of malaria RDTs: round 3 (2010-2011). Geneva: World Health Organization.

WHO. (2011c). Report on the regional workshop on strengthening quality management systems for parasitological diagnosis of malaria. Geneva: World Health Organization.

WHO. (2013). Intercountry meeting of national malaria programme managers from HANMAT and PIAM-net countries. Geneva: World Health Organization. http://applications.emro.who.int/docs/IC_Meet_Rep_2013_EN_14889.pdf?ua=1. Access date: 26 Apr 2016.

WHO. (2014a). World Malaria Report 2014. Geneva: World Health Organization.

WHO. (2014b). Status report on artemisinin resistance. Geneva: World Health Organization. http://www.who.int/malaria/publications/atoz/status-rep-artemisinin-resistance-sep2014.pdf. Access date: 26 Dec 2015.

WHO. (2015a). World Malaria Report 2015. Geneva: World Health Organization. http://www.who.int/malaria/publications/world-malaria-report-2015/report/en/. Access date: 10 Jan 2016.

WHO. (2015b). Guidelines for the treatment of malaria. Geneva: World Health Organization..http://apps.who.int/iris/bitstream/10665/162441/1/9789241549127_eng.pdf. Access date: 18 Jan 2016.

WHO. (2015c). Global Technical Strategy for Malaria 2016–2030. Geneva: World Health Organization.
http://apps.who.int/iris/bitstream/10665/176712/1/9789241564991_eng.pdf. Access date: 3 August 2016.

Wilson, C. M., Volkman, S. K., Thaithong, S., Martin, R. K., Kyle, D. E., Milhous, W. K., & Wirth, D. F. (1993). Amplification of pfmdr1 associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand. *Molecular and Biochemical Parasitology, 57*: 151-160.

Woehlbier, U., Epp, C., Kauth, C. W., Lutz, R., Long, C. A., Coulibaly, B., Kouyate, B., Arevalo-Herrera, M., Herrera, S., & Bujard, H. (2006). Analysis of antibodies directed against merozoite surface protein 1 of the human malaria parasite *Plasmodium falciparum*. *Infection and Immunity, 74*: 1313-1322.

Wongsrichanalai, C., Pickard, A., Wernsdorfer, W., & Meshnick, S. (2002). Epidemiology of drug-resistant malaria. *The Lancet Infectious Diseases, 2*: 209-218.

Wurtz, N., Fall, B., Bui, K., Pascual, A., Fall, M., Camara, C., Diatta, B., Fall, K., Mbaye, P., Diémé, Y., Bercion, R., Wade, B., Briolant, S., & Pradines, B. (2013). Pfhrp2 and pfhrp3 polymorphisms in *Plasmodium falciparum* isolates from Dakar, Senegal: impact on rapid malaria diagnostic tests. *Malaria Journal, 12*: 34.

Yamamoto, S. S., Louis, V. R., Sié, A., & Sauerborn, R. (2009). The effects of zooprophylaxis and other mosquito control measures against malaria in Nouna, Burkina Faso. *Malaria Journal, 8*: 283.

Young, M. D., Contacos, P. G., Stitcher, J. E., & Millar, J. W. (1963). Drug resistance in *Plasmodium falciparum* from Thailand. *The American Journal of Tropical Medicine and Hygiene, 12*: 305-314.

LIST OF PUBLICATIONS AND PAPER PRESENTED

Publications during candidature, directly arising from this thesis

1. **Atroosh, W. M**., Al-Mekhlafi, H. M., Al-Jasari, A., Sady, H., Al-Delaimy, A. K., Nasr, N. A., Dawaki, S., Abdulsalam, A. M., Ithoi, I., Lau, L. Y., Fong, Y. M., & Surin, J. (2015). Genetic variation of pfhrp2 in *Plasmodium falciparum* isolates from Yemen and the performance of HRP2-based malaria rapid diagnostic test. *Parasites & Vectors, 8*(1), 1-8. doi: 10.1186/s13071-015-1008-x.

