EPIDEMIOLOGY OF MALARIA AND THE DISTRIBUTION OF GENETIC MARKERS ASSOCIATED WITH DRUG RESISTANCE IN MAWZA DISTRICT, TAIZ GOVERNORATE, YEMEN

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ABSTRACT

In Yemen, malaria is still one of the most serious health problems, with 149,451 cases being reported in 2013. Of these, *Plasmodium falciparum* represents 99%. It is estimated that 25% of the population are at high risk and a total of 37,763 microscopy-confirmed and 29,750 rapid diagnostic tests (RDTs)-confirmed cases were reported in 2014.

The present study aimed to determine malaria prevalence and its associated risk factors in rural communities of Taiz governorate as well as to determine the knowledge, attitude and practices (KAP) of the local populations toward malaria. The study also aimed at evaluating the light microscopy (LM) and an RDT, combining both *P. falciparum* histidine-rich protein-2 (*Pf*HRP-2) and *Plasmodium* lactate dehydrogenase (pLDH), for falciparum malaria diagnosis against nested polymerase chain reaction (PCR) as the reference method. The last objective of the study was detecting the molecular markers (*Pfcrt* K76, *Pfmdr-1* N86, Kelch13, *Pfdhfr* and *Pfdhps*) of *P. falciparum* resistance to chloroquine, artemisinin and sulphadoxine/pyrimethamine respectively.

A household-based, cross-sectional malaria survey was conducted in Mawza District, a malaria-endemic area in Taiz governorate during the transmission season from October 2013 to April 2014. Blood specimens were collected from 488 participants and examined by LM and *Pf*HRP-2/pLDH RDT. Samples positive using LM and/or *Pf*HRP-2/pLDH RDT were confirmed using PCR to exclude the false positive results. Nested PCR followed by digestion was used for detection of mutations at position 76 in *pfcrt* and 86 in *pfmdr-1*. Mutations in the Kelch13, *dhfr* and *dhps* genes at specific codons were determined by nested PCR and followed by gene sequencing.

Malaria prevalence rate based on PCR-adjusted RDT was 25.5% (95% CI: 21.6-29.5). Sub-microscopic malaria was significantly more prevalent among non-febrile individuals (16.0%; 95% CI: 12.88–19.75).

Comparison between LM and *Pf*HRP-2/pLDH RDT showed that the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of *Pf*HRP-2/pLDH RDT were 96.0% (95% CI: 90.9-98.3), 56.0% (95% CI: 44.7-66.8), 76.3% (95% CI: 69.0-82.3) and 90.4% (95% CI: 78.8-96.8), respectively.

Fifty (50) falciparum positive samples were selected for detection of mutations at codon K76T of the *Pfcrt* gene and codon N86Y of the *Pfmdr-1* gene. The *Pfcrt* gene was exclusively mutant allele and *Pfmdr-1* gene was wild type allele. The *dhfr* 51I/108N double mutant allele was found in one isolate and regarding Kelch13 and *dhps*, all isolates were of wild type alleles.

In conclusion, the present study revealed a higher proportion of sub-microscopic malaria infections among rural populations of Mawza than that can be detected by LM, particularly among asymptomatic participants. This represents a significant reservoir of infection and its on-going transmission should be considered when tailoring future control and elimination strategies. The *Pf*HRP-2/pLDH RDT showed high sensitivity for the survey of falciparum malaria including asymptomatic malaria cases. artemisinin combination therapy (ACT); Artemisinin plus sulphadoxine/pyrimethamine (ART+SP) still has a high efficacy. The fact that all isolates had mutant allele of *pfcrt* 76T may indicate the continued usage of chloroquine (CQ) for treating malaria.

ABSTRAK

Di Yemen, malaria masih merupakan satu dari masaalah kesihatan yang paling serius, dengan 149,451 kes dilaporkan dalam tahun 2013. Dari kes-kes ini, 99% telah disebabkan oleh *Plasmodium falciparum*. Adalah di jangkakan 25% dari penduduk berada dalam risiko tinggi dan sejumlah 37,763 kes yang disahkan melalui mikroskopi dan 29,750 kes yang disahkan melalui ujian diagnostik pantas (RDT) telah dilaporkan dalam tahun 2014.

Kajian ini bertujuan untuk menentukan prevalens malaria dan faktor risiko yang berkaitan di komuniti luar bandar di wilayah Taiz dan juga untuk menentukan pengetahuan, sikap dan amalan (KAP) penduduk setempat terhadap malaria. Kajian ini juga bertujuan menilai mikroskop cahaya (LM) dan RDT, yang menggabungkan kedua-dua *P. falciparum* histidine-rich protein-2 (*Pf*HRP-2) dan *Plasmodium* lactate dehydrogenase (pLDH), untuk diagnosis malaria falciparum terhadap reaksi polimerase berangkai (PCR) nested sebagai kaedah rujukan. Objektif terakhir kajian ini adalah untuk mengesan penanda molekul (*Pfcrt* K76, *Pfmdr-1*N86, Kelch13, *Pfdhfr* dan *Pfdhps*) bagi *P. falciparum* yang masing-masing rintang terhadap chloroquine, artemisinin dan sulphadoxine/pyrimethamine.

Tinjauan malaria secara keratan rentas berasaskan seisi rumah telah dijalankan di daerah Mawza, kawasan endemik malaria di wilayah Taiz semasa musim penularan dari Oktober 2013 hingga April 2014. Spesimen darah telah dikutip dari 488 peserta dan telah diperiksa dengan LM dan *Pf* HRP-2/pLDH RDT. Sampel yang positif menggunakan LM dan/atau *Pf* HRP-2/pLDH RDT telah disahkan menggunakan PCR untuk menyingkirkan keputusan yang positif palsu. PCR nested yang diikuti dengan pencernaan telah digunakan untuk mengesan mutasi di posisi 76 dalam *pfcrt* dan 86 dalam *pfmdr-1*. Mutasi dalam gen Kelch 13, *dhfr* dan *dhps* di kodon spesifik telah ditentukan dengan PCR nested dan diikuti dengan penjujukan gen.

Kadar prevalens malaria berdasarkan pada “PCR-adjusted RDT” ialah 25.5% (95% CI: 21.6 – 29.5). Malaria sub-mikroskopik adalah lebih prevalen secara signifikant dikalangan individu yang tiada demam (16.0%, 95% CI: 12.88 - 19.75).

Perbandingan diantara LM dan *Pf* HRP-2/pLDH RDT menunjukkan yang sensitiviti, spesifisiti, nilai prediktif positif (PPV) dan nilai prediktif negatif (NPV) bagi *Pf*HRP-2/pLDH RDT masing-masing adalah 96.0% (95% CI: 90.9 – 98.3), 56.0% (95% CI: 44.7 – 66.8), 76.3% (95% CI: 69.0 – 82.3) dan 90.4% (95% CI: 78.8 – 96.8).

Lima puluh (50) sampel positif bagi falciparum telah dipilih untuk mengesan mutasi di Kodon k76T bagi gen *Pfcrt* dan Kodon N867 bagi gen *Pfmdr-1*. Gen *Pfcrt* adalah alel mutan secara eksklusif dan gen *Pfmdr-1* adalah jenis alel liar. Alel mutan ganda dua *dhfr* 511/108 N telah dijumpai dalam satu isolat dan mengenai Kelch 13 dan *dhps*, kesemua isolat adalah alel jenis liar.

Kesimpulannya, kajian ini menunjukkan kadar jangkitan malaria sub-mikroskopik yang lebih tinggi dikalangan penduduk luar bandar Mawza dari yang dapat dikesan oleh LM, khususnya dikalangan peserta tidak bersimptom. Ini merupakan sarang jangkitan yang signifikant dan penularan yang berterusan harus diambil kira apabila merangka strategi kawalan dan eliminasi. *Pf*HRP-2/pLDH RDT menunjukkan sensitiviti yang tinggi untuk tinjauan malaria falciparum termasuk kes malaria tidak bersimptom. Terapi penggabungan artemisinin (ACT); artemisinin campur sulphadoxine/pyrimethamine (ART + SP) masih mempunyai efikasi tinggi. Hakikat yang kesemua isolat mempunyai alel mutan bagi *Pfcrt* 76T mungkin menandakan penggunaan chloroquine (CQ) yang berterusan untuk merawat malaria.

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LIST OF ABBREVIATIONS

%	*Percentage*
µl	*Microliter*
°C	*Degree Celsius*
ACTs	Artemisinin-based combination therapies
AKI	Acute kidney injury
AL	Artemether-lumefantrine
An.	*Anopheles*
ARDS	Acute respiratory distress syndrome
ART	Artemisinin
AM	Amodiaquine
Bp	*Base pair*
CDC	United States Centers for Disease Control and Prevention
CI	Confidence interval
CQ	Chloroquine
CQR	CQ resistant
ddH_2O	Double-distilled water
DDT	Dichloro diphenyl trichloroethane
DHFR	Dihydrofolate reductase
Dhfr	Dihydrofolate reductase gene
DHPS	Dihydropteroate synthase
Dhps	Dihydropteroate synthase gene
DNA	*Deoxyribonucleic acid*
dNTPs	*Deoxynucleotide triphosphates*
EDTA	*Ethylene diamine tetra acetic acid.*
et al.	et alia (and other)

GMAP	Global Malaria Action Plan
GMS	*Greater Mekong subregion*
HRP-2	Histidine-rich protein 2
IM	Intramuscular
in vitro	*In cultured parasite-infected erythrocytes*
in vivo	Biological process occurring within a living organism
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
ITNs	Insecticide-treated nets
IV	Intravenous
KAP	Knowledge, attitude and practices
LM	Light microscopy
LTF	late treatment failure
MQ	Mefloquine
$MgCl_2$	*Magnesium chloride*
Min	*Minutes*
Ml	*Milliliter*
mM	*Millimolar*
NMCP	*National malaria control programme*
NPV	Negative predictive value
OR	Odds ratio
P	*Plasmodium*
P. f	*Plasmodium falciparum*
Pan	*Plasmodium* lactate dehydrogenase specific to *Plasmodium* species
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction

Pfcrt	*P. falciparum* chloroquine resistance transporter gene
*Pf*HRP-2	*P. falciparum histidine-rich protein-2*
Pfmdr-1	*P. falciparum* multi drug resistance-1 gene
pLDH	*Plasmodium* lactate dehydrogenase
PNG	Papua New Guinea
PPV	Positive predictive value
PYR	Pyrimethamine
RBCs	Red blood cells
RDTs	Rapid diagnostic tests
RFLP	*Restriction fragment length polymorphism*
Rpm	*Revolutions per minute*
S	*Seconds*
SERCA	Sarcoplasmic-endoplasmic reticulum Ca^{2+}- ATPase
SNPs	Single nucleotide polymorphisms
Sp	Species
SSU rRNA	Small subunit ribosomal RNA
SX	Sulfadoxine
vol/vol	Volume/volume
WHO	World Health Organization
YSH	Yemeni-Swedish Hospital

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Back ground

Malaria is a major cause of morbidity and mortality in Yemen, with *Plasmodium falciparum* being the predominant species responsible for almost 99% of cases. *Anopheles arabiensis* and *An. sergenti* are the main mosquito vectors (WHO, 2012; Bamaga *et al.*, 2014) and recently *An. algeriensis* (NMCP, 2014). However, in the Eastern governorate of Al-Maharah, *An. culicifacies* is the predominant vector (NMCP, 2014). It has been estimated that 78% of the population are at risk of malaria and approximately 25% at high risk with 78,336 reported cases in 2015 (NMCP, 2014; WHO, 2016).The National Malaria Control Program (NMCP) of Yemen is proactive in combating malaria through the implementation of a national strategy involving early diagnosis and prompt treatment, indoor residual spraying (IRS), distribution of insecticide-treated nets (ITNs) and case surveillance (NMCP, 2010).

In the national strategy of malaria control in Yemen, patient with fever should be diagnosed for malaria within 24 hours of the onset of the fever (NMCP, 2010). Although malaria diagnosis has been achieved using light microscopy (LM), Rapid Diagnostic Tests (RDTs) have been implemented by the NMCP since 2009 for detecting *P. falciparum* infections (WHO, 2011a).

Two RDTs were evaluated by researchers in Yemen. In 2012, Response® Malaria Ag. *P. falciparum* (HRP-2) RDT (Premier Medical Corporation Ltd, India) was evaluated against LM in an outbreak in Hadramout, Yemen. The study showed 74% sensitivity and 94% specificity of RDT during the field survey (Ghouth *et al.*, 2012). A recent study was conducted in Hodiedah and Al-Mihweet governorates to evaluate CareStart™ Malaria HRP-2 (Access Bio Inc., USA) in the diagnosis of malaria among febrile patients and reported 90.5% sensitivity and 96.1% specificity compared to LM as reference test (Atroosh *et al.*, 2015).

The old national antimalarial treatment policy in Yemen included chloroquine (CQ) and sulfadoxine/pyrimethamine (SP) as first- and second-line treatments for uncomplicated falciparum malaria, respectively (NMCP, 2006). Following the high-level CQ treatment failure of falciparum malaria concluded from efficacy studies in four sentinel sites covering the disease epidemiologic strata in the period from 2002 to 2005, the Yemeni NMCP revisited the national antimalarial treatment policy to artemisinin-based combination therapies (ACTs) in 2005, which was officially endorsed in 2009 (NMCP, 2010). The new policy includes artesunate (AS) plus SP and artemether-lumefantrine (AL) as first- and second-line treatments for uncomplicated malaria, respectively. However, CQ is still prescribed by physicians or bought by patients without medical consultation in Yemen (Bashrahil *et al.*, 2010; Al-Mekhlafi *et al.*, 2011; Ghouth, 2013). Such practices exacerbate the emergence and spread of resistance to the drug (Escalante *et al.*, 2009).

The *in vivo* clinical trial for monitoring the efficacy of antimalarial drugs has been considered the gold standard and widely used (WHO, 2010). A clinical trial showed that ACT still has a high efficacy.

The detection of molecular markers associated antimalarial drug resistance has been introduced as an important approach for early detection of the emergence of resistant malaria parasite (WHO, 2010). The mutation at codon 76 of the *P. falciparum* CQ resistance transporter gene (*pfcrt*), which results in a change of the amino acid lysine into threonine (K76T), is the key molecular markers of resistance to CQ (Djimdé *et al.*, 2001; Fidock *et al.*, 2000). On the other hand, the mutation at codon 86 of the *P. falciparum* multidrug resistance 1 gene (*pfmdr1*), which results in a change of the amino acid asparagine into tyrosine (N86Y), has been partially associated with resistance to CQ (Adagu & Warhurst, 2001; Babiker *et al.*, 2001; Djimdé *et al.*, 2001; Mu *et al.*, 2003).

Moreover, Picot *et al.* (2009) concluded the role of *pfmdr1* 86Y in CQ and Amodiaquine (AQ) treatment failure in a meta-analysis of 29 studies. The *pfmdr1* 86Y has also been associated with *in vitro* resistance to quinine among Thai *P. falciparum* isolates (Poyomtip *et al.*, 2012). In contrast, the *pfmdr1* 86Y has been associated with *in vitro* susceptibility of *P. falciparum* to other antimalarial drugs such as mefloquine (MQ) and artemisinin (ART) (Duraisingh *et al.*, 2000 a,b), whereas the *pfmdr1* N86 wild-type allele has been linked to a declined susceptibility of *P. falciparum* to AL (Venkatesan *et al.*, 2014).

In the context of resistance to SP, several mutations in *P. falciparum* dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*) genes have been associated with resistance to pyrimethamine and sulfadoxine, respectively (Kublin *et al.*, 2002; Peterson *et al.*, 1988; Wang *et al.*, 1997). Recently, mutations in the Kelch 13 (K13) propeller domain have been associated with resistance to artemisinin (Ariey *et al.*, 2014). In Yemen, several studies have used the molecular approach and detected mutations in *pfcrt*, *Pfmdr1, Pfdhfr* and *Pfdhps* genes of falciparum malaria isolates (Al-Mekhlafi, *et al.*, 2011; Mubjer, *et al.*, 2011; Al-Hamidhi, *et al.*, 2013; Abdul-Ghani, *et al.*, 2014; Bamaga, *et al.*, 2015). Recently, a study was conducted on K13 in Yemen and found no mutations (Atroosh *et al.*, 2016).

1.2 Study justification

Taiz governorate is located in the Southwest of Yemen and inhabited by more than 3 million people; representing about 12% of the total population of Yemen (CSO, 2013). This governorate has been classified as high transmission area, with approximately 4,048 malaria cases being reported in 2013 (NMCP, 2013). Besides, malaria was responsible for 40% of the paediatric admission (Al-Taiar *et al.*, 2009).

Light microscopy (LM) is still the cornerstone of malaria diagnosis in Yemen, especially in hospitals. However, LM has low sensitivity for detection of low parasite densities, is time-consuming and requires skilled technicians and good reagents (NMCP, 2011; Kahama-Maro *et al.*, 2011). Therefore, it may not reflect the sub-microscopic infectious reservoir in Yemen, which is still neglected and needs to be estimated if malaria elimination in the country is to be achieved (Abdul-Ghani, 2015).

RDTs have been introduced as an alternative to LM, especially when good LM practice cannot be maintained or is not available. RDTs that target *P. falciparum* histidine-rich protein-2 (*Pf*HRP-2) have the highest and most consistent detection rate (WHO, 2013). In contrast, *Plasmodium* lactate dehydrogenase (pLDH) detects all *Plasmodium* species and is usually combined with *Pf*HRP-2 for malaria screening in areas endemic with multiple species (Moody, 2002; WHO, 2011). The NMCP has been using RDTs for malaria diagnosis and field surveys since 2007 (NMCP, 2011; 2013). Although the World Health Organization (WHO) has provided comparative data on the performance of RDTs that can be used for procurement decision, it is well recognized that clinical sensitivity of RDTs depends on the epidemiology of malaria in the target population (WHO, 2013), which imposes field evaluation of such tests.

Malaria in Yemen is unstable, seasonal and affected by topography and rainfall. The country has been stratified with respect to malaria endemicity into four strata that are different in altitude, intensity, length, and season of transmission and even in the predominant vector species (NMCP, 2011). This heterogeneous epidemiology of malaria may affect the performance of RDTs, necessitating the need for their evaluation in the four strata.

In Yemen, only two previous studies evaluated the performance of *Pf*HRP-2-based RDTs against LM as the 'gold standard' including two brands; Response® Malaria Ag. *P. falciparum* (HRP-2) RDT (Premier Medical Corporation Ltd, India) (Ghouth *et al.,* 2012) and CareStart™ Malaria HRP-2 (Access Bio, Inc, USA) (Atroosh *et al.,* 2015). It is, however, noteworthy that false-negativity of LM limits its accuracy as reference method. Polymerase chain reaction (PCR) is more sensitive than LM and RDTs for detecting malaria in epidemiological studies assessing asymptomatic carriers in low endemicity settings (Coleman *et al.,* 2006; Ojurongbe *et al.,* 2010; WHO, 2013). Thus, this is the first community-based survey to evaluate the performance of LM and a *Pf*HRP-2/pLDH RDT for malaria diagnosis against PCR as the reference method during the transmission season in a malaria-endemic area in Taiz governorate.

Standard Diagnostics (SD) Bioline® Malaria Antigen *Pf/*Pan RDT was selected for the evaluation because it is available and widely used in the Yemeni market, has shown excellent performance (Ratsimbasoa *et al.,* 2008; Chaijaroenkul *et al.,* 2011; Tadesse *et al.,* 2016) and has not been evaluated in Yemen yet. Few studies have been published on the efficacy of SP alone or in combination with AS in Yemen. For instance, the NMCP reported an efficacy of 95–100% for SP in eight trials conducted in sentinel sites in endemic areas in the period between 2002 and 2005 (NMCP, 2010).

Al-Kabsi *et al.,* (2009) also reported the absence of treatment failure of falciparum malaria with SP in Tihama region. Since then, the effectiveness of the currently recommended first- and second-line treatments has been shown in eight drug efficacy trials of AS plus SP and AL for the treatment of uncomplicated falciparum malaria in the period from 2009 to 2013 (NMCP, 2013; Adeel *et al.,* 2015). It is noteworthy that *in vivo* drug efficacy trials measure the combined effect of both drug partners and do not differentiate between the efficacy of AS and its partner. Failure of the AS partner increases the risk of the emergence of AS-resistant parasites (WHO, 2015).

Therefore, surveillance of the molecular markers associated with resistance to antimalarial drug partners can be a useful tool for detecting and monitoring the emergence of resistant *Plasmodium* strains in a geographic region before treatment failure becomes clinically evident (Hastings *et al.*, 2002; Modrzynska, 2011). Although *in vivo* efficacy trials indicate that ACTs are still efficacious in Yemen (NMCP, 2013; Adeel *et al.,* 2015), detection of molecular markers associated with resistance to AS and SP is necessary as an early warning system for the evolution of drug resistance to their partner drugs. In addition, detection of the *pfcrt* 76T may demonstrate the trend in resistance to CQ after replacing CQ with AS plus SP combination. Thus, the present study aimed to detect mutations at the codons 76 and 86 of *pfcrt* and *pfmdr1*, respectively, as well as in K13, *pfdhfr* and *pfdhps* among *P. falciparum* isolates from Mawza district, Taiz governorate.

1.3 Objectives

1.3.1 General objective

This study aims to determine the epidemiology of malaria and anti-malaria drug resistance markers in the rural communities of Taiz Governorate, Yemen.

1.3.2 Specific objectives

1. To determine the prevalence and identify the risk factors of malaria among study population in this area.

2. To determine the knowledge, attitude and practice (KAP) toward malaria in the study area.

3. To evaluate light microscopy (LM) and rapid diagnostic test (RDT), combining both *P. falciparum* histidine-rich protein-2 (*Pf*HRP-2) and *Plasmodium* lactate dehydrogenase (pLDH), for falciparum malaria diagnosis against nested polymerase chain reaction (PCR) as the reference method.

