AN INVESTIGATION INTO THE PREVALENCE OF POINT MUTATIONS ASSOCIATED WITH ANTIMALARIAL DRUG RESISTANCE IN *PLASMODIUM FALCIPARUM* FOUND IN THE ZAMBEZI REGION OF NAMIBIA

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Abstract

Surveillance and monitoring of emerging drug resistance is important as Namibia moves towards malaria elimination. In 2005 artemisinin combination therapies (ACT's) were introduced as first-line treatment for uncomplicated *falciparum* malaria in Namibia. However, reduced ACT efficacy has been reported in Asia and in some parts of Western Africa, raising concerns around the efficacy of artemisinin. This study aimed to identify the different *plasmodium* species found in Zambezi region, Namibia as well as to investigate the prevalence of antimalarial drug resistance polymorphisms in the *pfcrt*, *pfmdr1* and Kelch 13 genes in the region. A QIAamp DNA mini-kit (Qiagen, Germany) was used to extract DNA from malaria positive Dried Blood Spots (DBS). Prior to investigating the prevalence of point mutations, multiplex PCR was performed to investigate the different *plasmodium* species found in the Zambezi region of Namibia. Seventy-two *P. falciparum* positive samples out of a total of 143 malaria positive samples were analysed by PCR-RFLP at codon N86Y in the *pfmdr1* whereas the prevalence of haplotypes at codons 72-76 in the *pfcrt* gene were analysed using Quantitative-PCR. Additionally, mutations at 25 codons in the Kelch 13 gene were analysed by n-PCR followed by sequencing. The study found that 76.2% of the cases were caused by *P. falciparum* which was less than expected. Additionally, 7.1% of the cases were found to be caused by *P. vivax* followed by 6.0% of *P. malariae* infections. In the *pfcrt* gene 92.5% samples contained the wild type haplotype, 3.8% contained the mutant haplotype while 3.8% samples had mixed haplotype. Furthermore, 18.5% mutant and 59.3% wild and type alleles were observed in *pfmdr1* gene at N86Y. No mutant alleles were observed in the Kelch 13 gene.

This study provides the first data on point mutations in the *pfcrt*, *pfmdr1* and Kelch 13 genes in Namibia. A low prevalence of mutations was observed in this study which shows that ACTs are still effective in Namibia. However, continued surveillance is recommended with similar studies in other endemic regions of Namibia. Additionally, it is recommended that further studies such as *in vivo* and *in vitro* responses to drug treatment be done to determine the role of these polymorphisms in drug resistance to support these findings.

Key words: *Plasmodium falciparum*; Artemisinin Combination Therapies; Antimalarial drug resistance; *Pfmdr1*; *Pfcrt*; *Kelch 13*; Namibia.

Table of Contents

Abstract i
List of Figures iv
List of Tables vi
List of Conferences and Posters vii
Acknowledgements viii
Declaration x
Dedication xi
List of Abbreviations xii
1. INTRODUCTION 1
1.1. Background of the study 1
1.1.1. Global Malaria Burden 1
1.1.2. The *Plasmodium* parasite, vector and its Life Cycle 2
1.1.3. Malaria Symptoms 7
1.1.4. Malaria Control 8
1.1.5. The Impact of Antimalarial Drug Resistance 13
1.2. Problem Statement 14
1.3. General Objectives of the study 15
1.3.1. Specific Objectives of this study were to: 15
1.4. Significance of the study 15
1.5. Limitations of the study 16
1.6. Delimitations of the study 16
2. Literature Review 17
2.1. Antimalarial drugs 18
2.1.1. Quinolines 19
2.1.2. Antifolates 24
2.1.3. Artemisinin 26
2.2. Antimalarial drug resistance 27
2.3. Development and Spread of Drug Resistance 28
2.4. Mechanisms of Antimalarial drug resistance 29
2.4.1. Resistance mediated by transporter mutations 29

2.4.2. Resistance to Antifolates 33
2.4.3. Artemisinin and Derivatives 34
3. Research Methods 37
3.1. Research Design 37
3.2. Methods 37
3.2.1. Study Population and Study Site 37
3.2.2. Sample Collection 38
3.3. Molecular Methods 39
3.3.1. Extraction of *plasmodium* parasite DNA using the QIAamp DNA mini-kit (Qiagen Germany) 39
3.3.2. *Plasmodium* Species Identification 39
3.3.3. SNP Analysis 40
3.3.4. Restriction Digest 42
3.3.6. Quantitative Real Time PCR for the Detection Of crt72-76 Haplotypes ... 43
3.3.7. Amplification and Sequencing of the K13 Propeller Gene 45
3.4. Data Analysis 47
4. Research Ethics 47
5. Results 48
5.1. Identification of *Plasmodium* species by Multiplex PCR 48
5.2. Prevalence of the *pfcrt* 72-76 haplotypes 50
5.3. Prevalence of the *pfmdr1* N86Y mutation 51
5.4. Prevalence of SNPs in the Kelch 13 propeller gene 52
6. Discussion 54
7. Conclusion 64
8. Recommendations 65
9. References 67
10. Appendices 92
Appendix 1: Carestart RDT Manufactures guideline 92
Appendix 2: Eiken Japan PAN/Pf LAMP guidelines 94
Appendix 3: New England Biolabs Restriction Digest Guidelines 97
Appendix 4: Qiagen DNA Extraction Protocol 101

List of Figures

Figure 1; Morphological differences between the four-plasmodium species in human blood smears Source: http://www.dpd.cdc.gov/dpdx 5

Figure 2: Plasmodium parasite life cycle, Source: http://www.dpd.cdc.gov/dpdx 7

Figure 3. The malaria distribution map showing the global effort to eradicate malaria (Feachem et al., 2009). 17

Figure 4. Chemical structure of Chloroquine (WHO, 2015b) 20

Figure 5. Chemical structure of Amodiaquine (Deshpande and Kuppast, 2016) 21

Figure 6. Chemical Structure of mefloquine (WHO, 2015b). 22

Figure 7. Chemical structure of lumefantrine (Amadi, Otuokere and Chinedum, 2017). 23

Figure 8. Chemical structure of Primaquine (Delves et al., 2012) 24

Figure 9. Chemical structures of both Type 1 and Type 2 Antifolates (Delves et al., 2012) 25

Figure 10. Chemical structure of Artemisinin and its derivatives (Staines and Krishna, 2012) 27

Figure 11. Structure of the the P-glycoprotein and amino acid positions (Ibraheem et al., 2014) 31

Figure 12. Structure of the transporter protein and amino acid positions (Pulcini et al., 2015) 33

Figure 13. Showing the Zambezi region of Namibia where this study was conducted (in dark green) 38

Figure 14. Representative pictures of RDTs (left) showing examples of a positive (A) and a negative (B) result. An example of a DBS (right) showing the collection of blood from a finger prick on filter paper. ... 39