2. **Atroosh, W. M.**, Al-Mekhlafi, H. M., Snounou, G., Al-Jasari, A., Sady, H., Nasr, N. A., Lau, Y.-L., & Surin, J. (2016). Sustained efficacy of artesunate-sulfadoxine-pyrimethamine against *Plasmodium falciparum* in Yemen and a renewed call for an adjunct single dose primaquine to clear gametocytes. *Malar J, 15*(1), 1-11. doi: 10.1186/s12936-016-1344-0.

3. **Atroosh, W. M**., Al-Mekhlafi, H. M., Al-Jasari, A., Sady, H., Dawaki, S. S., Elyana, F. N., Al-Areeqi, M. A., Nasr, N. A., Abdulsalam, A. M., Subramaniam, L. R., Azzani, M., Ithoi, I., Lau, Y. L., & Surin, J. (2016). Different patterns of pfcrt and pfmdr1 polymorphism in *Plasmodium falciparum* isolates from Tehama region, Yemen. *PeerJ, 4:e2191*. doi: https://doi.org/10.7717/peerj.2191.

Collaborating publications during candidature, not directly arising from this thesis

1. Al-Delaimy, A. K., Al-Mekhlafi, H. M., Lim, Y. A., Nasr, N. A., Sady, H., **Atroosh, W. M.**, & Mahmud, R. (2014). Developing and evaluating health education learning package (HELP) to control soil-transmitted helminth infections among Orang Asli children in Malaysia. *Parasites & Vectors, 7*(1), 1-18. doi: 10.1186/1756-3305-7-416

2. Al-Delaimy, A. K., Al-Mekhlafi, H. M., Nasr, N. A., Sady, H., **Atroosh, W. M.**, Nashiry, M., Anuar, T. S., Moktar, N., Lim, Y. A. L., & Mahmud, R. (2014). Epidemiology of Intestinal Polyparasitism among Orang Asli School

Children in Rural Malaysia. *PLoS Negl Trop Dis, 8*(8), e3074. doi: 10.1371/journal.pntd.0003074

3. Al-Mekhlafi, H. M., Al-Zabedi, E. M., Al-Maktari, M. T., **Atroosh, W. M.**, Al-Delaimy, A. K., Moktar, N., Sallam, A. A., Abdullah, W. A., Jani, R., & Surin, J. (2014). Effects of Vitamin A Supplementation on Iron Status Indices and Iron Deficiency Anaemia: A Randomized Controlled Trial. *Nutrients, 6*(1), 190-206. doi: 10.3390/nu6010190

4. Dawaki, S., Al-Mekhlafi, H., Ithoi, I., Ibrahim, J., Abdulsalam, A., Ahmed, A., Sady, H., **Atroosh, W.**, Al-Areeqi, M., Elyana, F., Nasr, N., & Surin, J. (2016). Prevalence and risk factors of schistosomiasis among Hausa communities in Kano State, Nigeria. *Rev. Inst. Med. Trop. Sao Paulo, 58*, 54. doi: http://dx.doi.org/10.1590/S1678-9946201658054

5. Dawaki, S., Al-Mekhlafi, H. M., Ithoi, I., Ibrahim, J., Abdulsalam, A. M., Ahmed, A., Sady, H., Nasr, N. A., & **Atroosh, W. M.** (2015). The Menace of Schistosomiasis in Nigeria: Knowledge, Attitude, and Practices Regarding Schistosomiasis among Rural Communities in Kano State. *PLoS ONE, 10*(11), e0143667. doi: 10.1371/journal.pone.0143667