4. To determine the frequency of antimalarial drug resistance (*Pfcrt* K76, *Pfmdr-1* N86, K13, *Pfdhfr* and *Pfdhps*) markers.

1.4 Hypotheses

1. The prevalence rate of malaria is high among the rural community during peak transmission season.

2. There is a significant association between the high prevalence rate of malaria with demographic and socioeconomic factors.

3. There is a low prevalence of CQ and SP resistance to *P. falciparum* infection after 6 years of shifting to ACTs.

4. Artemisinin is effective after implementation by NMCP following new drug policy.

CHAPTER 2: LITERATURE REVIEW

2.1 The global burden of malaria

Malaria remains an important cause of illness and death in poor countries throughout Africa, Latin America, and Asia. Global mortality and morbidity rate caused by malaria is very high. According to World Malaria Report (WHO, 2015a), 97 countries still suffering from malaria infection and approximately 214 million malaria cases resulting in 438000 malaria deaths globally, representing a decrease in malaria incidence (37%) and (60%) of death between 2000 and 2015 (Figure 2.1).

About 3.2 billion people, approximately half of the world's population, are at risk of malaria infection in 2015. Most of the burden and 88% of malaria-infected people live in sub-Saharan Africa; approximately 80% of malaria deaths are concentrated in 15 countries, mainly in Africa. Seventy-one percent (71%) of deaths are children under five years old in Africa. Moreover, according to the Global Malaria Action Plan (GMAP), funding for research, treatment and control of malaria has increased significantly over the years and reached approximately US $2.5 billion in 2014 (WHO, 2015a).

Effective, appropriate treatment and early diagnosis are imperative in easing the impact of malaria. Although control and elimination of malaria has been achieved in many countries, plans for worldwide eradication have been met with several obstacles. Insecticide resistant vectors, population movements, inadequate health service and drug resistance are problems in malaria control. Resistance *in vivo* has been reported against almost all antimalarial drugs (Zucker & Campbell, 1992; Sharma & Sood, 1997; WHO, 2013). Drug resistance in *P. falciparum* is not confined to chloroquine (CQ) alone, but also to the other currently used antimalarial and is widespread.

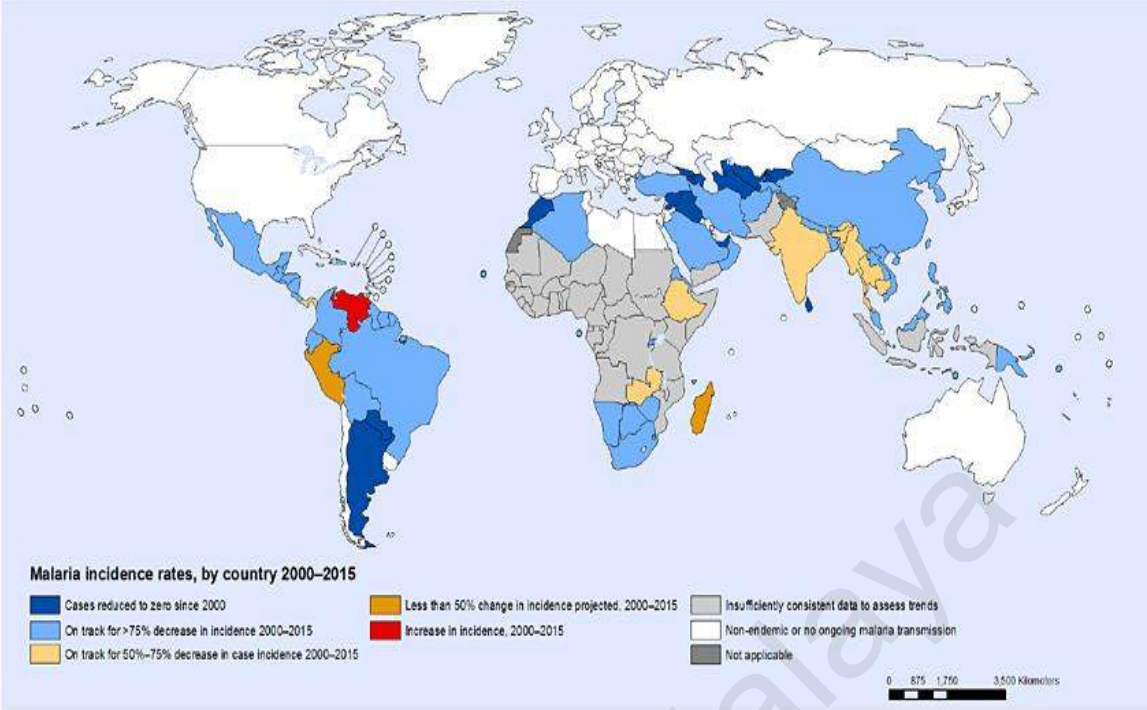


Figure 2.1: Global decrease in malaria incidence between 2000 and 2015 (Source: WHO, 2015). About 214 million malaria estimated cases worldwide in 2015, representing (37%) global decrease in malaria incidence and (60%) of deaths between 2000 and 2015.

2.2 Causative agent of malaria

2.2.1 *Plasmodium* species

Malaria, a term which originated from Medieval Italian: mala aria meaning 'bad air', is a disease which was formerly known as marsh fever due to its close association with swamps (Sachs & Malaney, 2002). *Plasmodium* specie is a sporozoan obligate intracellular parasite which lives in hepatocytes and erythrocytes. The number of species in the *Plasmodium* genus is large, which currently is above 250 species. Generally, the *Plasmodium* sp. undergoes sexual and asexual reproduction in its life cycle which requires both vector and human hosts respectively. Mosquito is the vector host. *Plasmodium* sp. are placed in the Kingdom Chromalveolata, Superphylum Alveolata, Phylum Apicomplexa, Class Aconoidasida, Order Haemospordia, Family Plasmodiidae and Genus *Plasmodium* (Levine, 1988).

The five *Plasmodium* species which cause malarial infections in human are *Plasmodium falciparum, P. vivax, P. malariae*, *P. ovale* (two species *wallikeri* and *curtisi*) and *P. knowlesi.* The latter is a simian malaria parasite and has only recently recognised as a *Plasmodium* species that can infect human (Cox-Singh *et al.*, 2008; White, 2008; Bronner *et al.*, 2009).

2.2.2 Vector and mode of transmission

Human malaria can be transmitted only by female *Anopheles* mosquitoes. There are about 430 species of *Anopheles* mosquitoes throughout the world (CDC, 2015). The genus *Anopheles* belongs to the order Diptera, suborder Nematocera, Family Culicidae, Subfamily Culicinae and tribe Anophelini. Within the tribe Anophelini the Genus *Anopheles* has six sub-genera (White, 1986; Breman *et al.*, 2004).

About 20 different *Anopheles* species are responsible for malaria transmission around the world and environment. This group of mosquito is typically found and breed

in fresh water, such as puddles, ponds, flowing stream and rice fields and the intensity of transmission depends on the parasite, the vector, the human host (CDC, 2015).

Malaria can also be transmitted via blood transfusion. This can happen if an infected person donates blood before the onset of clinical symptoms, after merozoites have entered the blood stream from the liver. Similarly, malaria may be transmitted by contaminated needles and syringes and organ transplantation. In congenital malaria, which is comparatively rare, parasites are transmitted from an infected mother to her unborn child before and/or during birth through the placenta (Filler *et al.*, 2003; Owusu-Ofori *et al.*, 2015).

2.2.3 Life cycle

Human *Plasmodium* sp. has a common life cycle. The life cycle is complex and consists of distinct stages in the *Anopheles* mosquito vector and human host (Figure 2.2). When an infected female *An.* mosquito bites the human skin during blood meal, sporozoites are passed along in the saliva with anticoagulant into the blood. The sporozoites readily travel (exo-erythrocytic cycle) through the blood stream to the liver, where they will infect the hepatocytes. In the liver cells, the sporozoites undergo an initial growth followed by asexual replication (liver schizogony), forming a mature liver schizont containing up to 30,000 merozoites (CDC, 2010).

The liver schizogony takes 5-7 days and during this time, the patient is asymptomatic. As the mature liver schizont rupture, merozoites are released. Majority are engulfed by liver macrophages i.e. Kupffer cells however, merozoites that escape, rapidly infect erythrocytes. In the erythrocytic cycle, the merozoite transforms to an immature trophozoite (ring form), then to a mature trophozoite, followed by asexual reproduction to a mature schizont containing around 10 to 20 merozoites. The merozoites are released upon erythrocyte rupture and instantly invade new erythrocytes

(CDC, 2010). The duration of the erythrocytic cycle lasts for 48 hours for *P. falciparum, P. vivax* and *P. ovale* (Garcia *et al.*, 2006).

Some merozoites do not undergo further asexual replication; instead they develop into male and female gametocytes. Gametocytes circulating in the blood are extracted by mosquito during its feeding on the human blood. Within the mosquito's gut, the gametocytes are triggered by the presence of specific mosquito factors and the drop in temperature to form male and female gametes (Liljander *et al.*, 2010).

In the sporogonic cycle, male and female gametes fuse to form a diploid zygote which undergoes meiosis and produces ookinete. The ookinete penetrates the mosquito's mid-gut wall, transforms into an oocyst that produces a large number of haploid sporozoites through repeated mitotic divisions. Sporozoites migrate and invade the salivary glands from which they can be injected into the human host when the mosquito takes a blood meal, thus starting the life cycle of the parasite again. The process of life cycle takes 10-18 days depending on the *Plasmodium* species. The mosquito remains infectious for 1-2 months (Liljander *et al.*, 2010).

2.2.4 Risk factors

Identification of factors-related to malaria can serve as the main principle for drawing up guidelines on innovative strategies that would help ensure better community participation and implementation of control measures. Socio-economic risk factors contribute to the increased occurrence and transmission of malaria, e.g., modest housing construction, population movements to endemic area, improper uses of mosquito nets, inadequacy with residual dichloro diphenyl trichloroethane (DDT) spraying (Van der Hoek *et al.*, 1998).

Environmental factors, such as temperature, rainfall, humidity, presence of stream, man-made reservoirs and vegetation cover are important for vector distribution

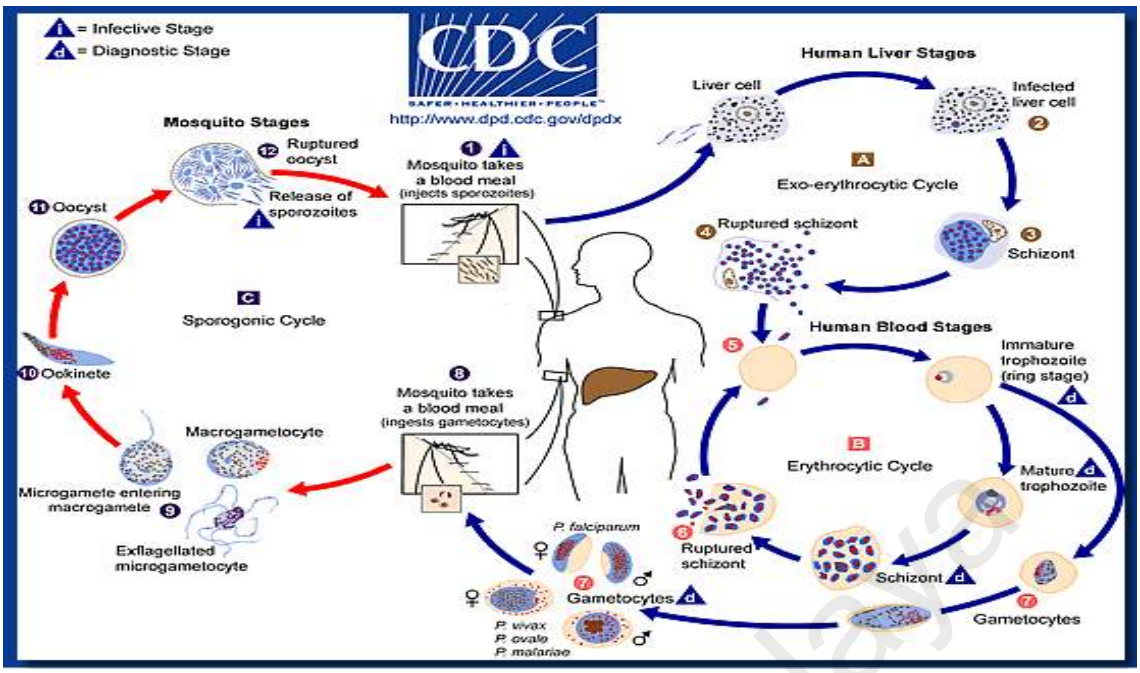


Figure 2.2: Sexual and asexual life cycle of *Plasmodium* species (Source: Centre for Disease Control and Prevention, 2010). Life cycle of *Plasmodium* species, which consists of three cycles; exo-erythrocytic cycle, erythrocytic cycle and sporogonic cycle.

and survival (Fungladda & Sornmani, 1986; Fungladda *et al.*, 1987; Yewhalaw *et al.*, 2009; Kar *et al.*, 2014). Other environmental factors including; living on flat land, lack ceiling in the home and open eaves were also associated with increase risk of malaria in endemic areas (Ghebreysus *et al.*, 2000; Ernst *et al.*, 2009).

Moreover, lack of knowledge about etiology, transmission and malaria prevention of an individual or a community plays an important role in the occurrence of the disease (Al-Taiar *et al.*, 2009; Baragatti *et al.*, 2009; Bamaga *et al.*, 2014). On the other hand, the relationship between malaria risk and the education level of female heads of household was reported (Ernst *et al.*, 2009; Fana *et al.*, 2015), and that the use of malaria prevention measures has been consistently related to higher maternal education (Keating *et al.*, 2005; Noor *et al.*, 2006).

Data have shown correlation between increase of occurrence of malaria and occupational staying outside especially at night without enough protective clothing (Arasu, 1991; Oemijati, 1992; Chuquiyauri *et al.*, 2012; Hiwat *et al.*, 2012; Bamaga *et al.*, 2014). Use of bed net during sleeping has significantly protected against mosquito bites (Tawrell, 2006). The role of human behaviour in the epidemiology of malaria focuses the need to identify risk factors that are related to infection, transmission and also severity of the disease.

Delay in treating malaria cases especially among people residing in malarias areas is a risk factor for complicated and severe malaria. This might be due to difficulty in accessing infected people in these areas or to the fact that people tend to treat themselves (Arasu, 1991; Oemijati, 1992).

2.3 Clinical symptoms and complications

Malaria is an acute febrile illness. In a non-immune individual, the early symptoms are fever paroxysms, myalgia, arthralgia, loss of appetite, chills, malaise,

fatigue, cold sweat, diarrhoea, nausea and vomiting. Symptoms typically begin approximately seven days or more (usually 10–15 days) after the infective mosquito bite. Parasite debris is released due to erythrocyte rupture and this causes host responses such as fever, cytokines release and the symptomatic phase of the infection (Sherman, 1998). The clinical manifestations vary from asymptomatic infections to severe life-threatening conditions.

Falciparum malaria may progress to severe illness and often death due to sequestration of *P. falciparum* infected red blood cells in the microvascular of organs. Sequestration is adherence of infected erythrocytes to endothelium in the deep vascular system during the asexual erythrocytic cycle. Accretions of electron-dense, histidine-rich parasite proteins are found in the 'knobs' present on the surface of the infected red blood cells (RBCs). These knobs extrude a strain specific, adhesive variant protein of high molecular weight that mediates red cell attachment to receptors on venular and capillary endothelium, causing cytoadherence (Beeson, 2000).

P. falciparum infected red cells also adhere to uninfected red cells to form rosettes. Cytoadherence and rosetting are central to the pathogenesis of *P. falciparum* malaria, resulting in the formation of red cell aggregates and intra vascular sequestration of RBCs in the vital organs like the brain and the heart (Mackintosh *et al.*, 2004). Thus, cytoadherence leads to obstruction of the microcirculation and results in dysfunction of multiple organs, typically the brain in cerebral malaria. This form of severe malaria is caused almost exclusively by *P. falciparum* (Trampuz *et al.*, 2003).

In adults, multi-organ failure may occur due to delay in accurate diagnosis and treatment. Children with severe malaria frequently develop one or more of the following symptoms like; severe anaemia (Uneke, 2007; Thapa *et al.*, 2009; Lee *et al.*, 2013), acute respiratory distress syndrome (ARDS) in relation to metabolic acidosis (Lee & Maguire, 1999; Tanios *et al.*, 2001; Lomar *et al.*, 2005; Anstey *et al.*,2007; Mohan *et*

al., 2008; Rojo-Marcos *et al.*, 2008; Deroost *et al.*, 2013; Hachimi *et al.*, 2013; Lau *et al.*, 2013), acute kidney injury (AKI) (Prakash *et al.*, 2003) and splenic rupture (Trampuz *et al.*, 2003; Das, 2008).

In malaria endemic areas, partial immunity is developed, allowing asymptomatic infections to occur. Uncomplicated malaria shows decrease in haemoglobin level and platelets count. The leukocyte count can be affected in two ways, towards hyper leucocytosis and leukopenia. Haemolysis parameters include a high reticulocyte count, a high indirect bilirubin and lactic dehydrogenase level. Transient albuminuria is possible in both complicated and uncomplicated *P. falciparum* malaria (Castelli *et al.*, 1997)**.**

Reappearance of malaria symptoms after varying symptom free periods depending on the cause and can be classified as recrudescence and relapse. Recrudescence is used to describe the situation in which parasitaemia of *P. falciparum* falls below detectable levels and then later increases to a patent parasitaemia (Beier, 1999), within two weeks of treatment from the initial infection, which is typically attributed to treatment failure (WHO, 2010). People may develop some immunity when exposed to frequent infections (Tran *et al.*, 2012).

In *P. vivax* and *P. ovale* infections, relapses may occur weeks to months after the first infection. These new episodes arise from dormant liver forms known as hypnozoites (Krotoski, 1985; Cogswell, 1992). Some *P. vivax* sporozoites do not immediately develop into exo-erythrocytic phase merozoites, but instead produce hypnozoites that remain dormant for 7–10 months to several years. After a period of dormancy, they reactivate and produce merozoites. Hypnozoites are responsible for long incubation and late relapses in *P. vivax* infections (White, 2011).

2.4 Pathophysiology

In the erythrocytic cycle, most merozoites undergo blood schizogony to form trophozoites, evolving to schizonts, which rupture to release new merozoites. These then invade new erythrocytes and the 48-hour cycle continues, sometimes resulting in periodicity of fever. The rupture of erythrocytes releases toxins that induce the release of cytokines from macrophages, resulting in the systemic manifestations of malaria and central nervous system manifestations (Clark *et al.*, 2006). Malaria toxins and inflammatory response; cytoadherence, rosetting and sequestration; altered deformability and fragility of parasitized erythrocytes; endothelial activation, dysfunction and injury, and altered thrombocytosis have been found to be involved in the development of severe malaria (Chen *et al.*, 2000; Miller *et al.*, 2002; Anstey *et al.*, 2009).

2.5 Diagnosis

Microscopic examination of malaria parasite in human is made by studying thick and fixed thin peripheral blood smears using Giemsa stain. Smears should be obtained at different times of the day from patients with suspected infection because parasitaemia may be intermittent and the number of circulating parasites may vary (Koneman *et al.*, 1997).

Microscopic examination of blood film is the gold standard diagnosis method for human malaria parasites. In *P. falciparum* infection, early trophozoites appear as ring forms with double chromatin dots, multiple infections and in a later stage of the disease, gametocytes are found in the peripheral blood. Schizonts in peripheral blood are rarely seen, since schizogony usually occurs in red blood cells sequestered in visceral organs, and its occurrence in peripheral blood is a sign of severe infection. While in *P. vivax* infection, all stages of the parasite developmental cycle may be seen in peripheral blood

because schizogony takes place in circulating infected red blood cells non adherent to the endothelium of internal organs (Castelli *et al.*, 1997). The limitation of microscopic examination is the difficulty in detecting the parasite in blood film when the parasitaemia level is low and is strongly dependent on the competence of the microscopist. False negative results may be reported.

RDTs are immuno-chromatographic tests for detecting parasite-specific antigens and recommended by WHO (2011a). RDTs are very important to reduce overuse of antimalarial drugs especially in endemic area. Malaria Ag *P.f*/Pan Cassette is designed to detect *Pf*HRP-2 and *Plasmodium* intracellular metabolic enzyme, pLDH for (*P. falciparum, P. malariae, P. vivax, and P. ovale*). It has high specificity in low parasite density and also does not need special equipment and electricity.

Polymerase chain reaction (PCR) technique described by Myjak *et al*., (2002) has been used to identify the species of malaria parasite and enhance malaria diagnosis in mixed infections especially in patients with low parasite densities and it is 10-fold more sensitive than microscopy (http:// www.Malaria site.com/malaria/Diagnosis of Malaria. PCR/htm; retrieved on 2 November 2015).

Nested PCR is a powerful variant of PCR technique. It was developed for the amplification of unexpected primer binding sites. It involves the use of two sets of primers, the second set intended to amplify a secondary target within the first run product. This allows amplification for a low number of runs in the first round, which allows running more total cycles while minimizing non-specific products. This is useful to amplify extreme low quantities of template DNA (Lok *et al.*, 2012).

Eight studies were reported with no misdiagnoses found in identification of *P. falciparum* by nested PCR (Morassin *et al.*, 2002; de Monbrison *et al.*, 2003; Calderaro *et al.*, 2004; Ndao *et al.*, 2004; Perandin *et al.*, 2004; Whiley *et al.*, 2004; Machouart *et*

al., 2006; Vo *et al.*, 2007). Nested PCR and real-time PCR have been used for detection of *P. vivax* infection in human (Genc *et al.*, 2010).

2.6 Control and prevention

Control and prevention of malaria involve three living beings: human (host), *Anopheles* mosquito (vector) and *Plasmodium* sp. (parasite) (Figure 2.3). People who live in malaria endemic areas should be educated to take protective measures against mosquito bites. Closure of doors and windows in the evening can prevent entry of nocturnal *Anopheles* mosquito into human dwellings.