Figure 15; Gel representative showing the identification of the four different Plasmodium species. Four positive controls (P. falciparum, P. malariae, P. ovale and P. vivax) were included during each run as well as a negative control. Sample 2 shows an example of a P. malariae infection, Sample 20 shows an example of a P. vivax infection whereas

samples 30-34 shows examples of P. falciparum infections. Additionally, lanes 9 and 26 contain no template samples. 49
Figure 15.1. Prevalence of the Plasmodium species in western Zambezi Region 50
Figure 16; Gel representative showing restriction digest PCR product from the pfmdr1 gene. Lane M=Molecular weight; NEC= negative control, Lane 3=no template control, Lane 4,5,7,8,9,10,11,12 and 13=P. falciparum malaria positive isolates carrying the wild type allele, Lane 6 and 14= P. falciparum malaria positive isolates carrying the mutant allele, PEC= Positive control 51
Figure 17. Prevalence of Drug resistance Polymorphisms, wild type and mutant alleles at codon N86Y in the pfmdr1 gene....................................52
Figure 18. Gel representative showing nested PCR product from the Kelch 13 propeller gene. Lane M=Molecular weight lane PEC= Positive control, Lane 1- 23= P. falciparum malaria positive isolates..................................53

List of Tables

TABLE 1 CURRENT MALARIA VACCINES UNDER DEVELOPMENT (COELHO ET AL., 2017). 12

TABLE 2; SUMMARY OF MOLECULAR MARKERS ASSOCIATED WITH ANTIMALARIAL DRUG RESISTANCE FOR THE DIFFERENT DRUGS 35

TABLE 3. SUMMARY OF PRIMER NAMES AND SEQUENCES USED TO AMPLIFY THE DIFFERENT PLASMODIUM SPECIES 40

TABLE 4. SUMMARY OF THE PRIMER NAMES AND SEQUENCES USED FOR THE AMPLIFICATION OF THE PFMDR1 GENE AT CODON 86 42

TABLE 5. PRIMERS USED IN THE MULTIPLEX REAL-TIME PCR FOR THE DETECTION OF TWO P, FALCIPARUM CRT ALLELES 44

TABLE 5.1. PROBES USED IN THE MULTIPLEX REAL-TIME PCR FOR THE DETECTION OF TWO P, FALCIPARUM CRT ALLELES 45

TABLE 6. PRIMER NAMES AND SEQUENCES USED DURING THE AMPLIFICATION OF THE KELCH 13 GENE 46

TABLE 7; PREVALENCE OF WILD TYPE MUTANT AND MIXED HAPLOTYPES FOUND IN THE PFCRT GENE 72-76 IN GENE FROM THE ZAMBEZI REGION OF NAMIBIA. 51

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1. Tambo M, Dausab L, Mukosha C, McCreesh P, Roberts K, Böck R, Cueto C, Hsiang MS, Gosling R and Mumbengegwi D (2017). Evaluation of Loop-mediated isothermal amplification (LAMP) as a surveillance tool for the detection of asymptomatic low-density malaria in the Zambezi region of Namibia. Paper presented at the 2nd Multi-disciplinary Research Centre Conference 2017, Windhoek, Namibia. Oral Presentation

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May God bless each one of you for being part of my journey because without Him, I wouldn’t have reached this far.

Declaration

I, Lucille Lorendana Dausab, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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.... Date..

Lucille Lorendana Dausab

Dedication

I dedicate this thesis to my parents. Baie dankie vir alles wat jul vir my gedoen mammie en pa.

List of Abbreviations

ACT: Artemisinin Combination Therapy

AL: Artemether-Lumefantrine

AQ: Amodiaquine

AS-AQ**:** Artesunate-Amodiaquine

BLAST: Basic Local Alignment Search Tool

bp**:** base pairs

CDC: Centre for Disease Control and Prevention

Ct: Cycle Threshold

CQ: Chloroquine

D (Asp): Aspartic Acid

DBS: Dried Blood Spots

DNA: Deoxyribonucleic Acid

DNTP’s: Deoxynucleotide Triphosphates

DV**:** Digestive vacuole

E (Glu): Glutamic Acid

G6PD**:** Glucose-6-Phosphate Dehydrogenase

K (Lys): Lysine

LAMP: Loop-mediated isothermal Amplification

IPT: Intermittent Preventive Treatment

IRS: Indoor Residual Spraying

ITN: Insecticide Treated Net

LLINs: Long Lasting Insecticide Treated Nets

MQ: Mefloquine

N (Asn): Asparagine

nPCR: nested Polymerase Chain Reaction

PCR: Polymerase Chain Reaction

PBS: Phosphate Buffered Saline

pfATPase6: Sarco-/endoplasmic reticulum Ca2+ - ATPase orthologue of P. falciparum gene

Pfcrt: P. falciparum chloroquine resistance transporter gene

Pfdhfr: P. falciparum dihydrofolate reductase gene

Pfdhps: P. falciparum dihydropteroate synthase gene

pfmdr1: P. falciparum multidrug resistance protein 1 gene

Pgh1**:** P-glycoprotein Homologue 1

QN: Quinine

qPCR: Quantitative PCR

RDTs: Rapid Diagnostic Tests

RFLP: Restriction Fragment Length Polymorphism

SNP: Single Nucleotide Polymorphism

SP: Sulfadoxine-pyrimethamine

T (Thr): Threonine

Y (Tyr): Tyrosine

1. INTRODUCTION

1.1. Background of the study

1.1.1. Global Malaria Burden

Malaria is the most important parasitic protozoan infection that poses a threat to human health globally (Feng *et al.*, 2015). This disease which is caused by protozoan parasites of the genus *Plasmodium*, is reported to affect 300-500 million people who reside in malaria endemic areas (Hasab *et al.*, 2012). Although malaria is preventable and treatable, the World Health Organization (WHO) reported approximately 216 million malaria cases and an estimated 445 000 deaths in the world in 2016 (WHO, 2017c). Nearly 3.3 billion people are at risk of malaria transmission with Africa being the most affected continent (Bayih *et al.*, 2016). Sub-Saharan Africa carries a disproportionally high share of the global malaria burden (World Health Organization, 2017). In 2016, Africa reported 90% of global malaria cases and 91% of global malaria deaths (WHO, 2017c). South East Asia and the Eastern Mediterranean Region represented 7% and 2% of global malaria cases respectively while 6% of all the malaria deaths in the world were reported in South East Asia (WHO, 2017c). Out of 91 malaria endemic countries, fifteen countries in sub-Saharan Africa are said to carry 80% of the global malaria burdern as well as 80% of the global malaria deaths excluding India (WHO, 2017c). In Namibia, 25 198 cases and 65 deaths were reported by WHO in 2016. (WHO, 2017c). Malaria in Namibia is confined to the northern parts of the country (Kavango (East and West), Kunene, Ohangwena, Omusati, Oshana, Oshikoto, Otjozunjupa, and the Zambezi region) where a high risk of infection is reported from November to June (Kamwi *et al.*, 2015).

Chloroquine was the first line treatment for malaria in several malaria endemic countries before resistant *P. falciparum* parasites were discovered along the Thailand and Cambodia border in 1957, resistance further spread to the rest of the world (Lucchi *et al.*, 2015). Following drug resistance to chloroquine, WHO recommended the use of Sulfadoxine-pyrimethamine (SP) as first-line treatment for *falciparum* malaria (Ashley *et al.*, 2014). However shortly after the introduction of SP, mutant strands were discovered which then led to the change in drug policy, subsequently leading to the introduction of artemisinin combination therapies (ACTs) (Winzeler and Manary, 2014). Thus, WHO recommends the routine monitoring of drug resistance to detect early emergence of resistance and using molecular makers as these tools is effective, especially in a low transmission setting such a Namibia where *in vivo* tests may be found difficult to conduct because of the lack of symptomatic participants.