6. Dawaki, S., Al-Mekhlafi, H. M., Ithoi, I., Ibrahim, J., **Atroosh, W. M.**, Abdulsalam, A. M., Sady, H., Elyana, F. N., Adamu, A. U., Yelwa, S. I., Ahmed, A., Al-Areeqi, M. A., Subramaniam, L. R., Nasr, N. A., & Lau, Y.-L. (2016). Is Nigeria winning the battle against malaria? Prevalence, risk factors and KAP assessment among Hausa communities in Kano State. *Malar J, 15*(1), 1-14. doi: 10.1186/s12936-016-1394-3

7. Elyana, F. N., Al-Mekhlafi, H. M., Ithoi, I., Abdulsalam, A. M., Dawaki, S., Nasr, N. A., **Atroosh, W. M.**, Abd-Basher, M. H., Al-Areeqi, M. A., Sady, H., Subramaniam, L. R., Anuar, T. S., Lau, Y. L., Moktar, N., & Surin, J. (2016). A tale of two communities: intestinal polyparasitism among Orang Asli and Malay communities in rural Terengganu, Malaysia. *Parasit Vectors, 9*(1), 1-17. doi: 10.1186/s13071-016-1678-z

8. Sady, H., Al-Mekhlafi, H. M., **Atroosh, W. M.**, Al-Delaimy, A. K., Nasr, N. A., Dawaki, S., Al-Areeqi, M. A., Ithoi, I., Abdulsalam, A. M., Chua, K. H., & Surin, J. (2015). Knowledge, attitude, and practices towards schistosomiasis

among rural population in Yemen. *Parasit Vectors, 8*(1), 1-13. doi: 10.1186/s13071-015-1050-8

9. Sady, H., Al-Mekhlafi, H. M., Ngui, R., **Atroosh, W. M.**, Al-Delaimy, A. K., Nasr, N. A., Dawaki, S., Abdulsalam, A. M., Ithoi, I., Lim, Y. A. L., Chua, K. H., & Surin, J. (2015). Detection of Schistosoma mansoni and Schistosoma haematobium by Real-Time PCR with High Resolution Melting Analysis. *International Journal of Molecular Sciences, 16*(7), 16085-16103. doi: 10.3390/ijms160716085

10. Sady, H., Al-Mekhlafi, H. M., Webster, B. L., Ngui, R., **Atroosh, W. M.**, Al-Delaimy, A. K., Nasr, N. A., Chua, K. H., Lim, Y. A. L., & Surin, J. (2015). New insights into the genetic diversity of Schistosoma mansoni and S. haematobiumin Yemen. *Parasit Vectors, 8*(1), 1-14. doi: 10.1186/s13071-015-1168-8

Atroosh et al. Parasites & Vectors (2015) 8:388
DOI 10.1186/s13071-015-1008-x

Parasites & Vectors

RESEARCH Open Access

Genetic variation of *pfhrp2* in *Plasmodium falciparum* isolates from Yemen and the performance of HRP2-based malaria rapid diagnostic test

Wahib M. Atroosh[1,2], Hesham M. Al-Mekhlafi[1,3,4*], Adel Al-Jasari[5], Hany Sady[1], Ahmed K. Al-Delaimy[1], Nabil A. Nasr[1], Salwa Dawaki[1], Awatif M. Abdulsalam[1], Init Ithoi[1], Yee Ling Lau[1], Mun Yik Fong[1] and Johari Surin[1]

Abstract

Background: The genetic variation in the *Plasmodium falciparum* histidine-rich protein 2 (*pfhrp2*) gene that may compromise the use of *pfhrp2*-based rapid diagnostic tests (RDTs) for the diagnosis of malaria was assessed in *P. falciparum* isolates from Yemen.

Methods: This study was conducted in Hodeidah and Al-Mahwit governorates, Yemen. A total of 622 individuals with fever were examined for malaria by *CareStart*™ malaria HRP2-RDT and Giemsa-stained thin and thick blood films. The *Pfhrp2* gene was amplified and sequenced from 180 isolates, and subjected to amino acid repeat types analysis.