Avoiding contact with mosquito during dust-to-dawn hours is an important malaria control measure. Insecticide-impregnated materials (permethrin-containing products) such as bed nets and curtains have been used. Insecticide bed nets can effectively reduce malaria morbidity and morbidity rate. Health authorities in malaria endemic countries organise campaigns for distribution of free insecticide-treated bed nets (ITNs) to their citizens (Thwing *et al.*, 2008; Hightower *et al.*, 2010). However, due to the low awareness and compliance of the nets owners, this strategy faces difficulties in implementation (Atieli *et al.*, 2011).

For travellers and workers in jungle, they should wear clothes which cover most parts of the body and use effective insect repellents like cream, lotion and coils. Chemoprophylaxis for prevention of malaria is taken one to two weeks before travelling to jungle and malaria endemic areas. A wide range of medication is used for chemoprophylaxis under consultancy of medical doctors. These medications include atovaquone/proguanil (malarone), chloroquine (aralen) and hydroxychloroquine (plaquenil), doxycycline, mefloquine, primaquine, and sulfadoxine/pyrimethamine (fansidar) (Fernando *et al.*, 2011). On the other hand, few strategies could be applied in mosquito vector control. Mosquito's larvae are controlled by using larvicide or

biological control, but in case of adult mosquitoes it is done by killing them with effective insecticides especially via indoor spraying (https://en.wikipedia.org/wiki/ Mosquito_control; retrieved on 20 August 2015).

Besides, accurate diagnosis and effective treatment are also crucial steps for control of malaria. Compliance of treatment is important to achieve complete clearance of parasitaemia and to prevent further spreading of infection to other humans. Other efforts on elimination and eradication of malaria include development of vaccines, and also genetic modifications of mosquito vectors to prevent transmission of parasites from mosquitoes to humans.

2.7 Treatment

2.7.1 Antimalarial drug policy according to World Health Organization (WHO)

Early diagnosis, coupled with prompt and effective treatment within 24-48 hr is very important as it can prevent complications and fatal outcomes. Artemisinin-based combination therapies (ACTs) are the recommended first line antimalarial treatments for uncomplicated malaria caused by *P. falciparum* in children and adults according to WHO guidelines.

Artesunate (AS) plus one of these drugs; amodiaquine (AM), mefloquine (MQ), sulfadoxine/ pyrimethamine (SP) and dihydroartemisinin plus piperaquine have also been found to effective for uncomplicated *falciparum* malaria as a first-line treatment. Artemether plus lumefantrine (AL), the second-line treatment is used when initial treatment is not effective. Any of these combinations are given for 7 days. In case of high parasitaemia and severely ill patients, intravenous (IV) or intramuscular (IM) artesunate is given. Quinine is given if artemisinin is not available. However, quinine may lead to many adverse effects such as cinchonism (quinism) or even death by pulmonary odema (WHO, 2015b).

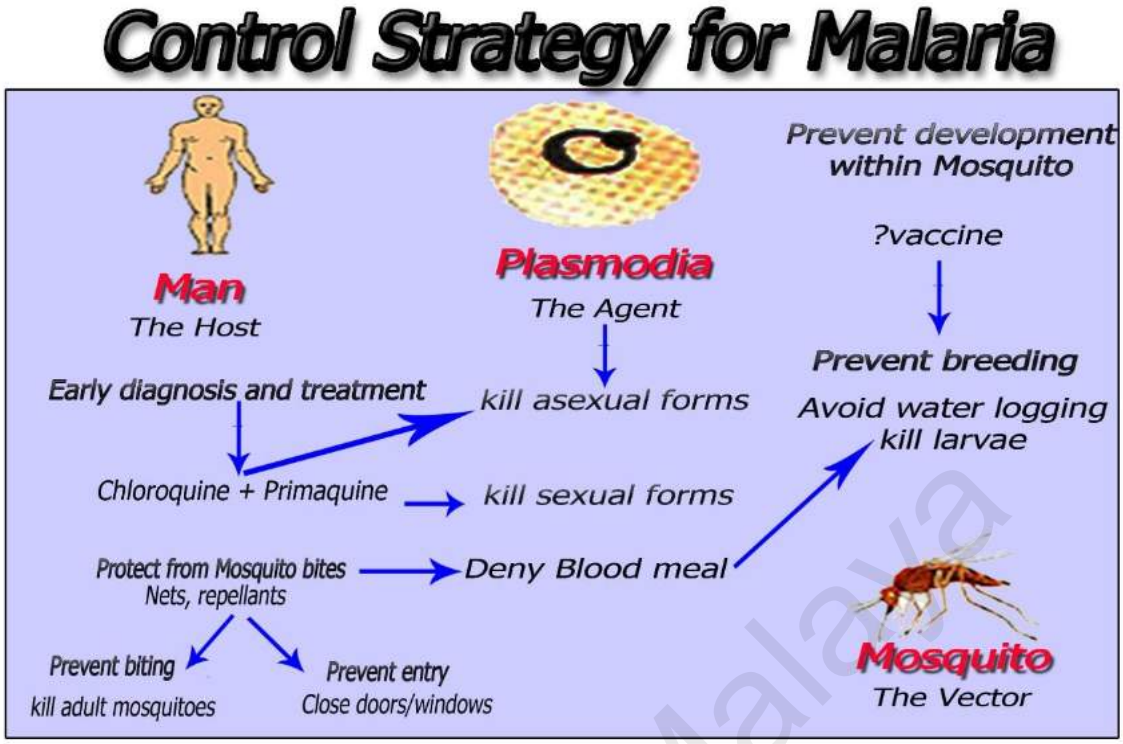


Figure 2.3: Chain map of control and prevention of malaria, which involves three parties: human, mosquito and *Plasmodium* species (Source: Malaria site, 2009). The control and prevention chain is summarized as use of personal protective measures and chemoprophylaxis. Control of adult mosquito and larvae by insecticides and larvicides respectively.

2.7.2 Antimalarial drug policy according to National Malaria Control Program (NMCP, 2005) in Yemen

Artemisinin combination therapies (ACTs); AS plus AS-SP is the first line treatment in the national new policy (NMCP, 2005), and the second line treatment is AL. Quinine (IV) or artemether (IM) remain the treatment of choice in severe and complicated cases of malaria. The old policy considered CQ as the first line of treatment for uncomplicated *P. falciparum*. SP was the second treatment line and the third was mefloquine and primaquine as a gametocytocidal drug. In severe and complicated falciparum malaria, quinine is given intravenously. Primaquine as an anti-relapse is used in *P. vivax* and *P. ovale* infections and as gametocytocidal drug in *P. malariae* (NMCP, 2005).

2.7.3 Antimalarial drug resistance

Due to the lack of an effective vaccine, the fight against malaria relies mostly on chemotherapy and chemoprophylaxis. However, resistance to currently available antimalarial drugs has seriously reduced the effectiveness of the drugs (WHO, 2013). In the middle of the 20th century during the late 1950s and the early 1960s, it was believed that malaria could be eradicated. This is because the *Plasmodium* parasite did not have an animal reservoir and efficacious drugs to interrupt transmission existed (Talisuna *et al.*, 2004) (Figure 2.4).

Five decades later, the characteristics of the parasite have changed, giving rise to antimalarial drug resistance. Drug resistance has been defined 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 limits of tolerance of the subject (WHO, 1973).

Drug resistance has become one of the greatest challenges pertaining to malaria control. It has been implicated in the spread of malaria to new areas and the re-emergence of the disease in areas where malaria had been eradicated (WHO, 2001b).

While the first report of CQ resistant (CQR) *P. falciparum* was in 1957 in the area of the Thai-Cambodia border (Payne, 1987), CQR *P. vivax* was not reported until 30 years later in 1989 in Papua New Guinea (PNG) (Rieckmann *et al.*, 1989). Since then, CQR in *P. vivax* has been detected in 10 other countries.

In African countries, a rapid increase in resistance to first line therapy was observed after the 1990s, with a consequent decrease in the efficacy of CQ (Talisuna *et al.*, 2004) and its replacement by SP (Myint *et al.*, 2004; Watkins *et al.*, 1993). Malawi was one of the first countries to adopt SP for *P. falciparum* as a first line treatment in 1993 (WHO, 2005). It is used within the approach of intermittent preventive treatment (IPT) in African countries with stable malaria transmission and low drug resistance (WHO, 2004; Peters *et al.*, 2007).

The development and spread of SP resistance poses a great public health problem as it is an effective, affordable, and well tolerated alternative to CQ, particularly in African countries (Wongsrichanalai *et al.*, 2002). Anti-folate-resistant *P. falciparum* populations have emerged unexpectedly in a quick pace even more than that of CQ (White, 1992; Winstanley, 2000).

Resistance to SP was reported in the same year of its introduction, in contrast to the first reports of CQR strains of *P. falciparum* two decades after its introduction (Wellems & Plowe, 2001; Wongsrichanalai *et al.*, 2002). Resistance to pyrimethamine (PYR) was reported after a short period of initial introduction in Africa and Southeast Asia (Clyde & Shute, 1954; Jones, 1954; Clyde & Shute, 1957; Jones, 1958; Archibald, 1960; Charles *et al.*, 1962; Young *et al.*, 1963; DeGowin & Powell, 1964; Peters, 1987), and it has been supposed to occur several years before that of sulfadoxine (SX) (Roper

et al., 2003; Mita *et al.*, 2009). It appears to be independently emergent, fast and simultaneous in different parts of the world (Bassat & Alonso, 2010).

To overcome this problem, it was potentiated by combining it with SX (Hurly, 1959). However, in the late 1960s, first reports of SP resistance were seen at the Thai-Cambodian border (Verdrager, 1986; Björkman & Phillips-Howard, 1990; Wernsdorfer & Payne, 1991; Wongsrichanalai *et al.*, 2002), and the highest-level of resistance was documented in other parts of Southeast Asia, Southern China, and the Amazon Basin (Wernsdorfer & Payne, 1991; WHO, 2001c; Wongsrichanalai *et al.*, 2002). In 1994, the emergence of SP resistance was first reported among Tanzanian children in Africa (Rønn *et al.*, 1996). ACTs are now recommended as first line treatment for falciparum malaria in malaria endemic countries by the WHO (2006) and the drug of choice also in case of CQR *P. vivax* (WHO, 2013).

In 2014, WHO reported that *P. falciparum* malaria parasite resistant to ACTs in five countries in the Greater Mekong sub-region (GMS) in Southeast Asia; Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand and Vietnam (WHO, 2014a). Reduced susceptibility towards artemisinin derivatives was noticed in 2005 in *P. falciparum* isolates from Senegal and French Guiana (Jambou *et al.*, 2005). Slower rate of parasite clearance in the presence of *in vivo* artemisinin has also been observed in Western Cambodia, Thailand, South eastern Myanmar and Vietnam. Another study in Western Thailand also demonstrated reduced susceptibility of *P. falciparum* to artesunate over time (Phyo *et al.*, 2012). There are reports of an increased proportion of cases of delayed parasites clearance after treatment with ACTs in therapeutic efficacy studies (TES) conducted in three South American countries (WHO, 2014b).

Artemisinin resistance could spread from these countries to other regions, including sub-Saharan Africa, where the incidence of malaria is highest, and where artemisinin resistance would have devastating consequences.

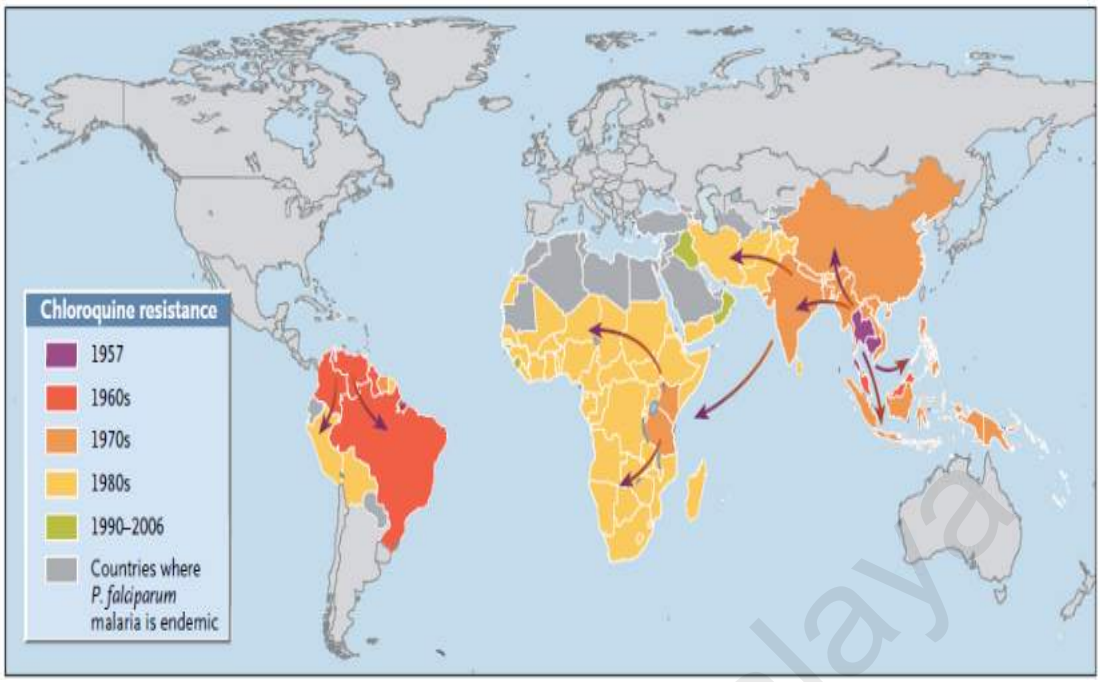


Figure 2.4: History of chloroquine-resistance in *P. falciparum* (Source: Worldwide Antimalarial Resistance Network, 2014).

Increased and uncontrolled travel between Asia and Africa may contribute to the spread of artemisinin-resistant malaria parasites (Van Hong *et al.*, 2015). Recently, K13 resistance-associated mutations have been confirmed in two African countries, Angola and Mozambique (Escobar *et al.*, 2015).

2.7.3.1 Chloroquine: Mode of action and mechanism of resistance

Chloroquine (CQ), a derivative of quinine, was synthesized in 1934 (Figure 2.5 a) and has been the most widely used antimalarial drug since it was clinically available in 1947. It was one of the most successful antimalarial drugs ever produced. It was cheap, effective against blood stages of the parasite and known for its good tolerability and low toxicity.

Moreover, its long half-life provided protection from early recurrence of malaria following treatment (Suwanarusk *et al.*, 2007). Due to its extensive use, *P. falciparum* developed resistance to CQ just within a decade of its introduction, with the first reports coming from the Thai-Cambodian borders in 1957. Then, the resistance spread to PNG in the 1960s and was observed in Africa by the 1970s (Payne, 1987; Wellems, 2002).

CQ accumulates in the food vacuole of *Plamodium* parasites and acts by interfering with the breakdown of the heme group (ferriprotoporphyrin IX) which is produced when haemoglobin is digested (Fitch, 2004). CQ in its uncharged state is membrane permeable. However, once the drug comes in contact with the acidic environment of digestive vacuole, it becomes positively charged and begins to accumulate to a concentration which is greater than the extracellular medium (Saliba *et al.*, 1998). Heme is detoxified into hemozoin via biocrystalyzation and the disruption of this process has detrimental effects to the parasite (Slater & Cerami, 1992; Chou & Fitch, 1993; Fitch, 2004).

CQR *P. falciparum* survives by reducing the amount of drug accumulates in its digestive vacuole by four to ten times less than chloroquine sensitive (CQS) parasites (Saliba *et al.*, 1998). It has been proposed that the initial accumulation rate of CQ in both resistant and susceptible parasites is the same, suggesting that the efflux rate of CQ may be the only significant difference between resistant and susceptible parasites. It appears that efflux is either absent or greatly reduced in a sensitive parasite (Krogstad *et al.*, 1988).

2.7.3.2 Sulphadoxine and Primethamine: Mode of action and mechanism of resistance

The antifolate drugs sulfadoxine (SX) and pyrimethamine (PYR) (Figure 2.5 b) are effective, safe and cheap antimalarial alternative to CQ (Basco *et al.*, 1998) and they are recommended by WHO (2004). Several countries particularly in Africa, have replaced CQ with SP as the first-line antimalarial treatment due to the emergence and spread of CQR *Plasmodium* species (White, 2004).

In the early 1950s, PYR was reported as a schizonticidal agent against malaria parasites in field trials (Archibald, 1951). It was used widely for treatment and for mass prophylaxis (Peters, 1987). More than a decade later, SX was shown to be an effective, slow-acting blood schizontocide (Laing, 1964, 1965). Subsequent studies in Southeast Asia and Africa revealed more rapid clinical response and action when combining SX with PYR (SP) against *P. falciparum* and *P. vivax,* including CQ-resistant strains (Richards, 1966; Harinasuta *et al.*, 1967; Laing, 1970).

`In 1967, SP was first used as a first-line treatment for falciparum malaria in Thailand (Wernsdorfer & Payne, 1991). Malawi was one of the first African countries to adopt the SP as a first-line treatment for falciparum malaria in 1993 (WHO, 2005). SP is the most effective and single-dose antimalarial prophylactic agent for malaria

prevention during pregnancy and in infants to reduce morbidity and mortality of malaria in sub-Saharan African countries. It is used within the approach of IPT in Africa with low drug resistance and stable malaria transmission (WHO, 2004; Grobusch *et al.*, 2007; Peters *et al.*, 2007; Aponte *et al.*, 2009). However, resistance to SP in many endemic areas has compromised its effectiveness and usefulness as a first-line antimalarial agent.

Malaria parasites are capable of *de novo* folate biosynthesis and the folate synthesis pathway is, therefore, an attractive drug target in *Plasmodium* sp (Mayers *et al.*, 2009). SP inhibits folate biosynthesis that is essential for pyrimidine synthesis and, in turn, for parasite's DNA replication (Foote & Cowman, 1994). Interruption of folate synthesis by dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) inhibitors decreases the levels of tetrahydrofolate that is an essential co-factor in transfer reactions in the purine, pyrimidine, and amino acid biosynthetic pathways (Ferone, 1977). SX targets the DHPS domain of the bifunctional 7, 8-dihydro-6-hydroxymethylpterin pyrophosphokinase-DHPS enzyme (Brooks *et al.*, 1994), while PYR targets the DHFR domain of the bifunctional DHFR-thymidylate synthase enzyme. The targeted enzymes are essential for parasite folate biosynthetic pathways. The combination of these drugs has a synergistic *in vitro* antimalarial activity against *P. falciparum* (Chulay *et al.*, 1984; Wang *et al.*, 1999).

2.7.3.3 Artemisinin: Mode of action and mechanism of resistance

Artemisinin (ART) (or qinghaosu), which is the active component of *Artemisia annua L* (qinghao or sweet wormwood) was isolated in 1972 and its structure was determined in 1979 (Figure 2.5 c). ART is a sesquiterpene trioxane lactone containing an endoperoxide bridge which is essential for its activity (Golenser *et al.*, 2006). ART is not only effective against all the asexual stages of the parasite, but they are also able to

exert their effects on the sexual stages (gametocytes) of *P. falciparum* which are essential for transmission.

The specific mechanisms of artemisinin and its derivatives are still uncertain. The first clue to the basis of its mechanism came from synthetic chemists who showed that the Endoperoxide Bridge was pivotal for antimalarial activity (Brossi *et al.*, 1988).

Artesunate (AS) is a semi-synthetic hemisuccinate derivative of artemisinin. This derivative is most effective because of its instantaneous bioavailability. It is water soluble and can be administered via several routes; oral, intramuscular, rectal, and intravenous. AS is an ideal candidate for the treatment of severe malaria due to its rapidity, the various available routes of administration, and also its convenient dosage regimens (Wilairatana & Looareesuwan, 1996). The first ACTs, artesunate-mefloquine was used in the northwest border of Thailand in 1994, an area where parasites had already developed resistance to MQ (White, 2008). This potent combination was also implemented at the Thai-Cambodia border in 2001 as the first line of treatment for falciparum malaria (WHO, 2001a). However, it was not long before a decrease in clinical efficacy was reported for this combination at the Thai-Cambodian border (Dondorp *et al.*, 2009).

2.7.3.4 Genetic markers of reduced antimalarial susceptibility

Molecular markers of drug resistance and genetic polymorphisms are essential and complimentary tools in the surveillance of drug susceptibility (WHO, 2003). They serve as valuable adjuncts to both *in vivo* clinical studies and *ex vivo* drug sensitivity testing, and may help in the early detection of emerging resistance before the treatment failure becomes clinically evident (Hastings *et al.*, 2002). With the aid of polymerase chain reaction (PCR) techniques, it is feasible to rapidly genotype a large number of

samples using stored material such as whole blood and blood spots as the source of DNA (Plowe *et al.*, 1995; Eboumbou *et al.*, 2009).

The discovery of the genetic basis of CQ resistance was a long and tedious process. Fidock *et al.* (2000) identified the *pfcrt* gene which is located on chromosome 7 and is highly polymorphic. The critical mutation which brings about CQ resistance is the substitution of lysine (K) at position 76 with threonine (T) which is located in a transmembrane region implicated in substrate recognition (Djimdé *et al.*, 2001) and concluded that the *pfcrt* K76 mutation can be used as a successfully marker for CQR surveillance (Djimdé *et al.*, 2001; Kublin *et al.*, 2003). Additional mutations have also been observed to be present together with the K76 mutation. These extra mutations however, are geographically dependent on the *P. falciparum* isolate (Bray *et al.*, 2005). These also appear to modulate the parasite's response to other antimalarial (Cooper *et al.*, 2007).