1.1.2. The *Plasmodium* parasite, vector and its Life Cycle

Malaria parasites are micro-organisms that belong to the genus *Plasmodium* (Gatc *et al.*, 2013). More than 100 *Plasmodium* species have been identified that can infect animal species such as reptiles, birds and there are a number of other mammals that are also infected (WHO, 2016). Four species of *Plasmodium* namely: *Plasmodium vivax* (*P.vivax*), *Plasmodium ovale* (*P. ovale*), *Plasmodium malariae* (*P. malariae*), and *Plasmodium falciparum* (*P. falciparum*) have been recognized to infect humans (Eyasu, 2015). Recently, a fifth new species, *Plasmodium knowlesi* (*P. Knowlesi*) which was known to only infect long-tailed and pig-tail macaque monkeys was discovered to cause human malaria (Zhang *et al.*, 2016) (Nkumama, O'Meara and Osier, 2017). Malaria parasites are differentiated by examination of thin blood smears

under a light microscope. These differences have been tabulated in figure 1 showing how the parasites differ at the various stages during the life cycle.

The most severe illness and deaths from malaria and the most drug resistant infections are due to infection with *Plasmodium falciparum* (Wongsrichanalai and Sibley, 2013). This parasite is known as the most virulent human malaria parasite and is responsible for the majority of malaria deaths globally (Kwenti *et al.*, 2017). *P. falciparum* is the most prevalent species in sub-Saharan Africa (WHO, 2016). In Namibia, *P falciparum* accounts for more than 90% of the malaria infections (Smith-Gueye *et al.*, 2014). The *P. falciparum* parasite is principally transmitted by the vector *Anopheles gambiae*. However, other vectors such as *Anopheles albimanus*, *Anopheles freeborni*, *Anopheles maculatus* and *Anopheles stephensi* among others have been found to transmit *P. falciparum* (Molina-Cruz and Barillas-Mury, 2014).

Plasmodium vivax is the second most significant species that infects humans causing 25-40% of malaria cases worldwide, and is most prevalent in Southeast Asia and Latin America (Golassa *et al.*, 2015). Previous studies observed that *P. vivax* remains dormant during the liver stage, but can be reactivated at a later stage even in the absence of a mosquito bite, thus leading to clinical symptoms (Le Bras and Durand, 2003) (WHO, 2015c).

Plasmodium ovale is also a species of parasitic protozoa of which two distinct sub species have been described; *P. ovale curtisi* and *P. ovale wallikeri* that cause tertian malaria in humans (*Plasmodium parasites | Scientists Against Malaria*, no date). Unlike *P. falciparum* and *P. vivax*, *P. ovale* has been reported to be less dangerous and the distribution of this parasite is said to be limited to West and Central Africa, Philippines, eastern Indonesia, and Papua New Guinea (Li *et al.*, 2016). Additionally,

it is also found in Bangladesh, Cambodia, India, Thailand and Vietnam (Li *et al.*, 2016). Although this parasite is found almost everywhere in the world, its prevalence has been reported to be low and ranges between 3-5%, though it is greater than 10% in areas of West and Central Africa (Strydom, Ismail and Frean, 2014).

Plasmodium malariae is considered as one of the minor parasites of the five human malaria species because of its low prevalence rates in endemic areas as well as to the disease's milder and chronic nature if compared to the other parasites (Li *et al.*, 2016). This parasite has been reported to cause quartan fever and is detected at low parasitaemia in mixed infections with either *P. falciparum* or *P. vivax* (Nakeesathit *et al.*, 2016). Previous studies have reported the presence of *P. malariae* in sub-Saharan Africa, Southeast Asia, Indonesia, on the islands of the Western Pacific and in areas of the Amazon Basin of South America and has been found to be in other endemic regions (Nakeesathit *et al.*, 2016) (Doctor *et al.*, 2016). However, the prevalence of this parasite has been found to range from less than 4% to more than 20% (Nakeesathit *et al.*, 2016).

The fifth species *Plasmodium knowlesi* is a species that infects primates, however they have recently found that this species can also lead to human malaria (Figtree *et al.*, 2010). The first case of *knowlesi* malaria was reported in 1965, additional cases of *knowlesi* malaria were later reported in 2004 in Sarawak, Malaysian Borneo (Zhang *et al.*, 2016). Further studies reported *knowlesi* malaria cases throughout Southeast Asian countries as well as in Europe (Calderaro *et al.*, 2013). In comparison to *P.malariae* and *P. ovale*, *P. knowlesi* has been reported to cause severe malaria and death in patients (Calderaro *et al.*, 2013).

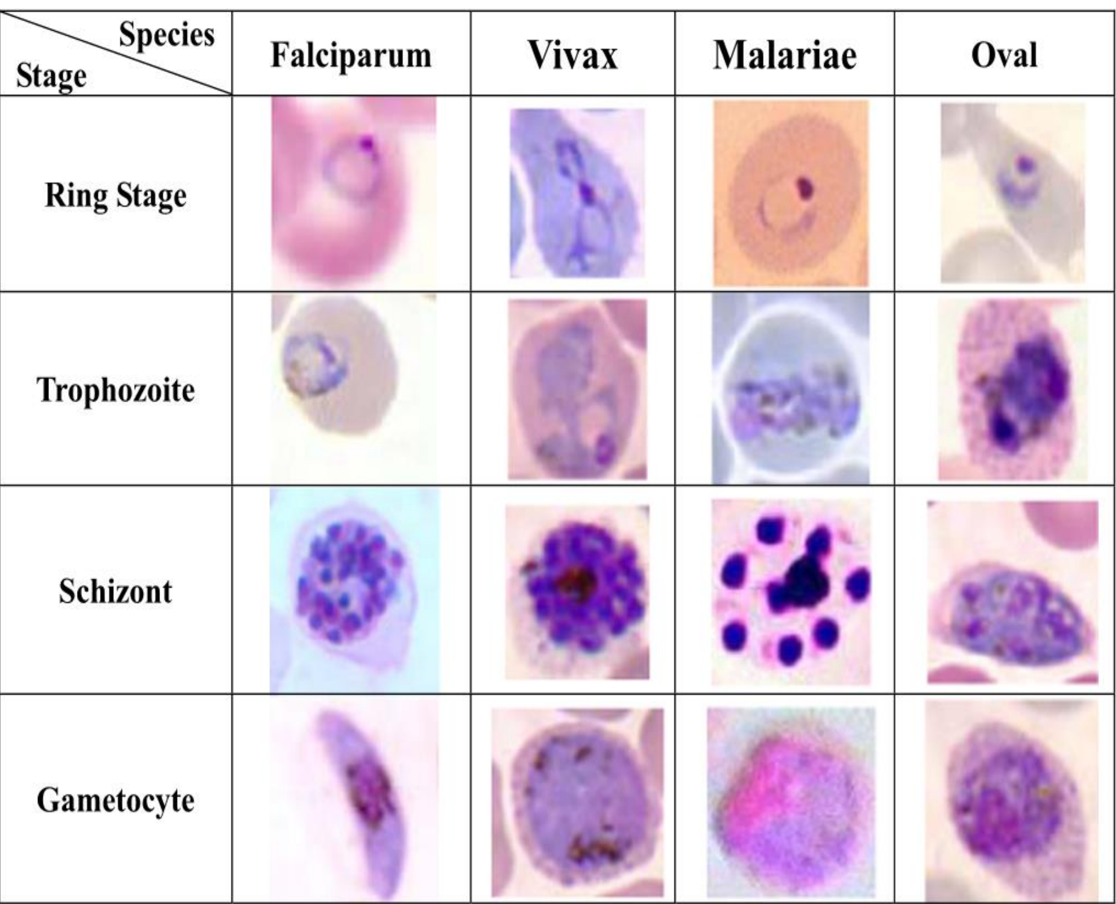


Figure 1; Morphological differences between the four-plasmodium species in human blood smears Source: http://www.dpd.cdc.gov/dpdx