Results: A total of 188 (30.2 %) participants were found positive for *P. falciparum* by the RDT. Overall, 12 different amino acid repeat types were identified in Yemeni isolates. Six repeat types were detected in all the isolates (100 %) namely types 1, 2, 6, 7, 10 and 12 while types 9 and 11 were not detected in any of the isolates. Moreover, the sensitivity and specificity of the used PfHRP2-based RDTs were high (90.5 % and 96.1 %, respectively).

Conclusion: The present study provides data on the genetic variation within the *pfhrp2* gene, and its potential impact on the PfHRP2-based RDTs commonly used in Yemen. *CareStart*™ Malaria HRP2-based RDT showed high sensitivity and specificity in endemic areas of Yemen.

Keywords: Malaria, *Plasmodium falciparum*, Rapid diagnostic test, *Plasmodium falciparum* histidine-rich protein 2, Yemen

Background

Malaria is still a major public health problem in Yemen, with almost 66 % of the population living in areas that suffer from stable malaria transmission [1]. *Plasmodium falciparum* is the predominant species and was responsible for almost 99 % of malaria cases in Yemen during 2012, a large number of which consisted of drug-resistant *P. falciparum* parasites [2, 3]. Among 17 countries with malaria-endemic areas in the Middle East and Eurasia region; Pakistan, Afghanistan and Yemen account for more than 99 % of the 56,000 regional deaths due to malaria [4].

The national malaria control programme in Yemen (NMCP) has achieved substantial success in controlling local cases of malaria, achieving a significant reduction in the number of malaria cases, dropping from 900,000 cases in the early 2000s to around 150,000 cases by 2013 [1]. However, Yemen is still classified among areas of high malaria transmission, making it the only country in the Arabian Peninsula and greater Middle Eastern region that is still plagued with malaria to the extent that residents still suffer from considerably high mortality and morbidity rates [2]. Imported malaria cases are still

* Correspondence: halmekhlafi@yahoo.com
[1]Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[3]Azal National Research Centre, Azal University for Human Development, 447 Sana'a, Yemen
Full list of author information is available at the end of the article

Atroosh *et al. Malar J (2016) 15:295*
DOI 10.1186/s12936-016-1344-0

Malaria Journal

RESEARCH Open Access

Sustained efficacy of artesunate-sulfadoxine-pyrimethamine against *Plasmodium falciparum* in Yemen and a renewed call for an adjunct single dose primaquine to clear gametocytes

Wahib M. Atroosh[1,2], Hesham M. Al-Mekhlafi[1,3,4*], Georges Snounou[5*], Adel Al-Jasari[6], Hany Sady[1], Nabil A. Nasr[1], Yee-Ling Lau[1] and Johari Surin[1,7]

Abstract

Background: In Yemen, artesunate plus sulfadoxine-pyrimethamine (AS + SP) has been used as first-line treatment for uncomplicated falciparum malaria, which accounts for about 99 % of malaria cases. There is evidence that resistance to SP is increasing, with potential negative impact on efficacy, and in particular on curbing transmission. This study aims: (a) to evaluate the therapeutic efficacy of AS + SP treatment for uncomplicated falciparum malaria in Yemen; (b) to investigate the frequency of mutations in *Plasmodium falciparum* genes associated with resistance to AS (Kelch 13 propeller domain, *pfK13*) and SP (dihydrofolate reductase, *pfdhfr*, and dihydropteroate synthase, *pfdhps*); and (c) to assess the adequacy of this ACT to clear gametocytes.

Methods: A 28-day in vivo evaluation of the clinical and parasitological response to three-day course of AS + SP was carried out in two areas of high endemicity (Hodeidah and Al-Mahwit provinces, Tehama region) in Yemen according to standard WHO protocol 2009. Clinical and parasitological indices were monitored over a 28-day follow-up, and the outcome was PCR-corrected. The frequencies of mutations in the *pfdhfr*, *pfdhps*, and *pfK13* genes were obtained by sequencing following amplification.