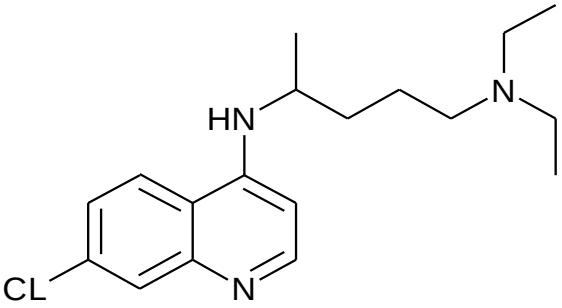
Resistance is also modulated by the *P. falciparum* multi drug resistance gene-1 (*Pfmdr*-1) (Conway, 2007). Earlier studies suggested an association between CQR and *Pfmdr*-1 gene located on chromosome 5 and encoding the P-glycoprotein 1 (Pgh-1) (Barnes *et al.*, 1992). CQR was thought to be linked to point mutations, such as asparagine (Y) to tyrosine (N) substitution at position 86 (Foote *et al.*, 1990). Djimde *et al.* (2001) proposed that the *Pfmdr*-1 N86Y mutation may be of an advantage to the parasite in the presence of CQ, either by compensating for fitness lost due to *Pfcrt* mutations or by further increasing the level of resistance. While the single nucleotide polymorphisms (SNPs) in this gene are associated with decreased susceptibility to CQ, mutation is also known to increase the parasite's susceptibility to other antimalarials such as lumefantrine, mefloquine, and artesunate (Reed *et al.*, 2000).

Monitoring of molecular markers may help in tracking the rates of resistance in a certain area after withdrawal or cessation of an antimalarial drug (Kublin *et al.*, 2003).

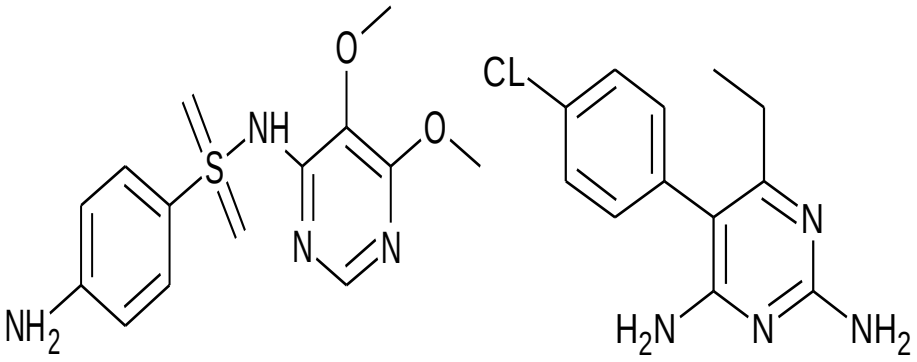
Mutations in the *dhfr* and *dhps* genes have been tested as molecular markers for anti-folate drug resistance and/or treatment failure. The role of many mutations in assessing resistance of *P. falciparum* to anti-folate drugs has been consolidated (Wernsdorfer & Noedl, 2003), and many genetic mutations have been reported in *dhfr* and *dhps* genes such as; mutations in *dhfr* include A16V, C50R, N51I, C59R, S108N/T and I164L. Those in *dhps* include S436A, A437G, K540E, A581G, and A613S/T.

The emergence of SP resistance is rapid and independent in several areas where the combination has been introduced (Talisuna *et al.*, 2004). According to the increasing reports of the widely distribution of SP resistance, researchers have begun to search for genetic backgrounds for such resistance in the individual components of the combination since the late 1980s. Parasite transfection experiments have proved the role of *dhfr* and *dhps* mutations in resistance to PYR and SX, respectively (Wu *et al.*, 1996; Triglia *et al.*, 1998). In addition, *in vitro* assays of parasite purified enzymes as well as *dhfr* allele expression in the yeast *Saccharomyces cerevisiae* support the role of gene mutations in SP resistance (Sirawaraporn *et al.*, 1997; Toyoda *et al.*, 1997; Wooden *et al.*, 1997; Cortese & Plowe, 1998). Then, studies began to determine *dhfr* and *dhps* haplotype frequency to quantify the resistance at the population level and to overcome the barrier of multiple infections.

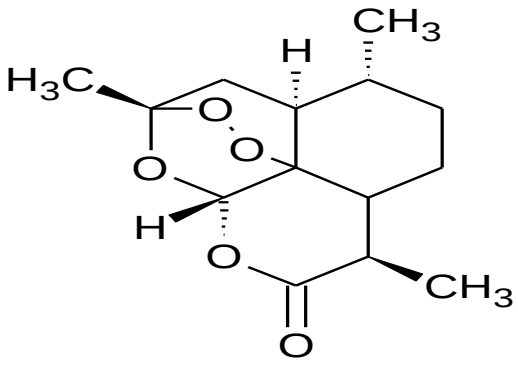
Pearce *et al.*, (2003) determined point mutation haplotypes in three Tanzanian districts where five *dhfr* and three *dhps* haplotypes were detected. In addition to the genetic mutations, efficacy of SP is also affected by nutritional folate intake and folate level in blood. Folic acid supplementation at standard therapeutic doses independently reduces antimalarial efficacy of anti-folate drugs (Carter *et al.*, 2005). Previous study by Dzinjalamala *et al.* (2005) reported association between late treatment failure (LTF) of SP and high serum folate concentration among children treated for malaria in Malaysia. Therefore, the effect of folate on SP efficacy should be considered while performing



(a)



(b)



(c)

Figure 2.5: Chemical structure of antimalarial drugs

(a): cholorquine

(b): sulphadoxine and pyrimethamine

(c): artemisinin

therapeutic efficacy tests or correlating SP treatment failure with the presence of mutant genotypes.

A study on a molecular marker of artemisinin resistance by Ariey *et al.* (2014) identified mutations in the propeller domain of the K13 gene that was associated with delayed parasite clearance *in vitro* and *in vivo*. It was revealed that the C580Y mutation was the most prevalent in parts of the Greater Mekong Sub-region (GMS), but many other mutations in and near the K13 propeller region were also found to be associated with artemisinin resistance (Y493H, R539T, I543T) (WHO, 2013).

About 17 mutations in the K13 propeller gene were reported in Cambodia (Ariey *et al.*, 2014). On the other hand, a study in Bangladesh reported that *P. falciparum* remains free of the C580Y mutation linked to delayed parasite clearance (Mohon *et al.*, 2014). Recent studies also reported that the sequencing of K13 gene is turning into a principal tool in the world reconnaissance of antimalarial drug resistance (Talundzic *et al.*, 2015; Tun *et al.*, 2015). Table 2.1 shows antimalarial molecular markers implicated in *P. falciparum* reduced drug susceptibility and delayed parasites clearance (http://www. tulane.edu/~wiser/malaria/ch15; retrieved on 5 July 2015).

Table 2.1 Antimalarial drug markers involved in *Plasmodium falciparum* drug resistance in the present study.

Antimalarial Drug	Protein	Gene	Subcellular Location	Primary function	Major Polymorphisms
Chloroquine	CRT	*Crt*	food vacuole	transporter	K76T
Chloroquine	MDR 1	*mdr-1*	food vacuole	transporter	N86Y
Pyrimethamine	DHFR	*Dhfr*	Cytoplasm	folate metabolism	A16V, C50R, N51I, C59R, S108N, I164L
Sulfadoxine	DHPS	*Dhps*	Cytoplasm	folate metabolism	S436A, A437G, K540E, A581G, A613S/T
Artemisinin	K13	K13	food vacuole	parasite clearance	C580Y, Y493H, R539T, I543T

2.8 Epidemiology

Malaria is one of the most common diseases worldwide. The total number of malaria cases globally is estimated to have dropped from 262 million in 2000 to 214 million in 2015 with a decline of (18 %) of malaria cases (WHO, 2015a). Most of the malaria cases (88%) are in the African Region, followed by the Southeast Asia Region (10%) and in the Eastern Mediterranean Region (2%). In total, 13 countries account for 75% of infections mainly in sub-Saharan Africa and 75% deaths globally (WHO, 2016). The Democratic Republic of the Congo and Nigeria accounting together for more than (35%) of the global total of estimated malaria deaths (WHO, 2015a).

Globally, the incidence of malaria is estimated to have decreased by (21 %) between 2010 and 2015 and malaria mortality rates among populations at risk decreased by (29%). Since 2001 about 6.8 million malaria deaths have been averted globally (WHO, 2016). It is estimated that 57 of 106 countries that had ongoing transmission in 2000 reduced malaria incidence by >75 %. A further 18 countries are estimated to have reduced malaria incidence by 50–75 % (WHO, 2015a). The number of malaria deaths globally fell from an estimated 839 000 in 2000 to 438 000 in 2015, a decline of (48 %) of malaria deaths. A bout (90%) of global malaria deaths in 2015 occurred in the African region, followed by the Southeast Asia Region (7%) and Eastern Mediterranean Region (2%). The mortality rate of malaria is estimated to have decreased by (60 %) globally between 2000 and 2015 (WHO, 2015a).

Children under five are mostly susceptible to infection, illness and death in areas with high transmission of malaria. The proportion of children aged 2–10 years infected with malaria parasites declined from (33 %) in 2000 to (16 %) in 2015 (WHO, 2015a). More than two thirds (70%) of all malaria deaths occur in this age group and as result between 2010 and 2015, the malaria death rate in this age group fell by (29%) globally.

(WHO, 2016). However, malaria is a major killer of children, particularly in sub-Saharan Africa, taking the life of a child every two minutes. An estimated 292,000 deaths occurred in children aged less than 5 years in the African Region. The global burden of mortality and morbidity is dominated by countries in sub-Saharan Africa, in which the Democratic Republic of the Congo and Nigeria together accounted for more than (35%) of the global total of estimated malaria deaths and (39%) of cases in 2014 (WHO, 2015a).

Moreover, recent study done by Dawaki, and co-workers (2016) confirmed the high prevalence of *P. falciparum* in Nigeria and the prevalence was (60.6%) and a similar high prevalence (48.2%) was reported in the Democratic Republic of the Congo among asymptomatic cases (Mvumbi, *et al.,* 2016).

The number of countries are moving towards elimination of malaria is increased. A bout, 33 countries are estimated to have fewer than 1000 malaria cases in 2015 with a substantial reduction in malaria cases have achieved in the Region of the Americas and Western Pacific Region and for first time the European Region reported zero indigenous malaria cases for the first time in 2015, while recent years, 16 countries have been certified by the WHO Director-General as having eliminated malaria; Argentina, Armenia, Azerbaijan, Costa Rica, Iraq, Georgia, Kyrgyzstan, Morocco, Oman, Paraguay, Sri Lanka, Tajikistan, Turkey, Turkmenistan, United Arab Emirates and Uzbekistan (WHO, 2015a).

2.8.1 Malaria in the Eastern Mediterranean region

Eight countries in the Eastern Mediterranean region are at some risk of malaria with about 279 million people are at risk and 111 million at high risk. Six countries have areas of high malaria transmission (Afghanistan, Djibouti, Pakistan, Somalia,

Sudan and Yemen). Most cases are due to *P. falciparum* except in Afghanistan, Iran and Pakistan, where *P. vivax* predominates. Four countries are continuing with the prevention of re-introduction phase; Egypt, since 1998; Oman, since 2004; and Syrian Arab Republic, since 2005; Iraq, since 2011. Saudi Arabia is entering the elimination phase. About 1.5 million in the Eastern Mediterranean region accounted for 91% of cases; 72% in the Sudan and 19% in Pakistan, and > 90% of death cases were in Sudan and Pakistan (WHO, 2015a).

Regarding malaria drugs resistance in the Middle East countries, a previous study done in Oman, Afghanistan, Pakistan and Yemen revealed that all *dhps P. falciparum* isolates were wild-type and all *dhfr* were double mutations except for two wild-type samples of Yemeni origin (Wang *et al.*, 1997). In Saudi Arabia, malaria is restricted to southwestern provinces of Aseer and Jazan (Dajem & Al-Qahtani, 2010) and previous studies done in this provinces reported a high prevalence of CQR in *P. falciparum* isolates (Zaher *et al.*, 2007; Dajem *et al.*, 2012). *dhfr* double mutations and absence of mutations in *dhps P. falciparum* isolates were observed in Jazan (Dajem *et al.*, 2012). However, another study done by Al-Farsi *et al.*, (2012) reported the predominance of wild-type *dhfr* and *dhps* alleles among parasite isolates from Jazan.

2.8.2 Malaria in Yemen

Yemen belongs to the Eastern Mediterranean Region. It is located in the Middle East at the southern tip of Arabian Peninsula in Southwest Asia. It is bordered on the north by Saudi Arabia, on the east by Oman, on the south by the Gulf of Aden and on the west by the Red Sea. The geography of Yemen can be divided into four main regions: the coastal plains in the west, the western highlands, the eastern highlands, and the Rub al Khali in the east. The total population is estimated at 23,584,000 people (CSO, 2013), within an area of about 555,000 km^2.

In Yemen, almost 62% of the population live in areas that suffer from stable malaria transmission. Yemen is still in the control phase, with approximately 25% of population at high risk of malaria (WHO, 2013). *P. falciparum* is the predominant species and is responsible for 99% of the malaria cases (NMCP, 2013; WHO, 2013) *Anopheles arabiensis, An. culicifacies* and *An. sergenti* are the main mosquito vectors (Knight, 1953; Kouznetsov, 1976; NMCP, 2002, 2010; WHO, 2013).

The national malaria control programme in Yemen (NMCP) has achieved substantial success in controlling local cases of malaria, with a significant reduction in the number of malaria cases from 900,000 cases in the early 2000s to around 150,000 cases in 2013(WHO, 2013). Mortality rates ranged from 2.1 to 4.7% in children (Al-Taiar *et al.*, 2006). Malaria prevalence in Yemen ranged between 12.8% and 18.8% (Al-Maktari & Bassiouny, 2003; Al-kadi *et al.*, 2006; Al-Taiar *et al.*, 2006; Mohanna *et al.*, 2007; Bamaga *et al.*, 2014).

In 2010, 78 (15.3%) out of 511 cases were positive for malaria in five governorates; Taiz and Hodeidah represent mountainous hinterland and coastal areas respectively, Raymah, Dhamar and Sana'a are highland area. These governorates have different climates, altitudes and seasonal transmission of malaria in Yemen. Three malaria species (*P. falciparum, P. vivax and P. malariae*) were detected, with *P. falciparum* predominating (83.3%) (Al-Mekhlafi *et al.*, 2010). In a recent study, prevalence rate of 18.8% was reported in Hadhramout governorate, and again *P. falciparum* (99.3%) was the predominant species (Bamaga *et al.*, 2014).

It was estimated that 40% of the paediatric malaria cases were admitted in some endemic areas during the rainy season (Al-Taiar *et al.*, 2006). Records at the Paediatric Health Centre in Sana'a from January 1998 to December 2000, showed that of the 753 children (484 boys and 269 girls) examined for suspected malaria, 130 (17.3%) were positive for malaria caused by *P. falciparum* (Azazy & Raja'a, 2003). A cross-sectional

study on asymptomatic primary-school children aged 6 to 11 years in Hajr valley recorded 60 of 469 asymptomatic school children had malaria parasitaemia, with malaria parasite rate of around 13% (Mohanna *et al.*, 2007). A study conducted in Makilla Children and Maternity Health Hospital, Hadhramout, showed that 163 of 400 children (40.8 %) were positive for malaria (Abdulhakeern *et al.*, 2009).

The problem of *P. falciparum* CQR in Yemen has gradually worsened since the detection of indigenous cases in February 1989 in Taiz and Hodeidah governorates (Mamser, 1989; Berga, 1999). Studies have been published in the last decade which aimed to evaluate the susceptibility of local Yemeni falciparum malaria isolates *in vivo* and/or *in vitro* to CQ, MQ and SP (Al-Maktari & Bassiouny, 2003; Al-kadi *et al.*, 2006; Al-Shamahy *et al.*, 2007; Al-Kabsi *et al.*, 2009).

Reem and co-workers (2011) confirmed the high prevalence of CQR and *dhfr* 59R mutant allele was reported among 5% of *P. falciparum* isolates in Lahj governorate and recommended further studies on antimalarial drug markers. Other studies have aimed to evaluate the effectiveness of CQ and SP from molecular aspect of view through detection of mutations in four governorates (Taiz, Hodeidah, Dhamar & Rymah) which showed high prevalence of CQR marker *pfcrt* 76T (Al-Mekhlafi *et al.*, 2011) and reported the double *dhfr* mutant 51I/108N among 54% of Yemeni *P. falciparum* isolates (Al-Hamidhi *et al.*, 2013).

In 2005, because of CQ and SP resistance, antimalarial drug policy was shifted from monotherapy to the combination of AS–SP as the first-line therapy and AL as a second line for uncomplicated malaria. However, this new policy was only implemented four years later in 2009, after proper training and distribution of the national guideline for antimalarial drugs were carried out (NMCP, 2010; WHO, 2012). Because of the low price of CQ, it is still being prescribed by doctors or bought by patients without doctor consultation (Al-Mekhlafi *et al.*, 2011).

In vivo studies have been carried out for the development of therapeutic efficacy of antimalarial drugs by NMCP, Yemen using WHO protocol for *in vivo* testing. Eight studies were conducted in four sentinel sites (Sadah, Hodeidah, Ibb & Lahj) from 2002 to 2005. The results showed high level treatment failure with CQ resistance rate of (39% to 57%), while SP was found to be effective (95% to 100%). Based on this information, WHO recommended change in the treatment policy (NMCP, 2010).

The last eight studies were carried out in 6 sentinel sites (Amran 2 sites, Haja, Hodeidah, Ibb & Dhamar) in 2009-2013, representing different epidemiological states in Yemen. Five studies were conducted for the evaluation of AS plus SP and three studies for the evaluation of AL. The studies were carried out according to the standard WHO protocol (2009) and reported the effectiveness of the new drug policy for uncomplicated falciparum malaria (Adeel *et al.*, 2015).

Data from Hodeidah governorate revealed high frequency of mutations in *pfcrt*-76 alleles and *dhfr* 108N and the new policy has been recommended for use in the governorate (Abdul-Ghani *et al.*, 2013a; Abdul-Ghani *et al.*, 2013b). Bamaga *et al.*, (2015a) recently reported high frequency of mutations in *pfcrt* 76 alleles but low prevalence (16.7%) of *pfmdr-1* 86Y mutant type among *P. falciparum* isolates in the Hadhramout governorate.

CHAPTER 3: METHODOLOGY

3.1 Study area and study population

The study was conducted in Mawza District, which is located about 97 km southwest of Taiz governorate (Figure 3.1). Its total area is about 665 km^2 and is inhabited by a total population of 119,818 people (CSO, 2013). It has a coastal climate which is warm in winter and hot in summer, with irregular heavy torrents of rainfall. The mean temperature is 29°C, and the humidity reaches to 67% (NIC, 2015). It is a malaria endemic area with a peak of transmission occurs in winter (October-April). The area is classified by the NMCP as belonging to stratum one, which has an altitude of less than 600 meters and is characterized by high malaria transmission (NMCP, 2011; 2013; NIC, 2015). The majority of the inhabitants are employed as daily labourers in agriculture, livestock and handicraft sectors.

3.2 Study design, sample size and sampling strategy

This study is a household-based cross-sectional study which was conducted during the malaria transmission season from October 2013-April 2014. Sample size was calculated using Epi Info™ 7.1.3 (Centers for Disease Control, Atlanta, USA) based on the following parameters: population size of 119,818 people, 5% confidence limits, 95% confidence level and an expected outcome frequency of 50.0% because several parameters were assessed. Accordingly, the minimum sample size required was 383. However, 488 participants were enrolled in the present study.

Household was the unit of sampling and all household members were invited to participate. The center of the district was selected for sampling because it was easy and safe to access and the visited houses were selected randomly.

3.3 Data collection

3.3.1 Blood sampling

Venous blood was collected from each participant using Vacutainer® Glass EDTA-tubes (Becton, Dickinson and Co., New Jersey, USA), and blood drops were spotted on Whatman® 3MM filter paper (Whatman International Ltd., Maidstone, Kent, UK).

3.3.2 Questionnaire

A pre-designed questionnaire was developed to collect data about demographic and socioeconomic characteristics of the study population as well as about their KAPs on malaria. In addition, the questionnaire also included questions relating to the clinical manifestations, prevention and control of the disease. Participants with history of fever in the last two weeks prior to the survey were also interviewed for fever treatment seeking behaviour.

The questionnaire was filled by face-to-face interview with participants. In case of children younger than 12 years the interview was conducted with their guardians and therefore the questions about knowledge and attitudes were excluded to avoid the duplicate opinions. Direct observation was made during the interview for the type of household building, wall, floor, the availability of functioning toilets, piped water, water containers, electricity, telephone, mosquito nets and the presence of nearby water or pools (Appendix 1).

3.3.3 Light microscopy and haemoglobin measurement

Thick and thin blood smears were prepared and stained with Giemsa for approximately 20 min according to standard procedures (Appendix 2). In the Yemeni-Swedish hospital laboratory, Taiz governorate, all thick and thin blood films were

examined using a light microscope by a qualified laboratory technician for a minimum of 100 high-magnification fields before being recorded as negative for malaria parasites. These films were then blindly examined by an independent malaria microscopist for the confirmation of the first examination. A third blinded examination was conducted for those films having discordant results between the first and second examiners. The results from the third examination were regarded as final.

Parasite density per μL of blood was estimated per 200 white blood cells (WBCs), assuming a standard mean WBC count of 8,000/μL (Moody, 2002; Singh *et al.*, 1999). Parasite density is defined as the total number of *Plasmodium* asexual forms per μL of blood. Parasite density was classified as low (1–999/μL), moderate (1000–9999/μL) or high (>10000/μL). Blood samples were also analyzed for haemoglobin estimation using a Sysmex KX-21N Hematology Analyzer (Sysmex Corp, Chuo-Ku, Kobe, Japan). Participants were categorized based on their haemoglobin concentrations as non-anaemic or anaemic (mild, moderate or severe) according to the recommendations of the World Health Organization (WHO), taking into consideration the variation between ages and gender (WHO, 2011b).