1.1.2.1. Life cycle

Malaria parasites are spread through bites by an *Anopheles* mosquito; the parasites require a human host to complete their life cycle as shown in figure 2 below.

The disease is caused when the female mosquito bites a human and injects saliva containing sporozoites into the blood stream of the human host (Malaria *et al.*, 2008). These move to the liver where they invade the hepatocytes, once they invade these liver cells the sporozoites undergo asexual replication which is known as exoerythrocytic schizogony to produce merozoites (Lindblade *et al.*, 2013). The

merozoites are then released into the bloodstream when the cell ruptures. However, with *P. ovale* and *P, vivax,* the merozoites remain dormant as hypnozoites for a few weeks to several years instead of immediately undergoing asexual reproduction but can reactivate even in the absence of a mosquito bite and cause the disease (Deshpande and Kuppast, 2016). The merozoites move through the bloodstream and invade the red blood cells (erythrocytes), they then mature into trophozoites and undergo a second phase of asexual replication which is known as erythrocytic schizongony (Soulard *et al.*, 2015). During this stage about 36 merozoites are released to invade new erythrocytes (Hawkins, Burton and Labarre, 2014). This process is repeated and is responsible for the disease. Some of the merozoites transform into male and female gametocytes that enter the peripheral blood where they are taken up into anopheles mosquito once it feeds on an infected individual (Lindblade *et al.*, 2013).

In the mosquito the gametocytes form male and female gametes that undergo sexual replication and produce a zygote (ookinete). The ookinete moves to the gut of the mosquito and develops into an oocyte, the oocyte in turn undergoes asexual replication and produce sporozoites (Lindblade *et al.*, 2013). These rounds of multiplication results in the rupture of the oocyte which releases the sporozoites that later migrates to the salivary gland of the mosquito, ready to infect another host (Ouattara and Laurens, 2015).

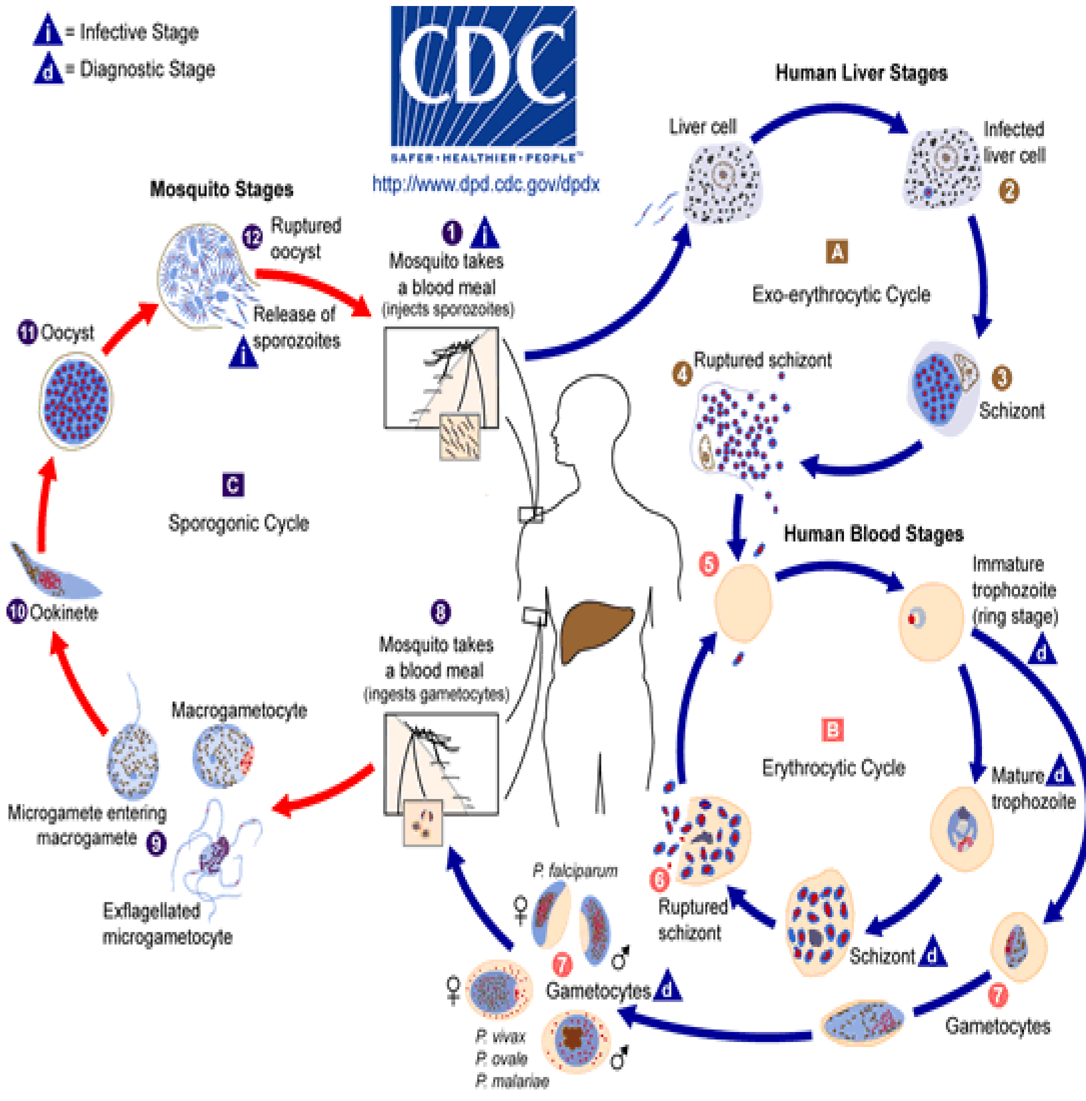


Figure 2: Plasmodium parasite life cycle, Source: http://www.dpd.cdc.gov/dpdx

1.1.3. Malaria Symptoms

The onset of malaria symptoms from the initial bite of a mosquito, to the appearance of symptoms is variable and it depends on the species of *plasmodium* (Lindblade *et al.*, 2013). For *P. falciparum* it takes 9 to 14 days whereas for *P. ovale* and *P. vivax* it takes about 12 to18 days however, some *P. vivax* strains may have an incubation period of 8 to 10 months or longer where they remain dormant. Additionally, it takes

P. malariae 18 to 40 days and *P. knowlesi* 9 to 12 days for symptoms to appear after the initial bite (*Malaria - including symptoms, treatment and prevention*, 2012).