Results: Eighty-six patients completed the study, with a cure rate of 96.5 % (94.2 % PCR-uncorrected). Whereas four (4.7 %) patients still showed parasitaemia on day 2 post-treatment, all were found negative for asexual malaria stages on days 3 and 7. The efficacy of gametocyte clearance was poor (14.5, 42.5 and 86.0 % on days 7, 14 and 28, respectively), with gametocytes persisting throughout the study in some patients. All the isolates sequenced had the *pfk13* propeller domain wild-type allele, and mutations associated with SP failure were observed only for *pfdhfr* with the double mutation (S108N + N51I) found in 65.4 % of the isolates sequenced.

Conclusion: In Yemen, AS + SP therapy remains effective for the treatment of uncomplicated falciparum malaria. Mutations were not detected in *pfk13* or *pfdhps*, though double mutations were observed for *pfdhfr*. The observed

*Correspondence: halmekhlafi@yahoo.com; georges.snounou@upmc.fr
[1] Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[5] UPMC Université Paris 06, Inserm (Institut National de la Santé et de la Recherche Medicale), Centre d'Immunologie et des Maladies Infectieuses (Cimi-Paris), UMR 1135, ERL CNRS 8255 (Centre National de la Recherche Scientifique), Sorbonne Universités, 91 Boulevard de l'Hôpital, F-75013 Paris, France
Full list of author information is available at the end of the article

Different patterns of *pfcrt* and *pfmdr1* polymorphism in *Plasmodium falciparum* isolates from Tehama region, Yemen

Wahib M. Atroosh[1,2], Hesham M. Al-Mekhlafi[1,3,4], Adel Al-Jasari[5], Hany Sady[1], Salwa S. Dawaki[1], Fatin N. Elyana[1], Mona A. Al-Areeqi[1], Nabil A. Nasr[1], Awatif M. Abdulsalam[1], Lahvanya R. Subramaniam[1], Meram Azzani[6], Init Ithoi[1], Yee Ling Lau[1] and Johari Surin[1,7]

[1] Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia
[2] Unit of Microbiology and Parasitology, Department of Para-Clinic, Faculty of Medicine and Health Sciences, University of Aden, Khormaksar, Aden, Yemen
[3] Endemic and Tropical Diseases Unit, Medical Research Centre, Jazan University, Jazan, Kingdom of Saudi Arabia
[4] Department of Parasitology, Faculty of Medicine and Health Sciences, Sana'a University, Sana'a, Yemen
[5] National Malaria Control Programme, Ministry of Health and Population, Sana'a, Yemen
[6] Department of Social and Preventive Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia
[7] Centre for Research and Innovation, Taylor's University, Subang Jaya, Selangor, Malaysia

ABSTRACT

Introduction. Despite the efforts of the malaria control programme, malaria morbidity is still a common health problem in Yemen, with 60% of the population at risk. *Plasmodium falciparum* is responsible for 99% of malaria cases. The emergence in Yemen of parasite resistance to chloroquine (CQ) prompted the adoption of artemisinin combination therapy (ACT) in 2009, which involves the use of artesunate plus sulphadoxine-pyrimethamine (AS + SP). However, CQ was retained as the drug of choice for vivax malaria. To assess the impact of the change in the malaria treatment policy five years after its introduction, the present study investigated the mutations in the CQ resistance transporter (*pfcrt*) and multidrug resistance 1 (*pfmdr1*) genes.

Method. A molecular investigation of 10 codons of *pfcrt* (72–76, 220, 271, 326, 356, and 371) and five codons of *pfmdr1* (86, 184, 1034, 1042, and 1246) was conducted on *P. falciparum* isolates from districts with the highest malaria endemicity in the Hodeidah and Al-Mahwit governorates in Tehama region, Yemen. A total of 86 positive cases of falciparum monoinfection were investigated for the presence of mutations related to CQ and other antimalarials using a PCR-RFLP assay.