3.3.4 Rapid diagnosis test (RDT)

The SD Bioline® Malaria Antigen *Pf*/Pan test, product code: SD05FK63 (Standard Diagnostics, Inc., Kyonggi, Korea) that combines the detection of *Pf*HRP-2 and pLDH was used based on WHO recommendations as being one of the ten top-performing RDTs and has not been evaluated in Yemen yet (WHO, 2008). Test kits were kept at room temperature and tests were performed following the manufacturer's instructions by using 5 μL of whole blood samples. RDT results were read and interpreted within 15–30 min according to the manufacturer's instructions (Appendix 3).

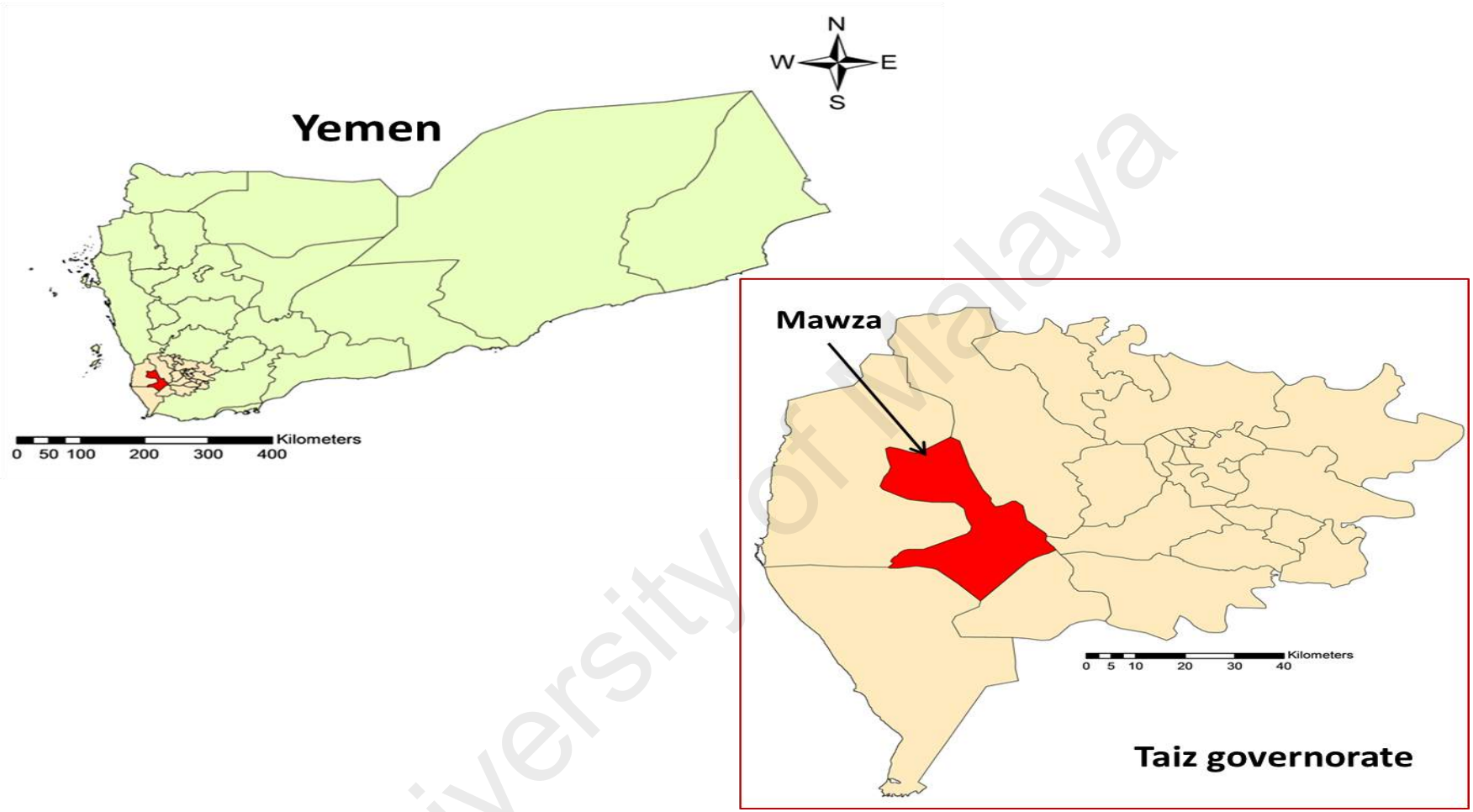


Figure 3.1: Map of Yemen showing the study area

3.4 Molecular analysis

3.4.1 DNA extraction

Parasite genomic DNA was extracted from blood spots on the filter paper. DNA was extracted using QIAgen DNA Mini Kit blood and tissue (QIAGEN, Hilden Germany) according to the manufacturer's instructions. Filter paper with blood spots were cut into smaller pieces and placed in a clean 1.5 ml microcentrifuge tube. To this, 180 µL of ATL buffer and 20 µL proteinase K were added to aid digestion of contaminating proteins as aforementioned. Then it was left to incubate at 56°C until completely lysed. The sample was vortexed occasionally during incubation. In order to ensure efficient lysis, 200 µL of AL Buffer was added to the mixture and this was left to incubate at 56°C for 10 min. To precipitate the DNA, 200 µL of absolute ethanol (96–100%) was added and was mixed thoroughly by vortexing. This mixture was then carefully applied to the DNasey Mini spin column in a 2 ml collection tube and centrifuged at 8000 rpm for 1 min. After centrifugation, the column together with the filter was discarded and the column was placed in a new 2 ml collection tube. Carefully, 500 µL of Buffer AW1 was added to the column and centrifuged for 1 min at 8000 rpm. The column then was placed in a new 2 ml collection tube. To this, 500 µL of Buffer AW2 was then added followed by a 3 min centrifugation at 14000 rpm. The DNasey Mini spin column was later placed in a clean 1.5 ml or 2 ml microcentrifuge tube. To this 50 µL of AE elution buffer was added. The column was left to stand at room temperature for 1 min before centrifugation for 1 min at 8000 rpm for elution of DNA. Extracted DNA was stored at -20°C for later use.

3.4.2 PCR for malaria detection and *Plasmodium* species identification using 18S *rRNA* gene

3.4.2.1 DNA amplification

Nested PCR assays based on the *Plasmodium* 18S *rRNA* gene were used to detect and identify *Plasmodium* species (Singh *et al.*, 1999). Nest 1 PCR was carried out using genus-specific primers (rPLU1 and rPLU5), while Nest 2 PCR was carried out using species-specific primers (FAL1 & FAL2 for *P. falciparum*; VIV1& VIV2 for *P. vivax*). The details for each primer are shown in Table 3.1. The nested PCR were carried out using GoTaq® Flexi DNA polymerase (Promega Corp., USA) with the following composition:

Reagents	**Volume (µL)**
ddH_2O	10.3
25 mM $MgCl_2$	4.0
10 mM dNTPs (2.5 mM each)	0.5
5X Go Taq® Flexi Buffer	5.0
Nest 1 F primer (rPLU1)-/Nest 2 F primer (FAL1 or VIV 1)	0.5
Nest 1 R primer (rPLU5)-/Nest 2 R primer (FAL 2 or VIV 2)	0.5
GoTaq® DNA polymerase (5 U/ µL)	0.2
DNA template/ddH_2O*	4.0
Total reaction	25.0

**Plasmodium* sp.DNA template was added into the sample reaction PCR tube, while ddH_2O was added into negative control PCR tube.

Nest 1 PCR was initiated with initial denaturing step at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min (step 2), and a final extension at 72°C for 10 min was added to the last cycle and the reaction was held at 4°C. PCR products were stored at -20°C until

analysis. Nest 2 PCR was performed under similar cycling conditions except that the annealing temperature in step 2 was increased to 58°C.

3.4.2.2 Agarose gel electrophoresis

Agarose gel, 1.5% was used for electrophoresis. Electrophoresis-grade agarose powder, 0.38 g was added into 25 ml of 1X TAE buffer. The agarose was melted in a microwave oven. Then, the melted gel was cooled to approximately 50°C under running tap water. SYBR safe DNA gel stain, 1 µL, was added to the cooled gel and the gel was poured onto a casting tray with inserted gel comb. The gel was allowed to harden for 30 min. The gel was placed in the electrophoresis tank and gel comb was removed. Sufficient 1X TAE buffer was poured into the tank until the gel was completely covered by the buffer. Gene ruler^{TM} 100 base pair (bp) DNA ladder was loaded into one of the wells for PCR product size estimation. PCR products were loaded into wells and electrophoresis was run at 100 V for 30 min. PCR products in the gel was visualized using Molecular Imager Gel Doc^{TM} XR+ system (Bio-Red Laboratories, U.S.A.).

Table 3.1: List of Oligonucleotide primers used for amplification of 18S rRNA, *Pfcrt* 76K, *pfmdr-1* 86N, *dhfr*, *dhps* and K13 genes

Genes	Purpose	Primer	Size (bp)	Sequence (5′ to 3′)	Reference
18S *Rrna*	Nest-1 PCR	rPLU1	1100	5′-TCAAAGATTAAGCCATGCAAGTGA-3′	Singh *et al.*, (1999)
		rPLU5		5′-CCTGTTGTTGCCTTAAACTCC-3′	
	Nest-2 PCR	FAL1	205	5′-TTAAACTGGTTTGGGAAAACCAAATATATT-3′	
		FAL2		5′-ACACAATGAACTCAATACTGACTACCCGTC-3′	
	Nest-2 PCR	VIV1	117	5′-CGCTTCTAGCTTAATCCA CATAACTGATAC-3′	
		VIV2		5′-ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3′	
Pfcrt 76K	Nest-1 PCR	TCRP-A	192	5'-ATGGCTCACGTTTAGGTGGAG-3'	Djimde *et al.*, (2001)
		TCRP-B		5'-CGGATGTTACAAAACTATAGTTACC-3'	
	Nest-2 PCR	TCRP-D1	145	5'-TGTGCTCATGTGTTTAAACTT-3'	
		TCRP-D2		5'-CAAAACTATAGTTACCAATTTTG-3'	
Pfmdr-1 86N	Nest-1 PCR	MDR1	603	5'-ATGGGTAAAGAGCAGAAAGA-3'	
		MDR2		5'-AACGCAAGTAATACATAAAGTC -3'	
	Nest-2 PCR	MDR3	521	5'-TGGTAACCTCAGTATCAAAGAA-3'	
		MDR4		5'-TATAACCTAAAAAGGAACTGG-3'	
K 13	Nest-1 PCR	K13-1	2097	5′-CGGAGTGACCAAATCTGGGA-3′	Ariey *et al.*, (2014)
		K13-4		5′-GGGAATCTGGTGGTAACAGC-3′	
	Nest-2 PCR	K13-2	849	5′-GCCAAGCTGCCATTCATTTG-3′	
		K13-3		5′-CGGAGTGACCAAATCTGGGA-3′	
Dhfr	Nest-1 PCR	AMP1	720	5'-TTTATATTTTCTCCTTTTTA-3'	Tinto *et al.*, (2007)
		AMP2		5'-CATTTTATTATTCGTTTTC-3'	
	Nest-2 PCR	SP1	700	5'-ATGATGGAACAAGTCTGCGAC-3'	
		SP2		5'-ACATTTTATTATTCGTTTTC -3'	
Dhps	Nest-1 PCR	O1	770	5'-GATTCTTTTTCAGATGGAGG-3'	Pearce *et al.*, (2003)
		O2		5'-TTCCTCATGTAATTCATCTGA-3'	
	Nest-2 PCR	R2	711	5′-AACCTAAACGTGCTGTTCAA-3'	Duraisingh *et al.*, (1998)
		R/		5′-AATTGTGTGATTTGTCCACAA-3'	

3.4.3 Molecular detection of K76T mutation in *Pfcrt* gene

3.4.3.1 DNA amplification

Nested PCR followed by restriction fragment length polymorphism (RFLP) was conducted according to the protocols of Djimde *et al.* (2001). Nest 1 PCR was carried out using primers TCRP-A and TCRP-B. Nest 2 PCR was carried out using primers TCRP-D1 and TCRP-D2 which cover the sequence region of the K76T mutation (Table 3.1). The nested PCR were carried out using GoTaq® Flexi DNA polymerase (Promega Corp., USA) with the following composition:

Reagents	**Volume (µL)**
ddH_2O	15.3
25 mM $MgCl_2$	2.0
10 mM dNTPs (2.5 mM each)	0.5
5X Go Taq® Flexi Buffer	5.0
Nest 1 F primer (TCRP-A)-/Nest 2 F primer (TCRP-D1)	0.5
Nest 1 R primer (TCRP-B)-/Nest 2 R primer (TCRP-D2)	0.5
GoTaq® DNA polymerase (5 U/ µL)	0.2
DNA template/ddH_2O*	1.0
Total reaction	25.0

**P. falciparum* DNA template was added into the sample reaction PCR tube, while ddH_2O was added into negative control PCR tube.

Primary PCR was initiated with denaturing step at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 1 min, extension at 65°C for 1 min, and a final extension at 65°C for 10 min was added to the last cycle and the reaction was held at 4°C. PCR products were stored at -20°C until analysis. Secondary PCR was performed under similar cycling conditions except that the annealing temperature in step 2 was increased to 50°C.

3.4.3.2 Restriction fragment length polymorphism

Restriction digestion was performed on the amplified products using restriction enzyme *Apo*I (New England Biolabs, UK) by incubating at 50°C for 20 min. *Apo*I which recognises the sequence 5'- RAATTY -3, will cut the wild type sample into two fragments but not the mutant sample. The digestion was carried out using the following reagents:

Reagents	**Volume (µL)**
ddH_2O	9.8
10X Buffer	2.0
*Apo*I	0.2
PCR product	8.0
Total reaction	20

The digested products were resolved on 2% agarose gel and agarose gel electrophoresis was performed as mentioned in section 3.4.2.2**.** Results were recorded on the Gel Doc™ XR+ system (Bio-Red Laboratories, U.S.A.).

3.4.4 Molecular detection of N86Y mutation in *Pfmdr-1* gene

3.4.4.1 DNA amplification

Nested PCR followed by restriction fragment length polymorphism (RFLP) was conducted according to the protocol of Djimde *et al.* (2001). Nest 1 PCR was carried out using primers mdr-1 and mdr-2. Nest 2 PCR was carried out using primers mdr-3 and mdr-4 which cover the sequence region of N86Y mutation (Table 3.1).

The nested PCR were carried out using GoTaq® Flexi DNA polymerase (Promega Corp., USA) with the following composition:

Reagents	**Volume (µL)**
ddH_2O	15.3
25 mM $MgCl_2$	2.0
10 mM dNTPs (0.2 mM each)	0.5
5X Go Taq® Flexi Buffer	5.0
Nest 1 F primer (mdr-1)/ Nest 2 F primer (mdr-3)	0.5
Nest 1 R primer (mdr-2)/ Nest 2 R primer (mdr-4)	0.5
GoTaq® DNA polymerase (5 U/ µL)	0.2
DNA template/ddH_2O*	1.0
Total reaction	25.0

**P. falciparum* DNA template was added into the sample reaction PCR tube, while ddH_2O was added into negative control PCR tube.

Primary PCR was initiated with denaturing step at 94°C for 3 min, followed by 45 cycles of denaturation at 92°C for 30 s, annealing at 48°C for 45 s, extension at 65°C for 1 min and a final extension at 65°C for 5 min was added to the last cycle and the reaction was held at 4°C. PCR products were stored at -20°C until analysis. Secondary PCR was performed under similar cycling conditions with only 20 cycles of PCR run in step 2.

3.4.4.2 Restriction fragment length polymorphism

Restriction digestion was performed on the amplified products using two restriction enzymes *Apo*I and *Afl*III (New England Biolabs, UK) by incubating at 37°C for 19 hours. *Apo*I which recognises the sequence 5'- RAATTY -3', cuts the wild type sample into three fragments but not the mutant sample and *Afl*III recognises the sequence 5'- ACRYGT -3', cuts the mutant type sample into two fragments but not the wild sample. The digestion was carried out using the following reagents:

Reagents	**Volume (µL)**
ddH_2O	9.8
10X Buffer	2.0
*Apo*I/*Afl*III	0.2
PCR product	8.0
Total reaction	20

The digested products were resolved on 3% agarose gel and agarose gel electrophoresis was performed as mentioned in section 3.4.2.2**.** Results were recorded on the Gel Doc™ XR+ system (Bio-Red Laboratories, U.S.A.).

3.4.5 Molecular detection of mutation in *Pfdhfr, Pfdhps* and K 13 propeller genes

3.4.5.1 Amplification of genomic DNA of *Pfdhfr* gene

The *Pfdhfr* gene was amplified using Nested PCR described by Tinto *et al.* (2007) (Table 3.1). Nest 1 and Nest 2 PCR were carried out using GoTaq® Flexi DNA polymerase (Promega Corp., USA) with the following composition:

Reagents	**Volume (µL)**
ddH_2O	15.3
25 mM $MgCl_2$	2.0
10 mM dNTPs (0.2 mM each)	0.5
5X Go Taq® Flexi Buffer	5.0
Nest 1 F primer (*dhfr-1)*/ Nest 2 F primer (*dhfr-3*)	0.5
Nest 1 R primer (*dhfr-2*)/ Nest 2 R primer (*dhfr-4*)	0.5
GoTaq® DNA polymerase (5 U/ µL)	0.2
DNA template/ddH_2O*	1.0
Total reaction	25.0

**P. falciparum* DNA template was added into the sample reaction PCR tube, while ddH_2O was added into negative control PCR tube.

Primary PCR was initiated with denaturing step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 45 s, extension at 72°C for 45 s, and a final extension at 72°C for 5 mins was added to the last cycle and the reaction was held at 4°C. PCR products were stored at -20°C until analysis. Secondary PCR was performed under similar cycling conditions except that the annealing temperature in step 2 was increased to 55°C and also extension at 74°C for 35 s and a final extension temperature increased to 74°C for 5 min. Agarose gel electrophoresis was performed as mentioned in section 3.4.2.2**.**

3.4.5.2 Amplification of *Pfdhps* gene

The *Pfdhps* gene was amplified using Nested PCR described by Duraisingh *et al.* (1998) and Pearce *et al.* (2003) (Table 3.1). Nest 1 and Nest 2 PCR were carried out using GoTaq® Flexi DNA polymerase (Promega Corp., USA) with the following composition:

Reagents	**Volume (µL)**
ddH_2O	9.6
25 mM $MgCl_2$	2.5
10 mM dNTPs (0.2 mM each)	0.5
5X Go Taq® Flexi Buffer	5.0
Nest 1 F primer (*dhps-1*)/ Nest 2 F primer (*dhps -3*)	2.5
Nest 1 R primer (*dhps -2*)/ Nest 2 R primer (*dhps -4*)	2.5
GoTaq® DNA polymerase (5 U/ µL)	0.4
DNA template/ddH_2O*	2.0
Total reaction	25.0

**P. falciparum* DNA template was added into the sample reaction PCR tube, while ddH_2O was added into negative control PCR tube.

Both primary and secondary PCR were performed under identical thermal cycling conditions: Initial denaturing at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, extension at 74°C for 1 min and a final extension at 74°C for 5 min (step 3) was added to the last cycle and the reaction was held at 4°C. PCR products were stored at -20°C until analysis. Agarose gel electrophoresis was performed as mentioned in section 3.4.2.2**.**

3.4.5.3 Amplification of K13 propeller gene

The K13 propeller gene was amplified using Nested PCR described by Ariey *et al.* (2014) (Table 3.1). Nest 1 and Nest 2 PCR were carried out using GoTaq® Flexi DNA polymerase (Promega Corp., USA) with the following composition:

Reagents	**Volume (µL) N1**	**Volume (µL) N2**
ddH_2O	10.1	9.6
25 mM $MgCl_2$	3.0	2.5
10 mM dNTPs (0.2 mM each)	0.5	0.5
5X Go Taq® Flexi Buffer	5.0	5.0
Nest 1 F primer (K13-4)/ Nest 2 F primer (K13-3)	2.5	2.5
Nest 1 R primer (K13-1)/ Nest 2 R primer (K13-2)	2.5	2.5
GoTaq® DNA polymerase (5 U/ µL)	0.4	0.4
DNA template/ddH_2O*	1.0	2.0
Total reaction	25.0	25.0

**P. falciparum* DNA template was added into the sample reaction PCR tube, while ddH_2O was added into negative control PCR tube.

Both primary and secondary PCR were performed under identical thermal cycling conditions: Initial denaturing at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s, extension at 72°C for 90 s, and a final extension at 72°C for 10 min was added to the last cycle and the reaction was held at 4°C. PCR products were stored at -20°C until analysis. Agarose gel electrophoresis was performed as mentioned in section 3.4.2.2**.**

3.4.5.4 Purification of PCR products

The PCR products were purified by using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) before being sent to a commercial laboratory for sequencing. Agarose gel electrophoresis was performed on PCR products using 0.8% agarose gel. The DNA fragment was excised from the agarose gel with a clean and sharp scalpel. The gel slice was weighed in 1.5 microcentrifuge tube. Three volumes of buffer PB was added to one volume of the gel (100 mg ~100 μL) and incubated at 50°C for 10 min (or until the gel slice has completely dissolved).

The mixture was mixed thoroughly by vortexing every 2–3 min during the incubation to aid the dissolving of gel. The mixture was pipetted into the QIAquick spin column, placed in a 2 ml collection tube (provided in the kit). The mixture was centrifuged at 13000 rpm for 1 min. The flow-through and the collection tube were discarded and the QIAquick spin column was placed in a fresh 2 ml collection tube. Buffer PE, 750 μL was added to the column and the mixture was centrifuged at 13000 rpm for 1 min. This ethanol-containing buffer acted as a wash buffer which removed any remaining contaminants. The flow-through was discarded and the column was placed back into the same collection tube and centrifuged again for 1 min. This step was necessary as it removed residual buffer PE. The QIAquick column was then placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 20 μL Buffer EB was added to the centre of the membrane and the column was left to stand for 1 min at room temperature. This was followed by a 1 min centrifugation process at 13000 rpm for elution of DNA.