The first symptoms of malaria are non-specific and are similar to that of a flu (CDC, 2013). These symptoms include: headaches, fatigue, abdominal discomfort and muscle and joint aches, followed by fever, chills, perspiration, anorexia and vomiting (WHO, 2015a). With treatment the symptoms and parasite should be cleared. However, if left untreated, especially with *P. falciparum* malaria, the disease could progress to severe malaria and cause cerebral malaria, metabolic acidosis, anaemia, hypoglycaemia, acute renal failure, acute pulmonary oedema and eventually death if not treated. (WHO, 2015a).

1.1.4. Malaria Control

The current goal of many malaria control programs and activities is to reduce the number of malaria cases and deaths to a level where malaria is no longer a public health problem in malaria endemic countries (CDC, 2014). In Africa the strategies that have been used to reduce malaria morbidity and mortality include, prevention through integrated vector control (LLINs and IRS), early diagnosis of all malaria cases, and the administration of effective and appropriate treatment once an individual has been diagnosed with malaria (Eyasu, 2015) (Korenromp *et al.*, 2016). Additional interventions that have reduced malaria over the years is the confirmation of malaria diagnostics through the use of rapid diagnostics tests (RDTs).

1.1.4.1. Vector Control

Karunamoorthi describes vector control as any kind of measures which are directed against a vector (mosquito) of a disease (malaria) and intending to limit its ability to transmit the disease (Karunamoorthi, 2011). Over the years, the use of Indoor residual

spraying (IRS) and long-lasting insecticidal nets (LLINs) have been reported to contribute greatly to reductions in malaria cases globally (Ngufor *et al.*, 2017). Both these interventions involve the use of insecticides by either spraying them on walls and ceilings of houses (IRS) or by treating mosquito nets with insecticides (LLINs) to prevent human-mosquito contact and thus preventing the onward transmission of the parasite (Karunamoorthi, 2011). The WHO recently reported an increase in household ownership of LLINs from 50% in 2010 to 80% in 2016 across sub-Saharan Africa (WHO, 2017c). In addition to the above mentioned vector control strategies, larviciding is another form of vector control which according to WHO, involves the killing of mosquito larvae or creating unfavourable conditions which prevents mosquitoes from breeding (WHO, 2012). Namibia has been using IRS, LLNs and larvaciding vector control interventions to reduce mosquito to human transmission of the parasite (WHO, 2016b).

1.1.4.2. Parasite Control

In order to treat all malaria effectively, it is important to detect all cases of malaria. Microscopy detects the presence of malaria parasites by the visualization of a patient's blood under light microscopy, this is the WHO recommended gold standard for detecting malaria (WHO, 2018). However, this method has some drawbacks, especially in a low transmission such as Namibia where there are asymptomatic patients (WHO, 2017c). When these cases are left undetected and not treated they contribute to the onward transmission of malaria (Hawkins, Burton and Labarre, 2014). Additionally, microscopy is not reliable in a field setting where highly skilled microscopists, high quality microscopes and electricity is needed (Ilesanmi *et al.*, 2017). Thus, it is important to introduce more cost-effective tools to effectively diagnose and treat all cases of malaria (M. L. McMorrow, M. Aidoo, 2015).

1.1.4.3. Case Detection and Rapid Diagnostic Tests (RDTs)

According to WHO after the introduction of Rapid Diagnostic Tests (RDTs) an increase in malaria diagnostics in Africa was reported from 36% of suspected malaria cases in 2005 to 65% of suspected cases (WHO, 2016a). As compared to microscopy, RDTs are more cost effective, results can be viewed within 15 minutes and they can be used in field settings (Jimenez *et al.*, 2017). RDTs are point of care lateral flow immunochromatographic assays and work by detecting species specific antigens in human blood (Thompson, 2012). In addition to RDTs being easy to use and cost effective, different types of RDTs can detect different antigens in malaria parasites (Jimenez *et al.*, 2017). Three main antigens are detected by RDTs, these are histidine-rich protein 2 (HRP2) which is specific to *P. falciparum,* the parasite specific *plasmodium* lactate dehydrogenase (pLDH) which is *P. falciparum* specific, pan-specific (can detect all species of *plasmodium*), and *P. vivax*-specific and the *plasmodium* aldolase which is pan-specific (Kakkilaya, 2015). Although standard RDTs have a detection limit of 200ul/parasite, which may result in missing some cases in low transmission settings, they are more affordable and easy to use as compared to microscopy (Kakkilaya, 2015). As early diagnosis is important for disease management and surveillance.

1.1.4.4. Antimalarial Treatment

In order to ensure that all the previously mentioned malaria control interventions are effective, equal efforts should be directed towards antimalarial treatment (WHO, 2015a). WHO recommends that all patients with uncomplicated malaria be treated with ACTs which is the recommended first line treatment, except for pregnant women in their 1st trimester, which are treated with quinine + clindamycin (WHO, 2015a). In patients with severe malaria, including pregnant women WHO strongly recommends

treatment with intravenous or intramuscular artesunate for at least 24 hours and until they are able to tolerate oral medication (WHO, 2015a). Once a patient has received at least 24 hours of parenteral therapy and can tolerate oral therapy, they should complete the treatment with 3 days of ACT with a single dose primaquine in areas of low transmission (WHO, 2015a). Although there have been previous reports on drug resistance in Sulfadoxin-Pyrimethamine (SP) as a first line treatment (Shah *et al.*, 2015), WHO recommends that SP should be provided with Intermittent Preventative Treatment (IPTp-SP) to pregnant women in Africa, who are in their 2nd trimester (WHO, 2014b).In addition to IPTp-SP WHO also recommends that Intermittent Preventive Treatment with SP be given to infants (< 12 months of age) (SP-IPTi) in moderate to high transmission areas where SP is still effective (WHO, 2015b). The use of chemoprophylactic regimens such as mefloquine, atovaquone/proguanil (Malarone) or doxycycline is recommended to people travelling from non-endemic to endemic to prevent malaria (Cui *et al.*, 2015)

1.1.4.5. Malaria Vaccines

According to WHO a vaccine is a "biological preparation that improves immunity to a disease which contains an agent that resembles a disease-causing microorganism, in its weakened or killed form. This agent activates the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can easily recognize and destroy any of these microorganisms that it later encounters" (WHO, 2017b). There are currently 10 vaccines, listed in table 1 below which are either in their preclinical development or their clinical trial development against malaria (Coelho *et al.*, 2017). Only one which is commonly known as Mosquirix (RTS,S/AS01) is in phase IV of its clinical trials and has been approved by the European Medicines Agency's Committee for Medicinal Products for Human Use

(CHMP) (Coelho *et al.*, 2017) (Greenwood *et al.*, 2017). RTS,S/AS01 is a Pre-erythrocytic vaccine which targets the clinically silent sporozoite and liver stages of the *P. falciparum* and aims to eliminate the parasite during its early stages (Coelho *et al.*, 2017). RTS, S is made up of hepatitis B surface antigen (HBsAg) (S) particles which are fused to the *Plasmodium falciparum* circumsporozoite protein which induces the humoral (R region) and cellular immune (T region) responses (WHO, 2016a). The combination is expressed in yeast cells (*Saccharomyces cerevisiae*) by recombinant DNA technology to produce non-infectious virus-like particles (WHO, 2016a). Because of its recombinant composition, RTS,S can also be used as a vaccine against Hepatitis B (Ouattara and Laurens, 2015).