Results. There was a wide prevalence of *pfcrt* gene mutations with the *pfcrt* 76T CQ resistance marker being predominant (97.7%). The prevalence of other *pfcrt* mutations varied from high (75E: 88%) to moderate (74I: 79.1%, 220S: 69.8%, 271E and 371I: 53.5%) or low (326S: 36%, 72S: 10.5%). Mutated *pfcrt* 72–76 amino acids haplotypes were highly prevalent (98.8%). Among these, the CVIET classic, old-world African/Southeast Asian haplotype was the most predominant, and was mostly found in the isolates from the Khamis Bani Saad district of Al-Mahwit (93.1%) and the AdDahi district of Hodeidah (88.9%). However, it was only found in 26.3% of the isolates from the Bajil district of Hodeidah. Surprisingly, the SVMNT new-world South American haplotype was exclusively detected in 9.3% of the isolates from the Bajil district of

Submitted 13 March 2016
Accepted 8 June 2016
Published 12 July 2016

Corresponding authors
Wahib M. Atroosh,
wahib_atrosh@yahoo.com
Hesham M. Al-Mekhlafi,
halmekhlafi@yahoo.com

Academic editor
Erika Braga

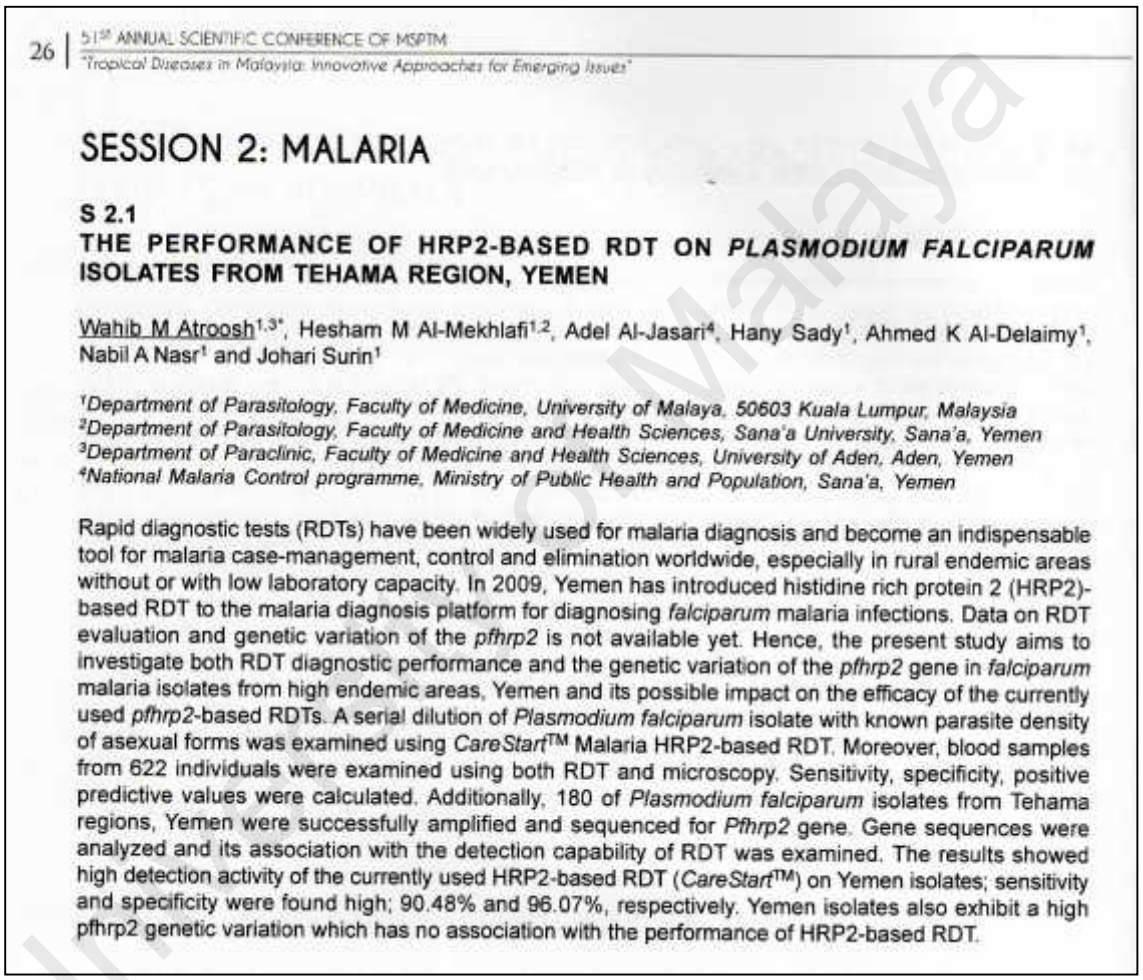
Additional Information and Declarations can be found on page 11