Genomic DNA of *P. falciparum* strain 3D7 was used as positive control. It was provided by the Malaria Research and Reference Reagents Resources Centre (MR4, ATCC, Manassas VA, USA).

3.4.5.5 DNA sequence analysis of purified DNA

Nucleotide sequence of each PCR product was determined by a commercial laboratory (MyTACG Bioscience Enterprise, Malaysia). The nest 2 primer pair was used as sequencing primers. The deduced amino acid sequences were assessed by using BioEdit Sequence Alignment Editor.

3.5 Data analysis

The Statistical Packages for Social Sciences (SPSS) software, version 20.0 (IBM SPSS Statistics for Windows, IBM Corp., Armonk, NY, USA) was used to analyse the data. Socioeconomic status (SES) was estimated based on the principal component analysis (PCA) of house structure, durable items owned by households and the source of household drinking water. The constructed PCA-based scores of households were divided into quintiles; the lowest 40%, the middle 20% and the highest 40% and classified as being of low, middle and high SES, respectively (Vyas & Kumaranayake, 2006).

Data were presented as proportions, and the associations between the outcomes and explanatory variables were tested using Pearson's chi-square test. Odds ratio (OR) and its 95% confidence intervals (CI) were calculated whenever needed. A multivariable analysis with logistic regression model was then developed for all variables, and adjusted ORs (AORs) were reported for the independent risk factors. Differences and associations were considered statistically significant at p-values < 0.05.

For evaluating the diagnostic tests, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the RDT and microscopy were calculated against the nested PCR with their 95% confidence intervals (CIs) by using MedCalc® software (https://www. medcalc. org/ calc/ diagnostic_test; retrieved on 21 March 2015). Cohen's kappa coefficient (*Kc*) was used to assess agreement between

results obtained by two different tests (Cohen, 1960), and the strength of agreement was scaled as follows: slight (*Kc* = 0.01-0.20), fair (*Kc* = 0.21-0.40), moderate (*Kc* = 0.41-0.60), substantial (*Kc* = 0.61-0.8) or almost perfect (*Kc* = 0.81-1) (Landis, 1977).

3.10 Ethical clearance

Participation was based on voluntary basis. Each participant was asked to sign or used thumb prints on informed consent form after a clear explanation of the study objectives (Appendix 4). In the case of children, informed consent was obtained from their guardians. All positive patients for malaria were treated following the national drugs policy, Ministry of Health and Population, Yemen.

The study protocol was approved by Ministry of Health and Population, Yemen (Appendix 5) and by the ethical committee of University of Science and Technology, Yemen (Reference Number: 2013/01) (Appendix 6).

CHAPTER 4: RESULTS

4.1 Characteristics of the study population

Table 4.1 summarises sociodemographic characteristics of the participants**.** Of the 488 participants, 316 (64.8%) were females and 172 (35.2%) were males. The age range of participants was 1 to 80 years old with a median age of 26 years old (interquartile range: 22–28). Of these, 28 (5.7%) were less than 5 years old, 36 (7.4%) between 5 and 9 years, 112 (23%) between 10 and 15 years, and 312 (63.9%) above 15 years old. Majority 269 (55.1%) of the participants were uneducated. About 330 (67.6%) of them were daily labourers, 20 (4.1%) farmers, while the rest were categorized as others such as students.

4.2 Prevalence and distribution of malaria infection

4.2.1 Prevalence and distribution of overall malaria based on LM, *Pf*HRP-2/pLDH RDT and PCR-adjusted *Pf*HRP-2/pLDH RDT

Of 488 participants, LM detected malaria among 10.7 % (95% CI: 8.22–13.71). Of these infected participants, 9.6 % (5/52) had low parasitaemia, 32.7 % (17/52) had moderate parasitaemia, 42.3 % (22/52) had high parasitaemia, and 15.4 % (8/52) showed only gametocytes. In terms of infecting species, 96.2% (50/52) were due to *P. falciparum* and 3.8% (2/52) due to *P. vivax*.

The prevalence based on *Pf*HRP-2/pLDH RDT was 32.8% (32.1 and 37.5% for ≥ 10 and <10 years old, respectively). The prevalence among children < 10 years old was four times higher with the RDT than with LM. Asymptomatic malaria among participants was found to be 1.6% with LM compared with 25.6% with RDT. However, 88.2% and 94.1% of infection with malaria were found among patients who reported fever in the 48 h prior to the survey by LM and *Pf*HRP-2/pLDH RDT, respectively (Table 4.2). After excluding cases positive by *Pf*HRP-2/pLDH RDT and negative by

PCR, the overall PCR-adjusted RDT-based malaria prevalence among the residents of Mawza District was 25.5% (95% CI: 21.84 –29.77) (Table 4.2).

4.2.2 Prevalence and distribution of microscopic and sub-microscopic malaria infections

Sub-microscopic malaria as detected by PCR-adjusted RDT was (15.0%; 95% CI: 12.81–19.28) compared to of infections being microscopic (10.7%; 95% CI: 8.22–13.71). Microscopic and sub-microscopic malaria rates were significantly higher among males compared to females, (15.7 *vs*. 7.9%) and (20.3 *vs.* 12.0%), respectively. However, the male gender was an independent risk factor for sub-microscopic malaria only as revealed by multivariable analysis. No significant difference in the distribution of microscopic and sub-microscopic malaria according to age (Table 4.3).

Microscopic malaria rates were higher among febrile patients compared to non-febrile participants (88.2 *vs.* 1.2%). Although bivariate analysis showed that sub-microscopic infection was significantly associated with non-febrile participants and those with past history of malaria or previous antimalarial drug intake, multivariable analysis identified only non-febrile infection as independent factor associated with sub-microscopic malaria (Table 4.3). All microscopy-confirmed malaria cases were anaemic. However, there was no significant association between sub-microscopic malaria and anaemia.

Table 4.1: Sociodemographic characteristics of the study participants (n = 488)

Variable		n (%)
Age (years)	< 5	28 (5.7)
	5–9	36 (7.4)
	10–15	112 (23.0)
	>15	312 (63.9)
Gender	Female	316 (64.8)
	Male	172 (35.2)
Family size (members)	≤ 5	203 (41.2)
	> 5	285 (58.8)
Education	Educated	219 (44.9)
	Uneducated	269 (55.1)
Occupation	Government employee	104 (21.3)
	Daily labourer	330 (67.6)
	Farmer	20 (4.1)
	Others	34 (7.0)
Economic status	Houses with electricity	182 (37.3)
	Availability of TV/radio	286 (58.6)
	Availability of telephone	223 (45.7)
	Availability of washing machine	33 (6.8)
	Availability of fridge	47 (9.6)
	Having motorcycle/car	102 (20.9)

Table 4.2: Prevalence of malaria based on LM, PfHRP-2/pLDH RDT and PCR –adjusted PfHRP-2/pLDH RDT screening among the study participants

		LM		*Pf*HRP-2/pLDH RDT		PCR –adjusted *Pf*HRP-2/pLDH RDT	
	No	**n**	**% (95% CI)**	**n**	**% (95% CI)**	**N**	**% (95% CI)**
Overall	488	52	10.7 (8.2-13.7)	160	32.8 (28.8-37.1)	124	25.5 (21.6-29.5)
Age (years)							
≥10	424	46	10.8 (8.2-14.2)	136	32.1 (27.8-36.7)	106	15.0 (20.9-29.4)
<10	64	6	9.4 (4.4-19.0)	24	37.5 (26.7-49.8)	19	29.7 (18.9-42.4)
Fever 48 hours prior to screening							
Yes	51	45	88.2 (76.6-94.5)	48	94.1 (84.1-98.0)	48	94.1 (84.1-98.0)
No	437	7	1.6 (1.0-3.3)	112	25.6 (21.8-29.9)	77	17.6 (14.2-21.5)
History of anti-malarial drug intake one week prior to the survey							
Yes	157	20	13.0 (9.0-19.0)	76	48.0 (41.0-56.0)	55	35.0 (27.6-43.0)
No	331	32	10.0 (7.0-13.0)	84	25.0 (21.0-30.0)	70	21.1 (16.9-25.9)

n, number positive; CI, confidence interval; LM, light microscopy; *Pf*HRP-2, *P. falciparum* histidine-rich protein-2; pLDH, *Plasmodium* lactate dehydrogenase; RDT, rapid diagnostic test

Table 4.3: Prevalence of microscopic and sub-microscopic malaria infections among rural populations in relation to certain population characteristics

Variable		Microscopic malaria			Sub-microscopic malaria		
	N	***n* (%)**	**OR** (95% CI)	**AOR** (95% CI)	***n* (%)**	**OR** (95%CI)	**AOR** (95% CI)
Gender							
Female	316	25 (7.9)	Reference		38 (12.0)	Reference	
Male	172	27 (15.7)	2.17 (1.21 – 3.87)*	2.15 (0.55 – 8.39)	35 (20.3)	1.87 (1.13–3.09)*	2.1 (1.24 – 3.48) *
Age (years)							
>15	312	35 (11.2)	0.76 (0.25 – 2.31)	0.20 (0.03 – 1.28)	45 (14.4)	1.01 (0.34 – 3.05)	1.48 (0.46 – 4.78)
11-15	99	7 (7.1)	0.46 (0.12 – 1.69)	0.08 (0.01 – 1.05)	13 (13.1)	0.91 (0.27 – 3.04)	1.09 (0.31 – 3.84)
6-10	49	6 (12.2)	0.84 (0.22 – 3.26)	0.13 (0.01 – 3.06)	11 (22.4)	1.74 (0.50 – 6.08)	2.31 (0.64 – 8.38)
≤ 5	28	4 (14.3)	Reference		4 (14.3)	Reference	
Fever (> 37.5^{o}C)							
Yes	51	45 (88.2)	Reference		3 (5.9)	Reference	
No	437	7 (1.2)	0.12 (0.06 – 0.25)*	0.001 (0.00–0.01)*	70 (16.0)	1.12 (1.04–1.21)*	4.49 (1.31–15.35)*
Anaemia status							
Mild/ moderate	334	48 (14.4)	NA	NA	52 (15.6)	1.24 (0.70 – 2.21)	2.68 (0.60 – 11.85)
Severe	15	4 (26.7)	NA	NA	3 (20.0)	1.68 (0.43 – 6.54)	1.36 (0.74 – 2.50)
No	139	00 (00.0)	Reference		18 (12.9)	Reference	
Past history of malaria							
Yes	164	21 (12.8)	1.39 (0.77 – 2.50)	0.83 (0.01–136.05)	34 (20.7)	1.91 (1.15 – 3.17)*	1.21 (0.21 – 7.17)
No	324	31 (9.6)	Reference	Reference	39 (12.0)	Reference	
History of antimalarial drug intake							
Yes	169	22 (13.0)	1.4 (0.80 – 2.59)	1.03 (0.01–165.7)	35 (20.7)	1.93 (1.17 – 3.20)*	1.67 (0.28 – 9.82)
No	319	30 (9.4)	Reference	Reference	38 (11.9)	Reference	

4.3 Factors associated with overall malaria detected by PCR-adjusted *Pf*HRP-2/pLDH RDT

Among the sociodemographic characteristics, gender was significantly associated with malaria in the rural populations of Mawza, where males showed about twice the risk of being infected with malaria compared to females (OR = 2.26; 95% CI: 1.49–3.43). On the other hand, absence of window screens in houses (OR = 2.74; 95% CI: 1.67–4.21) and presence of uncovered water near houses (OR = 2.18; 95% CI: 1.15–4.13) were significantly associated with malaria. Multivariable analysis showed that male (AOR = 2.00; 95% CI: 1.29–3.12), absence of window screens (AOR = 3.16; 95% CI: 1.86–5.37) and keeping uncovered water near houses (AOR = 2.67; 95% CI: 1.27–5.62) were independent risk factors for being infected with malaria (Table 4.4). All participants reported that they had neither slept under ITNs during the night preceding the survey nor sprayed residual insecticides inside their houses during the last year (Data were not presented in Table 4.4 because of the lack of "yes" responses during all interviews).

Table 4.4: Factors associated with malaria detected by PCR –adjusted PfHRP-2 /pLDH RDT among rural populations

Variable	*N*	n (%)	OR (95% CI)	AOR (95% CI)
Gender				
Female	316	63 (19.9)	Reference	
Male	172	62 (36.0)	2.26 (1.49–3.43)	2.00 (1.29–3.12)*
Age (years)				
>15	312	80 (25.6)	Reference	
11-15	99	20 (20.2)	0.73 (0.42–1.28)	0.63 (0.33–1.20)
6-10	49	17 (34.7)	1.54 (0.81–2.92)	1.44 (0.69–2.99)
≤ 5	28	8 (28.6)	1.16 (0.49–2.74)	1.23 (0.48–3.19)
Education level				
Secondary school or above	113	29 (25.7)	Reference	
Primary school	106	31 (29.2)	1.84 (0.72–4.62)	1.12 (0.57–2.21)
Uneducated	269	65 (24.2)	1.71 (0.76–3.85)	0.87 (0.45–1.68)
Occupation of the head of household				
Government employee	77	19 (24.7)	Reference	
Daily labourer	302	79 (26.2)	1.10 (0.61–1.93)	0.96 (0.51–1.82)
Farmer	20	9 (45.0)	2.50 (0.10–6.94)	2.10 (0.67– 6.58)
Other	69	13 (18.8)	0.71 (0.32–1.57)	0.62 (0.26–1.47)
Not working	20	5 (25.0)	1.02 (0.33– 3.17)	0.87 (0.26–2.93)
Family size (members)				
≤5	203	50 (24.6)	Reference	
>5	285	75 (26.3)	1.09 (0.72–1.65)	1.04 (0.66–1.63)
Socioeconomic status				
High	98	19 (19.4)	Reference	
Middle	195	54 (27.7)	1.59 (0.88–2.88)	1.66 (0.85–3.23)
Low	195	52 (26.7)	1.51 (0.84– 2.74)	1.73 (0.92–3.25)
Having window screens				
Yes	167	24 (14.4)	Reference	
No	321	101 (31.5)	2.74 (1.67-4.21)	3.16 (1.86–5.37)*
Presence of uncovered water near houses				
No	444	107 (24.1)	Reference	
Yes	44	18 (40.9)	2.18 (1.15-4.13)	2.67 (1.27–5.62)*

N, number examined; n, number infected; OR, odds ratio; AOR, adjusted odds ratio; CI, confidence interval; * statistically significant.

4.4 Knowledge, attitude and practices (KAP) toward malaria

Table 4.5 shows the results of KAP analysis in the study populations. The majority [316 (93.5%)] of the participants attributed the cause of malaria to mosquito bites. However, only 151 (44.7%) of them believed that sleeping under mosquito nets would protect against malaria. Thirty-four (10.1%) of the participants stated that elimination of breeding sites would protect against malaria, whereas 24 (7.1%) stated that house spraying with insecticide was a method of prevention. All participants reported that they did not use ITNs, and IRS was not conducted one year prior to the survey.

4.5 Fever treatment-seeking behaviours among people of rural Mawza

Of the 177 participants reporting fever in the last two weeks preceding the survey, the majority of participants (94.9%) reported home management of fever before seeking treatment at clinics or healthcare facilities.

Application of cold compresses was the most frequent home remedy of fever, being practiced by about 90.0% of feverish participants. This is very high compared to antimalarial drug intake, either single (5.4%) or in combination with antipyretics (4.8%). On the other hand, the vast majority of patients (98.9%) sought treatment at healthcare facilities, but the majority mentioned that they stayed at home for more than 24 hours before seeking treatment at the facilities. Delayed fever treatment in healthcare facilities for more than 24 hours was reported by 97% of the participants. No significant difference was seen in the prevalence of malaria between participants with correct knowledge and those with incorrect knowledge toward malaria. It is noteworthy that 96.6% of those seeking antimalarial treatment for fever were laboratory-confirmed malaria cases (Table 4.6).

Table 4.5: Knowledge, attitudes and practices of rural populations toward malaria during the transmission season

Variable	n (%)
Knowledge about malaria (*n* = 402)	
Correct knowledge about malaria	
Know malaria	338 (84.1)
Modes of transmission mentioned (*n* = 338)	
Mosquito bite	316 (93.5)
Symptoms of malaria mentioned (*n* = 338)	
Fever	334 (98.8)
Convulsion	281 (83.1)
Shivering	320 (94.7)
Sweating	306 (90.5)
Headache	326 (96.5)
Methods of prevention mentioned (*n* = 338)	
Sleeping under mosquito nets	151 (44.7)
Elimination of breeding sites	34 (10.1)
House spraying with insecticides	24 (7.1)
Taking antimalarial drugs	124 (36.7)
Incorrect knowledge about malaria	
Modes of transmission mentioned (*n* = 338)	
Bad sewage	22 (6.5)
Not having or delay the breakfast	15 (4.4)
Sleeping with infected person in the same bed	293 (86.7)
Breastfeeding	8 (2.4)
Attitudes toward malaria (*n* = 338)	
Positive attitudes	
Malaria is more serious for children than adults	5 (1.5)
Negative attitudes	
Malaria is more serious for adults than children	3 (0.9)
Malaria is equally serious for adults and children	321 (95)
I don't know	9 (2.7)
Good practices in respect with malaria (*n* = 488)	
Sleeping under ITNs the last night prior to the survey	0 (0)
Insecticide residual spray in the last year prior to the survey	0 (0)
Using window screens	167 (34.2)

*Only study participants older than 12 years old were interviewed for knowledge and attitudes. Sample size of variables was not equal.

Table 4.6: Fever treatment-seeking behaviours among participants who had fever in the last two weeks preceding the present survey

Behaviour	n (%)
Management of fever at home before visiting a clinic or a health facility	
Yes	168 (94.9)
No	9 (5.1)
Methods of home management of fever (n = 168)	
Applying a cold compress	151 (89.8)
Taking an antimalarial drug only	9 (5.4)
Taking an antipyretic in addition to an antimalarial drug	8 (4.8)
Seeking fever treatment at health facilities	
Yes	175 (98.9)
No	2 (1.1)
Delay in seeking fever treatment (hours) (n = 168)	
≤ 24	5 (3)
> 24	163 (97)
Seeking malaria treatment after laboratory diagnosis (n = 175)	
Yes	169 (96.6)
No	6 (3.4)

4.6 Evaluation of rapid diagnostic test and microscopy against nested PCR

4.6.1 Comparison between *Pf*HRP-2/pLDH RDT and LM

Table 4.7 shows that *Pf*HRP-2/pLDH RDT detected all LM-positive cases of *falciparum* malaria, irrespective of parasite density. Of the LM-negative samples, 108 (24.6%) were RDT-positive. RDT and LM showed fair agreement (77.9%; *Kc* = 0.379, $p < 0.001$) for the detection of *P. falciparum* among all participants. The two tests had substantial agreement (94.0%; *Kc* = 0.638, $p < 0.001$) for detecting the infection among febrile patients.

4.6.2 Sensitivity, specificity, positive, and negative predictive values of *Pf*HRP-2/pLDH RDT against nested PCR

Table 4.8 summarizes the evaluation of *Pf*HRP-2/pLDH RDT against nested PCR. In comparison with nested PCR, the *Pf*HRP-2/pLDH RDT had a sensitivity of 96.0% (95% CI: 90.9-98.3) and specificity of 56.0% (95% CI: 44.7-66.8), PPV of 76.3% (95% CI: 69.0-82.3), and NPV of 90.4% (95% CI: 78.8-96.8). The two types of tests showed a moderate degree of agreement (79.8%; *Kc* = 0.553, $p < 0.001$). In addition, the RDT maintained its high sensitivity for the detection of *P. falciparum* among children < 10 years old, asymptomatic participants and those with history of anti-malarial drug intake. However, it showed low specificity, which dropped to about 30% among people with history of anti-malarial drug intake.

Table 4.7: Comparison between *Pf*HRP-2/pLDH RDT and LM for detecting *Plasmodium falciparum* malaria

		LM				
	RDT	**Positive**	**Negative**	**Total**	**% Agreement (*Kc*)**	***p* value**
Overall	Positive	52	108	160	77.9 (0.390)	< 0.001
	Negative	0	328	328		
	Total	52	436	488		
Febrile	Positive	45	3	48	94.0 (0.638)	< 0.001
	Negative	0	3	3		
	Total	45	6	51		
Afebrile	Positive	7	105	112	76.0 (0.090)	< 0.001
	Negative	0	325	325		
	Total	7	430	437		

*Pf*HRP-2, *P. falciparum* histidine-rich protein-2; pLDH, *Plasmodium* lactate dehydrogenase; RDT, rapid diagnostic test; LM, light microscopy; *Kc*, Cohen's kappa coefficient; % agreement was calculated by summation of the number of positives and negatives by both RDT and LM divided by the total number of cases.