Table 1 Current malaria vaccines under development (Coelho *et al.*, 2017).

Parasite stage	Vaccine Classification	Status of Vaccine
Pre-erythrocytic Stage		
PfSPZ vaccine	Whole organism (radiation attenuated)	Phase II
GAP vaccines	Whole organism (genetic attenuated)	Phase I
RTS,S	Subunit	Phase IV
CVac	Whole organism (chemical Attenuated)	Phase I
Blood Stage		
Chemically attenuated parasites	Whole organism	Preclinical
AMA1-RON2	Subunit	Preclinical

PfRH5	Subunit	Phase I
Mosquito Stage		
Pfs25	Subunit	Phase I
Pfs230	Subunit	Phase I
Pfs47	Subunit	Preclinical

1.1.5. The Impact of Antimalarial Drug Resistance

Widespread *P. falciparum* resistance to the formerly used antimalarial medicines such as Chloroquine (CQ), Sulfadoxine-pyrimethamine (SP) and Amodiaquine (AQ) has led to changes in the malaria treatment policy in endemic countries, leading to the introduction of artemisinin-based combination therapies (ACTs) (Chilongola et al., 2014). WHO recommends that all uncomplicated *falciparum* malaria in both adults and children, except in pregnant women who are their 1st trimester be treated with either one of the following combination therapies: artemether + lumefantrine, artesunate + amodiaquine, artesunate + mefloquine, dihydroartemisinin + piperaquine and artesunate + sulfadoxine–pyrimethamine (SP) (WHO, 2015a). Recently there have been reports on declining efficacy of one of the ACTs which is the artesunate-mefloquine combination in Senegal, French Guiana, and on the Cambodia and Thailand border region of Southeast Asia, the same countries where emergence Chloroquine resistance originated from (Chilongola *et al.*, 2015). These findings are raising concerns around the efficacy of the other ACTs that are being used as first-line treatment.

The use of artemisinin in Namibia was introduced in 2005 and it is currently the first-line of treatment in Namibia, it is used in the combination therapy known as artemether + lumefantrine (Smith-Gueye *et al.*, 2014). This change in treatment was

made due to the high resistance levels of *P. falciparum* parasites to chloroquine in Namibia (Smith-Gueye *et al.*, 2014).

The development of antimalarial drug resistance is said to be associated with single nucleotide polymorphisms (SNPs) in genes such as the transporter genes which is the *Plasmodium falciparum chloroquine resistance transporter* (*pfcrt*) gene, or the increase in copy number of the *Plasmodium falciparum multidrug resistance1* (*pfmdr1*) gene (Petersen, Eastman and Lanzer, 2011). Additionally, antimalarial drug resistance may also be caused by the change in the parasite target of the antimalarial drug (Ouji *et al.*, 2018). These changes may be due to mutations at the cytosol level of genes encoding dihydropteroate synthase (*Pfdhps*) and dihydrofolate reductase (*Pfdhfr*) in sulfadoxine-pyrimethamine resistance, or at the mitochondrion level, cytochrome b which leads to atovaquone resistance (Ouji *et al.*, 2018). These markers can be an effective surveillance tools for monitoring the emergence of antimalarial drug resistance in malaria endemic countries such as Namibia.

1.2. Problem Statement

As countries move towards malaria elimination it becomes increasingly important to strengthen all malaria elimination strategies, including the effective use of antimalarial drugs as there are currently no new drugs available on the market. To date malaria control interventions have brought down malaria to a state of controlled low endemic malaria in Namibia. However, these control interventions effectiveness will be limited without equal efforts directed against antimalarial drug resistance. Hence, as Namibia is moving towards malaria elimination, the effectiveness of malaria case management without surveillance for resistance to antimalarial medicines will be limited. Therefore, monitoring and surveillance using molecular markers for drug resistance should be conducted to strengthen malaria control and

elimination efforts, as currently there is no data available on antimalarial drug resistance to ACTs and on Chloroquine among the *P. falciparum* population in Namibia.

1.3. General Objectives of the study

The main objective of this study was to determine the prevalence of *P. falciparum* point mutations in the Zambezi region of Namibia, that have been reported to be associated with resistance to the WHO recommended Artemisinin Combination Therarapy (ACT) and to Cholroquine, Amodiaquine and Lumefantrine. Artemether and lumefantrine is currently the first-line treatment in Namibia.

1.3.1. Specific Objectives of this study were to:

- Identify the different species of plasmodium parasites using multiplex PCR.
- Determine the prevalence of drug resistance polymorphisms at codon N86Y in the *pfmdr1* gene.
- Determine the prevalence of the three *Plasmodium falciparum* haplotypes formed by codons *crt*72-76 associated with antimalarial drug resistance in the *pfcrt* gene.
- Determine the prevalence of the 25 validated drug resistance markers in the Kelch 13 propeller protein encoded by the *PF3D7_1343700* gene.

1.4. Significance of the study

As many countries in Sub-Saharan Africa continue to scale-up malaria control measures, countries in Southern Africa are progressing towards malaria elimination (WHO, 2013). Thus, WHO recommends the routine monitoring of these antimalarial drugs using molecular markers as surveillance tools amongst other surveillance strategies to detect early emergence of drug resistance. This study will provide the

data on antimalarial drug resistance polymorphisms among the *P. falciparum* population in Namibia, in addition will attempt to provide baseline data on the level mutations associated with antimalarial drug resistance in the country. Such data will ensure the appropriate and effective use of antimalarial drugs in the country.

1.5. Limitations of the study

This study will be limited by the fact that only samples from Zambezi region, of Namibia will be analysed and will not be representative of the whole country. In addition, it will only focus on known mutations associated with artemisinin, chloroquine and lumefantrine resistance and not on the new and emerging ones.

1.6. Delimitations of the study

This study will only focus on drug resistance in *P. falciparum* and not on the other *plasmodium* species in the Zambezi region of Namibia. Quantitative PCR will not be conducted on all markers although copy number of markers may be a key factor in determining resistance.

2. Literature Review

Malaria is caused by protozoan parasites of the genus *Plasmodium*, it is highly infectious in tropical and subtropical countries causing severe illness and death (Chauhan, Pande and Das, 2014). The disease is transmitted by the bite of the female Anopheles mosquitoes (Winzeler and Manary, 2014). Malaria has been on the decline globally as shown in figure 3, nearly half of the world's population is of risk of malaria with sub-Saharan Africa reportedly carrying most of this burden (WHO, 2016). However, an estimated malaria case incidence decreased by 22% between 2010 and 2015, additionally the estimated malaria mortality rate has reduced by 22% between 2010 and 2015. To date, in Southern Africa zero countries have eliminated malaria since 2010 (WHO, 2016).