DOI **10.7717/peerj.2191**

ow to cite this article Atroosh et al. (2016) Different patterns of *pfcrt* and *pfmdr1* polymorphism in *Plasmodium falciparum* isolates from Tehama region, Yemen. **PeerJ 4:e2191; DOI 10.7717/peerj.2191**

LIST OF CONFERENCE PRESENTATIONS:

1. The performance of HRP2-based RDT on *Plasmodium falciparum* isolates from Tehama region, Yemen. As oral presentation for the 51st Malaysia Society of Parasitology and Tropical Medicine (MSPTM), 3rd - 4th March 2015-Kuala Lumpur-Malaysia. (Oral presenter).



SESSION 2: MALARIA

S 2.1
THE PERFORMANCE OF HRP2-BASED RDT ON *PLASMODIUM FALCIPARUM* ISOLATES FROM TEHAMA REGION, YEMEN

Wahib M Atroosh[1,3*], Hesham M Al-Mekhlafi[1,2], Adel Al-Jasari[4], Hany Sady[1], Ahmed K Al-Delaimy[1], Nabil A Nasr[1] and Johari Surin[1]

[1]*Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[2]*Department of Parasitology, Faculty of Medicine and Health Sciences, Sana'a University, Sana'a, Yemen*
[3]*Department of Paraclinic, Faculty of Medicine and Health Sciences, University of Aden, Aden, Yemen*
[4]*National Malaria Control programme, Ministry of Public Health and Population, Sana'a, Yemen*

Rapid diagnostic tests (RDTs) have been widely used for malaria diagnosis and become an indispensable tool for malaria case-management, control and elimination worldwide, especially in rural endemic areas without or with low laboratory capacity. In 2009, Yemen has introduced histidine rich protein 2 (HRP2)-based RDT to the malaria diagnosis platform for diagnosing *falciparum* malaria infections. Data on RDT evaluation and genetic variation of the *pfhrp2* is not available yet. Hence, the present study aims to investigate both RDT diagnostic performance and the genetic variation of the *pfhrp2* gene in *falciparum* malaria isolates from high endemic areas, Yemen and its possible impact on the efficacy of the currently used *pfhrp2*-based RDTs. A serial dilution of *Plasmodium falciparum* isolate with known parasite density of asexual forms was examined using *CareStart*™ Malaria HRP2-based RDT. Moreover, blood samples from 622 individuals were examined using both RDT and microscopy. Sensitivity, specificity, positive predictive values were calculated. Additionally, 180 of *Plasmodium falciparum* isolates from Tehama regions, Yemen were successfully amplified and sequenced for *Pfhrp2* gene. Gene sequences were analyzed and its association with the detection capability of RDT was examined. The results showed high detection activity of the currently used HRP2-based RDT (*CareStart*™) on Yemen isolates; sensitivity and specificity were found high; 90.48% and 96.07%, respectively. Yemen isolates also exhibit a high pfhrp2 genetic variation which has no association with the performance of HRP2-based RDT.

APPENDIX A

FLOW CHART OF STUDY METHODOLOGY