4.6.3 Sensitivity, specificity, positive, and negative predictive values of LM against nested PCR

Table 4.5 gives the evaluation of LM against nested PCR. In comparison with nested PCR, LM had a sensitivity of 37.6% (95% CI: 29.6-46.3), specificity of 97.6% (95% CI: 91.7-99.7), PPV of 95.9 (95% CI: 86.3-98.9), and NPV of 51.3% (95% CI: 43.2-59.2). The two types of tests showed fair degree of agreement (61.7%; *Kc* = 0.307, p <0.001). A reduction in LM sensitivity was observed among children < 10 years old, asymptomatic participants and those with history of anti-malarial drug intake. Although LM showed high sensitivity (93.0%) for detecting symptomatic malaria, such sensitivity dropped to 8.5% in the case of asymptomatic malaria. On the other hand, LM maintained its high specificity for the detection of *P. falciparum.*

Table 4.8: Sensitivity, specificity, PPV and NPV of *Pf*HRP-2/pLDH RDT for detecting *P. f*alciparum against nested PCR as a reference method

	PCR+ve RDT+ve	PCR+ve RDT-ve	PCR-ve RDT-ve	PCR-ve RDT+ve	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	% Agreement* (*Kc*)
Overall	119	5	47	37	96.0 (90.9-98.3)	56.0 (44.7-66.8)	76.3 (69.0-82.3)	90.4 (78.8-96.8)	79.8 (0.553)
Age (years) (n = 208)									
≥ 10	100	5	41	32	95.2 (89.3-97.9)	56.2 (44.1-67.8)	75.8 (67.8-82.3)	89.1 (76.4-96.4)	79.2 (0.545)
< 10	19	0	6	5	100 (82.4-100)	54.6 (23.4-83.3)	79.2 (57.9-92.9)	100 (54.1-100)	83.3 (0.603)
Fever 48 hours prior to survey (n = 208)									
Yes	42	0	2	2**	100 (92.3-100)	50.0 (6.8-93.2)	95.5 (84.9-98.7)	100 (15.8-100)	95.3 (0.646)
No	77	5	45	35	93.9 (86.3- 98.0)	56.3 (44.7- 67.3)	68.8 (59.3-77.2)	90.0 (78.2-96.7)	75.3 (0.504)
History of anti-malarial drug intake one week prior to the survey (n = 207)									
Yes	52	2	9	21	96.3 (87.5-99.0)	30 (14.7-49.4)	71.2 (60.0-80.3)	81.8 (48.2-97.7)	72.6 (0.306)
No	66	3	38	16	95.7 (88.0-98.5)	70.4 (57.2-80.9)	80.5 (70.6-87.6)	92.7 (80.6-97.5)	84.6 (0.678)

CI, confidence interval; *Pf*HRP-2, *P. falciparum* histidine-rich protein-2; pLDH, *Plasmodium* lactate dehydrogenase; RDT, rapid diagnostic test; PCR, polymerase chain reaction; PPV, positive predictive value; NPV, negative predictive value; *Kc*, Cohen's kappa coefficient; % agreement was calculated by summation of the number of positives and negatives by both RDT and PCR divided by the total number of cases.* The agreement between RDT and PCR was significant for all categories with $p<0.001$; **Parasite densities of these two cases were 80 parasites and 400 parasites/ µL.

Table 4.9: Sensitivity, specificity, PPV and NPV of LM for detecting *P. falciparum* against nested PCR as a reference method

	PCR+ve LM+ve	PCR+ve LM-ve	PCR-ve LM-ve	PCR-ve LM+ve	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	% Agreement* (*Kc*)
Overall	47	78	82	2**	37.6 (29.6-46.3)	97.6 (91.7-99.7)	95.9 (86.3-98.9)	51.3 (43.2 - 59.2)	61.7 (0.307)
Age(years) (n = 209)									
≥10	41	65	71	2	38.7 (30.0-48.2)	97.3 (90.5-99.7)	95.3 (84.5-98.7)	52.2 (43.5 -60.8)	62.6 (0.329)
<10	6	13	11	0	31.6 (12.6-56.6)	100 (71.5-100)	100 (54.1-100)	45.8 (25.6 - 67.2)	56.7 (0.253)
Fever 48 hours prior to study(n = 209)									
Yes	40	3	2	2	93.0 (81.4-97.6)	50.0 (6.8-93.2)	95.2 (84.2-98.7)	40.0 (5.3- 85.3)	89.4 (0.386)
No	7	75	80	0	8.5 (3.5-16.8)	100 (95.5-100)	100 (59.0-100)	51.6 (43.5-59.7)	53.7 (0.084)
History of anti-malarial drug intake one week prior to the survey (n = 208)									
Yes	19	37	30	0	33.9 (22.9-47.0	100 (88.4-100)	100 (83.2-100)	44.8 (33.5-56.6)	57.0 (0.264)
No	28	40	52	2	41.2 (30.3-53.0)	96.3 (87.5-99)	93.3 (78.7-98.2)	56.5 (46.3-66.2)	75.6 (0.349)

CI, confidence interval; PCR, polymerase chain reaction; LM, light microscopy; PPV, positive predictive value; NPV, negative predictive value; *Kc*, Cohen's kappa coefficient; % agreement was calculated by summation of the number of positives and negatives by both LM and PCR divided by the total number of cases. * The agreement between LM and PCR was significant in all categories with $p < 0.05$. **Parasite densities of these two cases were 80 parasites and 400 parasites/ µL.

4.7 Analysis of molecular markers associated with *P. falciparum* antimalarial drugs resistance

4.7.1 18S *rRNA* gene detection and identification by nested PCR

A total of 212 blood specimens were examined by nested PCR. These included 160 specimens which were positive by RDT, 52 included RDT positives by LM, and 52 negative controls selected randomly to evaluate RDT and LM. According to Singh *et al.* (1999), Nest 2 genus specific primers would generate amplicon size of 205 bp and 117 bp, *P. falciparum* (Appendix 7) and *P. vivax* (Appendix 8) respectively.

Of the 212 blood specimens examined by nested PCR, 127 were positive for *P. falciparum.* Majority of the positive RDT specimens were found to be positive for *P. falciparum* (122/160) by nested PCR. Five samples that were negative by RDT and LM (5/52) were positive by nested PCR. Two PCR-negative samples were positive by LM and RDT.

4.7.2 Molecular markers of antimalarial drugs resistance

4.7.2.1 *Plasmodium falciparum* chloroquine resistance (*Pfcrt* &*Pfmdr-1)*

Fifty falciparum positive samples were selected for detection of single nucleotide polymorphism (SNPs) at codon K76T of the *Pfcrt* gene and codon N86Y of the *Pfmdr1*gene. The *pfcrt* gene PCR product (145 bp) was digested by restriction enzyme *Apo*I and the digested products were resolved on 2% agarose gel. *Apo*I digests the *pfcrt* wild-type allele K76 but not the CQ resistant mutant allele T76 (Appendix 9) While *pfmdr-1* (521 bp) was digested with RE *Afl*III and *Apo*I. *Afl*III was unable to digest the *pfmdr-1* mutant allele Y86, whereas *Apo*I digested the wild type *pfmdr-1* allele N86 and generated two fragments of 299 bp and 222 bp. The digested products were resolved on 3% agarose gel (Appendix 10).

4.7.2.2 *Plasmodium falciparum* Sulphadoxine/Pyrimethamine resistance (*Pfdhfr &Pfdhps)*

Fifty falciparum positive samples by microscopy and nested PCR were selected for amplification and sequencing analysis of *dhfr and dhps.* Fourty five were successfully amplified with PCR size product of 700 bp, 711 bp respectively (Appendix 11) and (Appendix 12).

The *dhfr* sequences of *P. falciparum* were multiple-aligned against the nucleotide sequence of *dhfr* of *P. falciparum* (GenBank Accession number XM_001351443.1) and the sequences of *dhps* were also multiple-aligned against the nucleotide sequence of *dhps* of *P. falciparum* (GenBank Accession number XM_001349382.1).

4.7.2.3 *Plasmodium falciparum* artemisinine resistance Kelch13

Out of 50 falciparum positive samples by microscopy, 47 were successfully amplified with PCR size product of 849 bp. The product was sequenced for the propeller region of the gene (Appendix 13).

The 47 K13 sequences were multiple-aligned against the nucleotide sequence of K13 of *P. falciparum* (GenBank Accession number XM_001350122.1).

4.7.2.4 Prevalence of *pfcrt* 76T, *pfmdr* 86Y, K13, *pfdhfr* and *pfdhps* mutations

Table 4.10 shows the prevalence of *pfcrt* 76T, *pfmdr* 86Y, K13, *pfdhfr* and *pfdhps* mutations. All the *pfcrt* gene of the isolates was found to carry the mutant allele 76T. In contrast, all the *Pfmdr-1* gene of the isolates was of the N86 wild type allele.

As for K13 polymorphism, codons associated with artemisinin resistance (Y493H, R539T, I543T and C580Y) were investigated. All isolates were observed to have wild type alleles, with no polymorphism in this region of the gene.

Molecular markers for SP resistance were investigated by examining four codons of the *pfdhfr* gene (N51I, C59R, S108N and I164L) and five codons of the *pfdhps* gene (S436A, A437G, K540E, A581G and A613T). Sequencing of the *pfdhfr* gene was successful in 45/50 (90.0%) of the isolates. Of the total, one isolate was found to have double mutant, ICNI (amino acids at positions 51 and 108, mutations underlined), whereas the other isolates had the wild type NCSI (Table 4.10).

Fourty five (45) of 50 *pfdhps* gene isolates (90.0%) were successfully amplified and sequenced. No mutation was observed at all codons and the wild type SAKAA genotype was common in the area (Table 4.10).

Table 4.10: Prevalence of *pfcrt* 76T, *pfmdr* 86Y, *pfK*13, *pfdhfr and pfdhps* mutations

Gene	Loci	Amino acid		
		Wild /Mutant	Wild type (%)	Mutant type (%)
Pfcrt (n=50)				
	76	K/T	0 (00)	50 (100)
Pfmdr 1 (n=50)				
	86	N/Y	50 (100)	0 (00)
K13 (*n= 47)				
	493	Y/H	47 (100)	0 (00)
	539	R/T	47(100)	0 (00)
	543	I/T	47 (100)	0 (00)
	580	C/Y	47(100)	0 (00)
dhfr (*n= 45)				
	51	N/**I**	44 (97.8)	1 (2.2)
	59	C/R	45 (100)	0 (00)
	108	S/**N**	44 (97.8)	1 (2.2)
	164	I/L	45 (100)	0 (00)
dhps (*n= 45)				
	436	S/A	45 (100)	0 (00)
	437	A/G	45 (100)	0 (00)
	540	K/E	45 (100)	0 (00)
	581	A/G	45 (100)	0 (00)
	613	A/S	45 (100)	0 (00)

*n = Number of successful amplified and sequenced genes

CHAPTER 5: DISCUSSION

5.1 Prevalence and distribution of malaria

5.1.1 Prevalence and distribution of overall malaria

In the present study, the prevalence of malaria was estimated using LM and *Pf*HRP-*2/*pLDH RTD. The overall prevalence of malaria was three times higher when using *Pf*HRP-2/pLDH RDT compared with LM and 16 times higher among asymptomatic patients. In this context, Mappin *et al.* (2015) reported a strong, non-linear relationship between malaria prevalence rates derived from the LM and RDTs. Higher RDT-based prevalence rates were also reported from Ethiopia and Tanzania, being two and three times higher than those by LM, respectively (Golassa *et al.,* 2013; Mwingira *et al.,* 2014). The better performance of RDTs over LM in field surveys has also been reported from the Brazilian Amazon (Andrade *et al.,* 2010) and Angola (Fançony *et al.*, 2013). Although the sensitivity of RDTs may, to some extent, reflect the true prevalence of symptomatic as well as asymptomatic malaria that cannot be detected by LM in the field, the prevalence estimates by RDT need to be standardized if they are to be used for epidemiological purposes, such as mapping (Mappin *et al.,* 2015) due to the false positive results (Hastings &Watkins, 2005).

In a country with limited resources, it would be rather difficult to use PCR as a screening tool for malaria prevalence. Therefore, in addition to the detection of microscopic infections by LM, the present study adopted a cost-effective approach to further investigate RDT-positive cases by PCR in an attempt to rule out its false-positivity disadvantage and to adjust the estimate of malaria burden after excluding false positives. This approach was called PCR-adjusted *Pf*HRP-*2/*pLDH RTD, by which the malaria prevalence was reduced by (6.9%) compared to the RDT-based prevalence and increased by (15.0%) compared to the LM-based prevalence. It has been well documented that all RDT-positive malaria that are confirmed by PCR are true malaria

(Bell *et al.,* 2005). Similar PCR-corrected RDT approach has been recommended by Gitonga *et al.* (2012) for school malaria surveys in low transmission settings, where PCR is performed on all RDT-positive samples and on pools of five RDT-negative samples. It is noteworthy that prevalence of malaria as estimated by the adopted approach is not comparable with previous LM-based prevalence in Yemen but confirms the classification of the study area as high transmission area (NMCP, 2013).

5.1.2 Prevalence and distribution of microscopic malaria compared to sub-microscopic malaria

In Yemen, the burden of sub-microscopic malaria infections has not been assessed in a way that could reflect the impact of the continuing efforts to eliminate the disease because diagnosis relies mainly on LM and/or *Pf*HRP/pLDH RTDs. Although LM and RDTs could have a role in malaria surveillance for the assessment of control interventions, detection of sub-microscopic infections using tools of higher detection limits becomes a pressing need for assessing malaria elimination (Zimmerman & Howes, 2015; Ferreira & Castro, 2016). It would be impractical to regularly screen for malaria in mass screening surveys in endemic areas of Yemen using PCR. The PCR-adjusted *Pf*HRP/pLDH RTD approach adopted the use of PCR for the detection of malaria parasites among RDT-positive cases to exclude RDT false positivity. This approach seems to be cost-effective and practical.

Of an overall rate of (25.5%) of malaria among the rural populations of Mawza, sub-microscopic infections represented the majority of cases (15.0%). This could partly be a consequence of the interventions of case treatment and mass distribution of ITNs as part of the efforts devoted by the NMCP. Although such interventions reduce the infectious parasite reservoir, they may lead to a shift towards the sub-microscopic parasite population (Gerardin *et al.*, 2015).

In the present study, the majority of participants with sub-microscopic infections were asymptomatic, with a significant association between sub-microscopic malaria and absence of fever. In Hadhramout, a coastal governorate southeast of Yemen, more than half of malaria-positive blood smears were found to be afebrile with low parasites densities (Bamaga *et al.,* 2014). It is noteworthy that acquired immunity against malaria in endemic areas may significantly reduce malaria parasite densities, increasing the proportion of sub-microscopic and asymptomatic infections (Drakeley *et al.,* 2005; Okell *et al.,* 2012). Therefore, asymptomatic participants harbouring sub-microscopic infections can represent a major reservoir for the on-going transmission of disease in such situations, particularly in areas of low endemicity (Lindblade *et al.,* 2013; Bousema *et al.,* 2014; Lin *et al.,* 2014), because these patients are not usually diagnosed or treated. Moreover, there is evidence that sub-microscopic infections can efficiently initiate gametocyte carriage and induce infectivity of mosquitoes (Schneider *et al.,* 2007; Ouedraogo *et al.,* 2009; Okell *et al.,* 2012). The present study reported a microscopy-positive rate of (10.7%). Individuals with sub-microscopic infections may represent an important hidden reservoir for sustaining malaria transmission, particularly in settings where the microscopy-positive rate is less than 10–20% (Okell *et al.,* 2012).

Although all microscopic malaria cases in this study were anaemic, there was no significant difference in the prevalence of sub-microscopic malaria between anaemic and non-anemic participants. This finding is concurrent with several previous studies that reported significant association between microscopic malaria and anaemia among different population categories in Yemen (Elbadr *et al.,* 2010; Abdulsalam *et al.,* 2010; Bamaga *et al.,* 2014). However, this is the first study in Yemen to report the occurrence of anaemia with respect to both microscopic and sub-microscopic infection. In contrast, sub-microscopic malaria infections have been associated with anaemia among children (Gudo *et al.,* 2013; de Mast *et al.,* 2015) and pregnant women (Cottrell *et al.,* 2015).

Above all, a recently published study concludes that malaria parasites at any densities have serious manifestations, including anaemia, and that asymptomatic malaria is a chronic and debilitating condition that requires treatment (Chen *et al.,* 2016).

On the other hand, several previous studies revealed that prevention and control interventions, such as the combination of ITNs and chemoprophylaxis, could lead to the reduction of anaemia among children in malaria endemic areas (D'Alessandro *et al.,* 1995; Shiff *et al.,* 1996; Korenromp *et al.,* 2006; Kirby *et al.,* 2006), therefore reflecting the need for determination of anaemia status among those sub-microscopic carriers not only to avoid undesirable consequences but also to assess the impact of on-going interventions on malaria-related anaemia. In this context, there is a pressing need for deploying highly sensitive and specific molecular techniques to detect sub-microscopic infections in low transmission areas following control and elimination interventions. However, this may not be possible in resource-limited countries like Yemen. Therefore, the cost-effective approach of PCR-adjusted RDT-screening approach adopted in the present study could be one possible alternative that requires further investigation in large-scale studies.

The finding that past history of malaria and previous antimalarial drug intake were significant predictors of sub-microscopic infections in the present study is crucial. This is because ineffective antimalarial drugs, due to either incomplete treatment courses or antimalarial resistance to the drug, may lead to persistent sub-microscopic infections (Roper *et al.,* 1996). This is particularly important with the widespread practice of self-medication in malaria-endemic areas of Yemen for several reasons, including the cost and the lack of confidence in health services (Abdo-Rabbo, 2003; Al-Maktari & Bassiouny, 2003). Moreover, physicians may not adhere to the national guidelines recommended by the NMCP, leading to the prescription of antimalarial

which are not effective in clearing parasitaemia and to the persistence of chronic, sub-microscopic infections.

5.2 Factors associated with malaria detected by PCR–adjusted *Pf*HRP-2/pLDH RDT

Socio-economic and environmental factors could be significant barriers of effective malaria control. Thus, this study aimed at identifying factors associated with malaria. In this study, gender was a predictor of malaria among rural residents in Mawza, where males were about twice more likely to be infected with malaria than females. This may reflect the higher exposure to mosquito bites due to the more frequent outdoor activities of the male population at night. Furthermore, males usually sleep outdoors without enough protective clothing during the hot season of malaria transmission. This is in contrast to the finding of a previous study from Hadhramout, where no significant association was found between gender and malaria infection (Bamaga *et al.*, 2014).

In the present study, however, no association was found between age and malaria. This is in contrast to a previous finding by Abdulsalam *et al.*, (2010), who reported that severe malaria among febrile patients with microscopy-confirmed malaria in Yemen was age-dependent, being more severe among young children. This could be explained by the fact that patients recruited in the latter study were symptomatic and that malaria detection was by LM.

Okell *et al*. (2012) reported common carriage of sub-microscopic infections by adults in low-endemicity settings. The detection of malaria parasites in blood films decreases with age as a result of cumulative exposure and acquired immunity (Sama *et al.,* 2006; Okell *et al.,* 2012). Nevertheless, the age-stratified distribution of malaria needs further analysis that should take into account the changing patterns of age-related malaria epidemiology and severity as a result of the implementation of control

interventions. In addition, the association of age with malaria in the present study may be unlike that in hospital-based studies, where admissions due to clinically severe malaria are mainly of paediatric patients.

Poor preventive practices still afflict malaria-endemic areas in Yemen and pose another challenge to the elimination of the disease. In this respect, living in houses with unscreened windows and the existence of uncovered water collections in their vicinity were statistically associated with malaria among the rural populations of Mawza. Moreover, no participant reported sleeping under an ITN the night preceding the present survey or IRS spraying during the last year. Unscreened windows provide easy indoor access of vector mosquitoes, which essentially breed in the nearby uncovered water collection and ITNs were not used to prevent mosquito bites. These poor practices of leaving unscreened windows open and keeping uncovered water in plastic or concrete tanks near houses were also reported from Hadhramout, although sleeping under ITNs was minimally practised by 11% of the participants (Bamaga *et al.*, 2014).

Al-Taiar *et al.* (2009) reported that living near streams and freshwater marshes was a significant predictor of malaria acquisition among Yemeni children. On the other hand, screened windows are associated with reduction of indoor mosquito bites and disease transmission. This is important for disease vector control and elimination and this finding has been noted in other endemic areas such as in North-eastern Ethiopia, Gambia and Tanzania (Kirby *et al.*, 2009; Ogoma *et al.*, 2010; Abate *et al.*, 2013). This, in turn, reflects the role of poor preventive practices related to the prevention and control of vector mosquitoes in challenging the efforts of the NMCP for disease control and elimination.

5.3 Knowledge, attitudes and practices toward malaria, and fever treatment-seeking behaviors in the study population

Identification of the knowledge, attitudes and practices (KAPs) of the rural populations of malaria-endemic areas about malaria is needed to uncover the role of such KAPs in the continuing transmission of the disease and propose suitable educational interventions. In this study population, good knowledge that a mosquito bite is a possible cause of malaria among participants is consistent with other previous reports from Tanzania and Eastern India (Legesse & Deressa, 2009; Sabin *et al.*, 2010). However, poor knowledge on the transmission of malaria by flies and bad sewage was also mentioned by a minority of participants. Such misconceptions have also been reported by other studies from Ethiopia, India and Tanzania (Legesse *et al.*, 2007; Legesse & Deressa, 2009; Kinung'hi *et al.*, 2010; Sabin *et al.*, 2010; Abate *et al.*, 2013). In contrast to knowledge, practices toward malaria were poor and showed significant association with its prevalence, where people live in houses without window screens and keep uncovered water near houses as mentioned above. Although the Yemeni government is currently undertaking control measures, including the distribution of ITNs and IRS in endemic regions, the present study found a lack of their usage. Therefore, free distribution of ITNs through campaigns is not an effective way to increase their usage. The assessment of the association between ITNs usage and malaria prevalence was not feasible because all participants reported no usage. Thus, it appears that implementing appropriate health education for correcting misconceptions about the cause and transmission of malaria is not enough unless combined with actions to eliminate the poor practices of the community toward the disease.

The present study also reveals the magnitude of inappropriate fever treatment-seeking behaviours practised by the rural population of Mawza, where the majority (94.9%) of febrile patients sought home management of fever prior to being admitted to

healthcare facilities, predominantly by applying cold compresses and less frequently by taking antimalarial drugs and/or antipyretics. Alleviation of symptoms or waiting for the results of such home remedies usually lead to a longer delay in seeking treatment in healthcare facilities, as evidenced by more than 24-hour delay after the onset of fever in a high proportion (97.0%) of febrile patients, even though a major proportion reported seeking antimalarial treatment at healthcare facilities after laboratory confirmation.