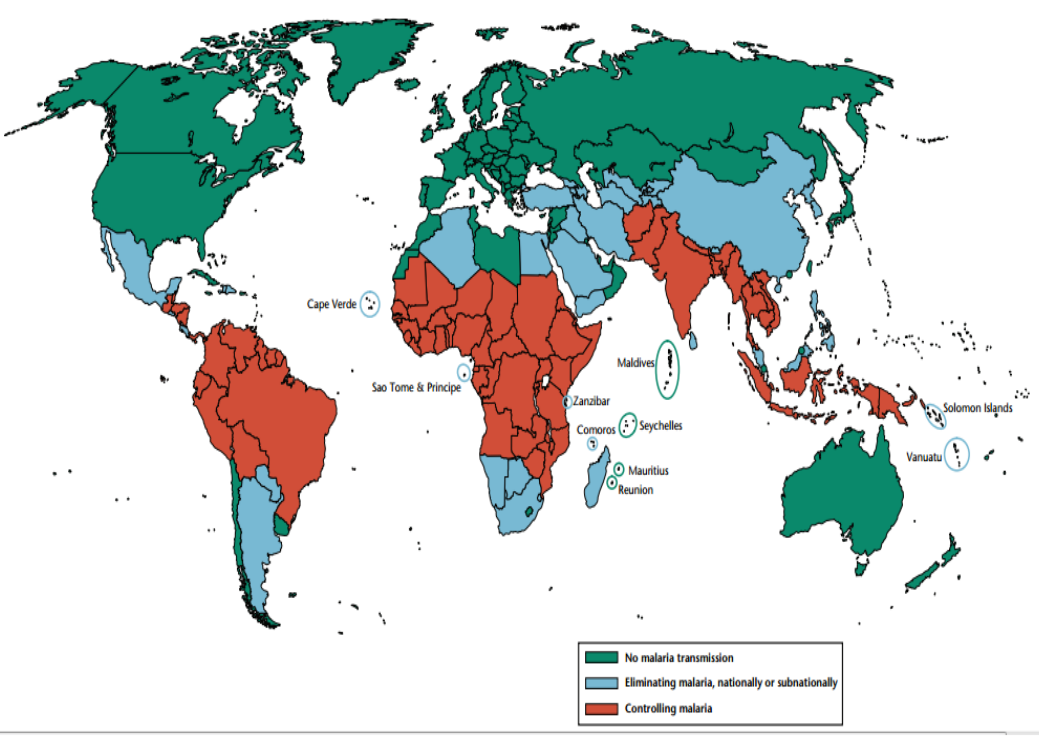


Figure 3. The malaria distribution map showing the global effort to eradicate malaria (Feachem et al., 2009).

Among the five species (*P.vivax, P.ovale, P.malaria, P.knowlesi* and *P.falciparum*) that infect humans, *P. falciparum* is the most severe form of the disease (Winzeler and Manary, 2014). Therefore, the development and spread of *P. falciparum* resistance to antimalarial drugs represents a major threat to global malaria control (Ghanchi *et al.*, 2011).

Following results of drug resistance to (CQ) and (SP), ACTs are now recommended by the (WHO) as first-line treatment of uncomplicated falciparum malaria in all areas in which malaria is endemic (Dondorp *et al.*, 2009). ACTs are the current treatment for *P. falciparum* malaria globally and they have reduced the morbidity and mortality associated with malaria. However, studies have shown signs that the efficacy of artemisinin-based combination therapy and artesunate monotherapy recently have declined in Southeast Asia (Dhorda *et al.*, 2015).

2.1. Antimalarial drugs

The currently available antimalarial drugs fall into three broad categories according to their chemical structure and mode of action (Fig 4):

1. Aryl amino alcohol compounds (Quinolines): quinine, quinidine, chloroquine, amodiaquine, mefloquine, halofantrine, lumefantrine, piperaquine, tafenoquine
2. Antifolate compounds (“antifols”): pyrimethamine, proguanil, chlorproguanil, trimethoprim
3. Artemisinin compounds (artemisinin, dihydroartemisinin, artemether, artesunate) (Deshpande and Kuppast, 2016).

2.1.1. Quinolines

2.1.1.1. Chloroquine (CQ)

Chloroquine is a 4-aminoquinoline as shown in figure 4 below and was introduced in the late 1940s and used on a massive scale for malaria treatment and prevention (Petersen, Eastman and Lanzer, 2011). Since this was affordable, non-toxic and effective against all strains of the plasmodium parasite, CQ was made the gold standard treatment of malaria for many years, until resistance was developed by *P. falciparum* to an extent that chloroquine has been rendered virtually unusable in most endemic areas (Eyasu, 2015).

According to Awasthi and Das (2013) CQ acts by reaching high concentrations in the vacuoles of the parasite, which, due to its alkaline nature, raises the internal pH of the parasite vacuole. CQ is said to control the conversion of toxic heme to hemozoin by inhibiting the biocrystallization of hemozoin, thus poisoning the parasite through excess levels of toxicity in the vacuole (Awasthi and Das, 2013).

Figure 4. Chemical structure of Chloroquine (WHO, 2015b)

2.1.1.2. Amodiaquine (AQ)

Amodiaquine, also a 4-aminoquinoline, is structurally similar to chloroquine shown in figure 5 and has been in use for more than 70 years (Shah *et al.*, 2015). It has a short half-life of 3 hours, therefore its antimalarial activity is thought to be used by the primary metabolite, monodesethylamodiaquine, which has a reported half-life of 9– 18 days (Fröberg *et al.*, 2012). Amodiaquine is said to have a similar mode of action as chloroquine and inhibits heme detoxification which further accumulates within the digestive vacuole of the parasite (Shah *et al.*, 2015). In previous *in vitro* studies, cross-resistance between chloroquine and amodiaquine have been observed and decreased susceptibility to both drugs has been said to be associated with mutations in the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) and *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*) genes (Shah *et al.*,

2015). However, when cross-resistance is incomplete some chloroquine resistant parasites remain susceptible to amodiaquine (Rosenthal, 2013).

Amodiaquine is a potent blood schizonticide that has been used for the treatment of uncomplicated malaria particularly in Africa. As AQ can cause neutropenia in patients it was not used for many years. However, it has recently been revived as part of an ACT (Petersen, Eastman and Lanzer, 2011).

Figure 5. Chemical structure of Amodiaquine (Deshpande and Kuppast, 2016)

2.1.1.3. Mefloquine (MQ)

Mefloquine is an aryl amino alcohol as pictured below (Figure 6) it is a blood schizonticide which mode of action is similar to that of chloroquine (Cui *et al.*, 2015). Mefloquine also acts by creating toxic compounds with the free heme in the parasite vacuole which poisons the parasite (‘Antimalarial Drugs_ Modes of Action snd Mechanisms of Resistance’, no date). It is active against the asexual blood stages of all malaria parasites and was introduced to treat patients with CQ resistant parasites. Additionally, MQ has also been reported to be active against the gametocyte stages of

P. vivax, P. ovale and *P. malariae* (Cui *et al.*, 2015). MQ was initially used as monotherapy in areas of low malaria transmission however due to resistance it is now principally used in combination with artesunate to treat *P. falciparum* in Southeast Asia (Phompradit *et al.*, 2014).