Therefore, the delay in properly seeking antimalarial treatment could be the worst outcome of several factors that need to be uncovered using in-depth studies. In a previous study, Al-Taiar *et al.,* (2009) reported that financial constraints and the absence of males from houses were among the determinants of delayed antimalarial treatment seeking for febrile children in Taiz governorate. Delay in seeking treatment is against the WHO guidelines for effective disease management, which include early diagnosis and prompt treatment, for disease control and elimination strategies (WHO, 2015b).

5.4 Evaluation of *Pf*HRP-2/pLDH RDT and LM for the diagnosis of malaria against nested PCR

Prompt malaria diagnosis is a key component of the national malaria control strategy in Yemen, which relies on the use of LM and RDTs. One of the study objectives was to evaluate the diagnostic accuracy of these two methods against nested PCR in Mawza District, during the peak seasonal transmission. In the present study, the *Pf*HRP-2/pLDH RDT and LM showed a fair level of agreement in their performance to detect *P. falciparum* in the field, despite approaching 80 %. However, a substantial agreement was observed between RDT and LM for the detection of *P. falciparum* among febrile patients. This is consistent with a recent study (Atroosh *et al.,* 2015) that reported a very good level of agreement between LM and CareStart™ HRP-2

RDT results among febrile patients. In the present study, RDT proved effective in detecting all LM-positive cases and in detecting a large proportion of LM-negative cases. The investigated RDT showed higher sensitivity than LM compared with nested PCR (96.0 vs 37.6 %), which is also higher than the sensitivity recommended by the WHO (2014c).

The good performance of the *Pf*HRP-2/pLDH RDT in the field is evidently shown by its ability to detect *P. falciparum* in all different degrees of microscopic parasite densities. Moreover, its sensitivity exceeds that of LM for parasite detection (93.9% vs 8.5%) among afebrile participants, indicating its utility in active case detection. This could help in strategies for reducing malaria transmission by identifying asymptomatic carriers and their subsequent treatment. Similar findings of higher RDT sensitivity have been reported previously (Laban *et al.*, 2015). The WHO has recently reported a good level of sensitivity of RDTs in low parasitaemia (WHO, 2014c). However, one should consider that not all RDT-positive cases correlate with those obtained by PCR. This in turn indicates that despite the better performance of RDT compared with LM, false positivity of RDT should not be ruled out. However, its performance is still superior to that of LM. In this respect, a moderate agreement (about 80 %) exists between RDT and PCR in detecting falciparum malaria among Yemeni patients in the field compared with a fair agreement (about 62 %) between LM and PCR. Most importantly, the *Pf*HRP-2/pLDH RDT showed a higher NPV than LM (90.4 vs 51.3 %) during the peak seasonal transmission of malaria.

This is advantageous for the definite exclusion of malaria among patients, and the avoiding of unnecessary presumptive treatments. Given that the NPV is (100.0 %) for the RDT and (40.0 %) for LM among febrile patients, RDT-negative results for patients experiencing fever will be straightforward and will rationalize the prescription of anti-malarial drugs. Similarly, a very recent study (Umbers *et al.*, 2015) recommends the use

of RDTs for diagnosis of suspected malaria among symptomatic pregnant women but not for asymptomatic cases in Papua New Guinea.

The superiority of RDTs compared with LM could be explained by the fact that sequestered *P. falciparum* missed by LM can be detected by RDTs because of the release of *Pf*HRP-2 by parasites and its circulation in the blood (Howard *et al.*, 1986; Goldring, 2004). Meanwhile, the low sensitivity of LM in the present study could also be attributed to the high proportion of asymptomatic, very low-parasite density malaria cases. RDTs targeting *Pf*HRP-2 has been suggested as a better alternative to LM in areas of low-density parasitaemia and their false positives compared with LM have been confirmed by PCR to be cases below the threshold detection of LM (Bell *et al.*, 2005). In addition to the diagnostic limitation imposed by microscopist expertise, the poor-quality LM in developing countries contributes to its low sensitivity in detecting low-parasite density infections. In Yemen, poor performance of LM for malaria diagnosis has been ascribed to low quality reagents, laboratory equipment and supplies (NMCP, 2011). LM of low quality has been reported to influence its sensitivity and specificity for malaria diagnosis (Kahama-Maro *et al.*, 2011).

In contrast, LM had higher overall specificity than RDT (97.6% vs 56%) compared with the reference method. LM is still the gold standard for species identification and detecting the severity of malaria by quantifying parasitaemia and for differentiation of transmissible stages from those responsible for clinical disease (Wongsrichanalai *et al.*, 2008). Low specificity of the *Pf*HRP-2/pLDH RDT in the present study is in contrast to the high specificity (96.1%) recorded by the CareStart™ HRP-2 RDT test in an earlier study (Atroosh *et al.*, 2015) which compared with microscopy for the detection of falciparum malaria among febrile patients. However, such low specificity is in agreement with a previous study comparing four brands of *Pf*HRP2-based RDTs for

falciparum malaria diagnosis among febrile patients in Malawi, where specificity of (39–68%) was reported (Chinkhumba *et al.*, 2010) It is noteworthy that two cases were positive with both LM and *Pf*HRP-2/pLDH RDT but negative by the PCR reference method. Although these were considered as false-positive results compared to nested PCR as the reference method, unperceived factors contributing to the inhibition of PCR could not be ruled out. Moreover, PCR false-negativity has been documented in the literature (Barker *et al.*, 1994; Singh *et al.*, 1996; Coleman et al., 2006; Harris *et al.*, 2010). The false positivity of the RDT in the present study could overestimate the prevalence rate by (about 25 %) as indicated by the PPV (76.9 %) compared with nested PCR. This is in agreement with the high false-positive rates of *P. falciparum* using *Pf*HRP-2-based RDTs reported in Congo (Swarthout *et al.*, 2007; Ilombe *et al.*, 2014) and Burkina Faso (Kattenberg *et al.*, 2012). The *Pf*HRP-2-based RDT false positivity and its relatively low PPV could be attributed to the persistence of *Pf*HRP-2 antigenaemia in the blood circulation for 4–5 weeks after parasite clearance with successful treatment (Mayxay *et al.*, 2001; Swarthout *et al.*, 2007; Kattenberg *et al.*, 2012). The possible impact of persistent antigenaemia on the specificity of RDT investigated in the present study could, in part, explain its dropped specificity to (30 %) among patients with history of anti-malarial drug intake.

Furthermore, malaria survey at the peak seasonal transmission, when prevalence rate is >10 %, may partially account for the low specificity of *Pf*HRP-2/pLDH RDT in the present study. Previous studies showed a negative correlation between the specificity of RDTs and malaria prevalence (Swarthout *et al.*, 2007; Abeku *et al.*, 2008; Laurent *et al.*, 2010; Mtove *et al.*, 2011). False-positive results by *Pf*HRP-2/pLDH RDTs can lead to overdiagnosis and subsequent overtreatment, which may contribute to the emergence and spread of drug resistance (Hastings IM & Watkins WM, 2005). Therefore, its combination with a more specific test is recommended. Furthermore, in

addition to genus-specific pLDH, *P. falciparum*-specific LDH-based RDTs should be evaluated for screening of falciparum malaria in Yemen. This may help to avoid the drawback of *Pf*HRP-2 RDTs resulting from persistent antigenaemia in blood after treatment and cure, minimizing the false positivity rate to reasonable and acceptable levels. However, *Pf*HRP2 positivity in the absence of *P. falciparum*-specific LDH or pan-specific LDH does not necessarily mean a false-positive result due to persistent antigenaemia (Gatton *et al.,* 2015). Although PCR is the most sensitive and specific tool for malaria diagnosis (Snounou *et al.,* 1993), it is not practical for routine use in Yemen due to the limited resources.

5.5 Frequency of antimalarial drug resistance markers

Early treatment of malaria with ACTs is a mojor component of the national control strategy of malaria in Yemen and therefore the efficacy of the national drug policy is crucial for the success of malaria control. Athough *in vivo* clinical trial is the gold standard method for monitoring the efficacy of antimalarial drugs, monitoring the mutations in molecular markers associated with AS and SP resistance is necessary as an early alarm system for the evolution of drug resistance particularly SP resistance, the AS partner in ACTs. A genetic analysis has shown that CQR is associated with a point mutation and substitution of lysine (K) by threonine (T) at amino acid position 76 (K76T) in the *pfcrt* gene. It has been identified as the primary marker conferring CQR *in vitro* and *in vivo* (Fidock *et al.*, 2000; Djimdé *et al.*, 2001).

In the current study, the *pfcrt* 76T mutation has been associated with increased resistance to CQ and is considered as a useful marker for the field surveillance of resistance to the drug (Djimdé *et al.,* 2001; Wellems and Plowe, 2001; Wongsrichanalai *et al.,* 2002; White, 2004). Saturation of all investigated *P. falciparum* isolates with the *pfcrt* 76T mutant allele reflects the high level of resistance to CQ in Mawza District. Despite CQ being officially withdrawn several years ago (NMCP, 2010), the high

frequency of this key mutation of resistance to CQ could be attributed to the drug pressure as a result of its continued self-medication or prescription, its cheap price and over-the-counter availability in several areas in the country. For instance, Bashrahil *et al.* (2010) reported that CQ is the most frequently prescribed antimalarial by general practitioners after the new ACT policy in Mukalla, an urban city in Hadhramout. Meanwhile, the situation may even be worse in rural areas such as Mawza in the present study. Poor knowledge of physicians about the ACT policy may be one among several reasons for continued prescription of the drug (Ghouth, 2013).

The frequency of the *pfcrt* 76T among parasite isolates from Mawza is higher than that reported in a recent study in Taiz governorate (Al-Hamidhi *et al.*, 2013), where 50.9% (55/108) of parasite isolates harboured the mutant allele. It is also higher than those reported from different geographically-dispersed, malaria-endemic areas in the country, 85.2–90.5% in Hodeidah, west of Yemen (Abdul-Ghani *et al.*, 2013b; Al-Mekhlafi *et al.*, 2011) and 73.9% in Hadhramout, east of Yemen (Bamaga *et al.*, 2015a) in its pure and mixed-type alleles. However, it is close to the *pfcrt* 76T frequency of 98% reported among *P. falciparum* isolates from Lahj, south of Yemen (Mubjer *et al.*, 2011).

The saturation level of *pfcrt* 76T among *P. falciparum* isolates in the present study is consistent with the high frequencies (98.7–100%) reported for the mutant allele among Ugandan isolates of *P. falciparum* several years after incomplete CQ withdrawal (Kiwuwa *et al.*, 2013; Nsobya *et al.*, 2010). On the other hand, the fixation of the mutant allele among parasite isolates in the present study is in contrast to its decline after CQ withdrawal from a number of African countries. Several previous reports showed a significant *pfcrt* 76T frequency decline after CQ withdrawal in Malawi (Kublin *et al.*, 2003; Laufer *et al.*, 2006), Tanzania (Alifrangis *et al.*, 2009; Kamugisha *et al.*, 2012; Malmberg *et al.*, 2013; Mohammed *et al.*, 2013), Kenya (Mang'era *et al.*,

2012; Mwai *et al.,* 2009), Mozambique (Thomsen *et al.,* 2013) and Ethiopia (Mekonnen *et al.,* 2014).

In the present study, the complete absence of *pfmdr1* 86Y mutant allele was found among the *P. falciparum* isolates from Mawza, Taiz. This finding is discordant with the low *pfmdr1* 86Y frequency of 16.7% that has been recently reported among parasite isolates from Taiz (Al-Hamidhi *et al.,* 2013) and Hadhramout (Bamaga *et al.,* 2015a). The absence of this mutant allele could be attributed to the widespread use of AL (artemether-lumefantrine) as a second-line treatment within ACTs; possibly selecting for the *pfmdr1* N86 wild-type allele. In this context, several studies suggested that AL contributes to the selection of *pfmdr1* N86 wild-type allele among parasite isolates in Africa (Sisowath *et al.,* 2005; Happi *et al.,* 2009; Raman *etal.,* 2011; Thomsen *et al.,* 2011, 2013; Kamugisha *et al.,* 2012; Malmberg *et al.,* 2013).

Pooled analysis of 31 clinical efficacy studies identified *pfmdr1* N86 as an independent predictor of recrudescence in patients treated with AL and recommended its routine detection for monitoring resistance to the combination (Venkatesan *et al.,* 2014). However, lack of baseline surveys for the molecular markers of resistance before adopting AL as a second-line treatment for uncomplicated falciparum malaria in Yemen makes it difficult to assume that the high frequency of the wild-type allele is due to AL pressure. Moreover, the possible role of the *pfmdr1* N86 wild-type allele as a predictor of resistance to AL among Yemeni isolates of *P. falciparum* has yet to be established.

There are increasing concerns about the emergence and spread of artemisinin-resistant *P. falciparum* in Southeast Asia (Dondorp *et al.,* 2009; Phyo *et al.,* 2012). Recently, a strong association between K13 propeller mutations and delayed clearance of *P. falciparum* parasites after treatment with artemisinin has been documented in Southeast Asia (Ariey *et al.,* 2014; Ashley *et al.,* 2014). Although their association with resistance to artemisinin has not been validated outside Southeast Asia, K13 mutations

have also been reported from a number of countries in sub-Saharan Africa (Kamau *et al.,* 2014). Thus far, there is no report on K13 propeller gene marker in Yemen. In the present study, none of the investigated isolates showed mutations in the K13 sequences. This finding supports those reported by Adeel *et al.* (2015) on the effectiveness of the ACTs adopted for malaria treatment by the NMCP against uncomplicated falciparum malaria in Yemen.

Resistance to non-artemisinin partners within ACTs is alarming, possibly cancelling the goal of the combination therapy and increasing the risk of emergence and spread of resistance to artemisinin derivatives (WHO, 2015a). In the present study, all *pfdfr* and *pfdhps* alleles were of the wild-type except for one isolate showing the *pfdhfr* 51I/108N double mutant allele. This frequency is lower than those reported from Taiz, Hodeidah, Dhamar and Hadhramout governorates (Al-Hamidhi *et al.,* 2013; Bamaga *et al.,* 2015b), Saudi Arabia (Dajem *et al.,* 2012) and Sudan (Gadalla *et al.,* 2013; Marma *et al.,* 2010; Osman *et al.,* 2007). The *pfdhfr* 59R mutant allele was not detected in parasite isolates from Mawza, which is consistent with that reported by Al-Hamidhi *et al.* (2013) among parasite isolates from Taiz, Hodeidah and Dhamar. In contrast, low frequencies of 5% (5/99) and 0.8% (1/102) of the *pfdhfr* 59R mutant allele were reported from Lahj (Mubjer *et al.,* 2011) and Hadhramout (Bamaga *et al.,* 2015b), respectively.

It is noteworthy that the emergence of resistance to antimalarial drugs is multi-factorial, including the heterogeneous epidemiology of malaria in Yemen that has been classified into three different strata in altitude, seasonality and intensity of transmission (NMCP, 2010). The impact of intensity of transmission and seasonality on the emergence of antimalarial drug resistance has been well documented (Babiker *et al.,* 2005; 2009; Escalante *et al.,* 2009). In general, all studies conducted in Yemen so far have not reported the quadruple *pfdhfr* 51I/59R/108N/164L and the triple *pfdhps*

436A/437G/540E mutant genotypes associated with SP treatment failure, supporting the sustained efficacy of the non-artemisinin partner of the first-line ACT in the country.

5.6 Study limitation

The present study unveils high prevalence of malaria and a number of factors that are challenging to malaria control and elimination efforts in rural settings, including the circulation of sub-microscopic infections among asymptomatic carriers, poor preventive practices and delayed treatment seeking. It also confirms the efficacy of the national antimalarial drug policy and evaluates the current diagnostic tests used. However, a number of limitations do exist.

1. First, the magnitude of sub-microscopic infections missed by RDT screening remains unclear because of restriction in the use of PCR on RDT-positive cases. This can be justified by the fact that the *Pf*HRP-2/pLDH RDT used in the present study showed high sensitivity among asymptomatic patients and high negative predictive value for the exclusion of malaria in febrile patients under field conditions. Therefore, adjusting its results by re-testing malaria parasite-positive specimens using PCR would provide a cost-effective screening approach for estimating the true burden of malaria, including those not detected by LM, in such resource-limited setting. An alternative approach that could be adopted is the PCR-corrected RDT approach recommended by Gitonga *et al.*, (2012) for school malaria surveys in low transmission settings, where PCR is performed on all RDT-positive samples and on pools of five RDT-negative samples. Validation of the performance of such cost-effective approaches for community malaria surveys should be considered as part of the elimination strategies in low-intensity settings.

2. Second, the study is limited by the fact that it focused on community's knowledge and certain preventive practices but ignores the determination of some practices and attitudes towards malaria in the study area. However, the association of the poor practices to malaria in the study highlights the need for extensive KAP studies to explore the gaps of incorrect knowledge, negative attitudes and poor practices that may have an association with microscopic and sub-microscopic infections.
3. Third, the study did not explore the reasons for delaying fever treatment seeking at health facilities among rural populations. Therefore, highlighting the existence of the problem in the present study could guide the design of detailed studies investigating the reasons and proposing solutions related to poor treatment-seeking behaviours.
4. Fourth, the present study included only a subset of the negative samples for molecular analysis. However, the large difference between RDT-positive and LM-positive samples, which is still of suspected positivity and could be due to persistent antigenaemia (108 samples of the RDT-positive ones), helps to avoid or, at least, reduce any possible bias.
5. Fifth, this study is limited by the small number of isolates screened for the drug resistance markers although they are consistent with *in vivo* clinical efficacy trials conducted in Yemen.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

In summary, this study showed high prevalence of malaria with high proportion of sub-microscopic malaria infections among asymptomatic rural populations of Mawza. Therefore, cost-effective surveillance approaches, such as PCR-adjusted RDT screening, could be helpful in overcoming the drawback of RDT false positivity and, hence, determining and mapping the true estimate of malaria as a measure of the effectiveness of ongoing interventions to eliminate the reservoir of infection in low-intensity settings. Besides the problem of sub-microscopic infections, poor preventive practices of not screening windows, leaving water collections near houses uncovered, not sleeping under ITNs and not spraying residual insecticides indoors threaten malaria elimination efforts in rural areas.

Although seeking antimalarial treatment after laboratory confirmation at health facilities has been reported by the majority of rural people, delayed treatment seeking for more than 24 hours represents a major problem that might have serious consequences on the predominance of sub-microscopic infections and emergence and spread of drug resistance. Asymptomatic malaria carriers with sub-microscopic infections in the study area, represent a significant reservoir of the ongoing malaria transmission, should be considered when tailoring future control and elimination strategies.

The *Pf*HRP-2/pLDH RDT showed better performance than LM in field survey for malaria, even in asymptomatic cases. It also showed a moderate degree of agreement with nested PCR with a high sensitivity. A major drawback of the RDT is that the high false-positivity rate may limit its use as an independent diagnostic tool for malaria. However, its negative results totally exclude falciparum malaria among febrile patients as indicated by its 100 % NPV. This may have public health implications in educating healthcare providers and patients in endemic areas to perform RDTs in all cases of fever

to exclude falciparum malaria before prescribing or taking anti-malarial drugs. The low sensitivity of LM indicates that a high proportion of malaria cases is missed, leading to an underestimation of the true malaria prevalence in the community. However, LM remains indispensable for species identification, differentiation of gametocytes from asexual stages and the assessment of the severity of the disease. Furthermore, it is still the gold standard for the diagnosis of symptomatic malaria. RDTs should be further investigated as rapid malaria-excluding diagnostic test among febrile patients in endemic areas.

Regarding the prevalence of *P. falciparum* drug resistance-associated polymorphisms, the current data provide baseline information and highlight the fixed frequency of the *pfcrt* 76T mutant allele which indicates the high level of resistance to the drug, and entails its strict control in the Yemeni market because of its effectiveness in the treatment of vivax malaria. In addition, the absence of mutant alleles associated with resistance to artemisinin derivatives and SP strongly necessitate strict adherence of physicians to the ACT policy adopted by the NMCP to sustain the effectiveness of this policy in the long run.

Regular large-scale surveillance of molecular markers of resistance to the antimalarial components of the current antimalarial treatment policy is recommended, preferably at sentinel sites. Meanwhile, educational programmes on the effects of poor treatment-seeking behaviours, antimalarial drug misuse, emergence and spread of drug resistance should be launched for physicians, pharmacists and general population in endemic areas.

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LIST OF PUBLICATIONS AND PAPER PRESENTED

a) Publications

Lina M.Q. Alareqi, Mohammed A. K. Mahdy, Yee-Ling Lau, Mun-Yik Fong, Rashad Abdul-Ghani, Arwa A Ali, Fei-Wen Cheong, Rehab Tawfek, Rohela Mahmud (2016). Field evaluation of a *Pf*HRP-2/pLDH rapid diagnostic test and light microscopy for diagnosis and screening of *falciparum* malaria during the peak seasonal transmission in an endemic area in Yemen. *Malaria Journal*, *16*(49).

Lina M.Q. Alareqi, Mohammed A. K. Mahdy, Yee-Ling Lau, Mun-Yik Fong, Rashad Abdul-Ghani, Rohela Mahmud (2016). Molecular markers associated with resistance to commonly used antimalarial drugs among Plasmodium falciparum isolates from a malaria-endemic area in Taiz governorate, Yemen during the transmission season. *Acta Tropica*, *162*(174–179).

b) Conference presentation made during the candidature period

Lina M.Q. Alareqi, Mohammed A. K. Mahdy, Yee-Ling Lau, Mun-Yik Fong, Nada Abdullah Alshebani, Rohela Mahmud. Malaria in the rural communities of Taiz governorate, Yemen: an assessment of current Prevalence, Risk factors, Knowledge, Attitude and practices. Participate in the 52nd Annual scientific conference of the Malaysian Society of Parasitology and Tropical medicine on 2nd-3rd March (2016). Kuala Lumpur. Malaysia.