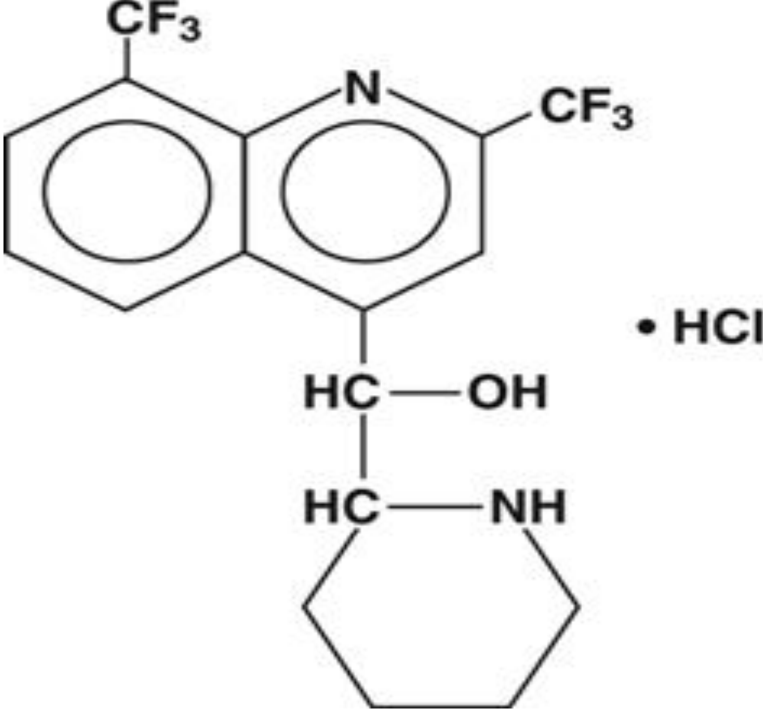


Figure 6. Chemical Structure of mefloquine (WHO, 2015b).

2.1.1.4. Lumefantrine

Lumefantrine is also a blood schizonticide that was first synthesized in China it was previously known as benflumetol (Petersen, Eastman and Lanzer, 2011). It is only available as an oral preparation and is used together with artemether (Study, 2015). The mechanism of action of lumefantrine is not yet fully understood but its mode of action is said to take place in the cytoplasm whereas the mode of action of many other antimalarial drugs such as Chloroquine takes place in the parasite the digestive vacuole (Morris, 2015). In recent studies it has also been shown that lumefantrine

inhibits haemozoin formation in the parasite cell, which shows that lumefantrine may have a similar mechanism to chloroquine (Petersen, Eastman and Lanzer, 2011).

Figure 7. Chemical structure of lumefantrine (Amadi, Otuokere and Chinedum, 2017).

2.1.1.5. Primaquine

Primaquine is an 8-aminoquinoline (figure 8), was introduced as an antimalarial in 1950 (Bhattacharjee and Shivaprakash, 2016). Primaquine works by exerting its action over the exoerythrocytic stages in the liver and is effective against intrahepatic forms of all types of malaria parasites. Additionally it is also gametocytocidal against *P. falciparum* (Bassat *et al.*, 2015). Although primaquine is recommended by WHO for treatment, patients with G6PD deficiency as well as pregnant and breastfeeding women should not be administered this drug unless the G6PD status of the infant is known as this drug can travel through the placenta (WHO, 2015b).

Figure 8. Chemical structure of Primaquine (Delves *et al.*, 2012)

2.1.2. Antifolates

Antifolates were reported to act on the folate metabolism of the parasite. Following resistance to CQ, Sulfadoxine/pyrimethamine (SP) was introduced to treat uncomplicated malaria (Ako *et al.*, 2012). Antifolates including proguanil and Sulfadoxine-pyrimethamine, were introduced in 1948 and 1967 respectively (Bhattacharjee and Shivaprakash, 2016). These drugs work by targeting two critical enzymes, the dihydrofolate reductase (DHFR) and the dihydropteroate synthase (DHPS) in the folate pathway (Sharma *et al.*, 2015).

However not long after their introduction it was found that the parasite had developed resistance to these antimalarial drugs, which led to their replacement by ACTs (Dondorp *et al.*, 2009).

Type 1

dapsone

sulfadoxine

sulfadiazine

sulfalene

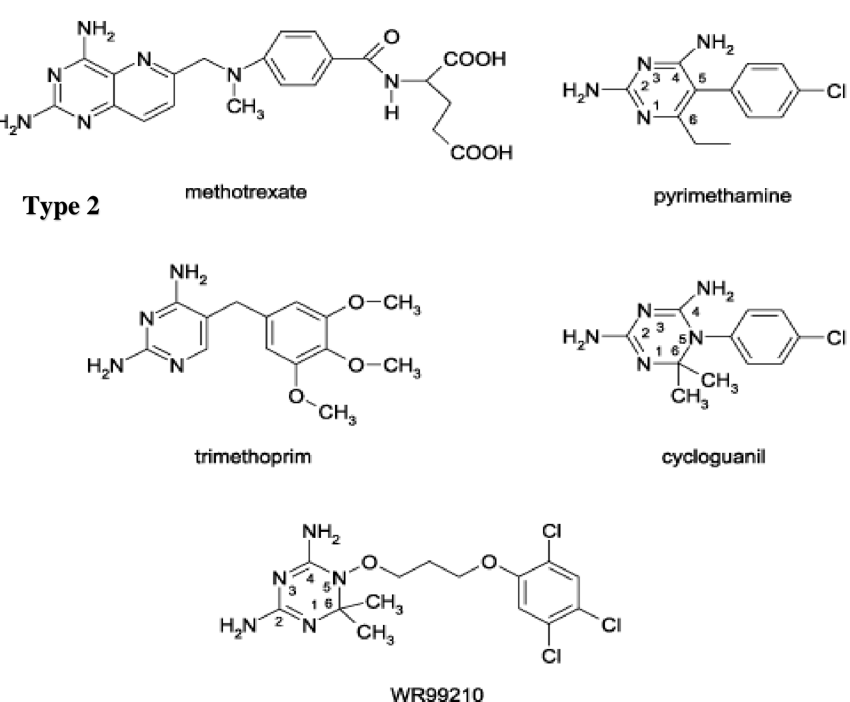


Figure 9. Chemical structures of both Type 1 and Type 2 Antifolates (Delves *et al.*, 2012)

2.1.3. Artemisinin

Artemisinin is a potent antimalarial, and their short half-life of around 1-2 hours helps prevent the selection of resistant parasites, by acting very fast and reducing the parasite load quickly (Dhorda *et al.*, 2015). They are active against the asexual stages of the parasites and are said to act on the young gametocyte stage in the life cycle of the parasite thus prevents the transmission of mature gametocytes (Aminake and Pradel, 2013). Artemisinin-based combination therapy (ACT) is the recommended standard first-line treatment for individuals with uncomplicated malaria (Bassat *et al.*, 2015). However, it is recommended that these drugs are used in combinations of artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, dihydroartemisinin-piperaquine, artesunate-pyronaridine, artesunate-sulfadoxine-pyrimethamine to protect drugs from resistance. The chemical structures of artemisinin and its derivatives (artesunate, artemether, arteether and dihydroartemisinin) are shown in figure 10 (Olasehinde et al., 2014).

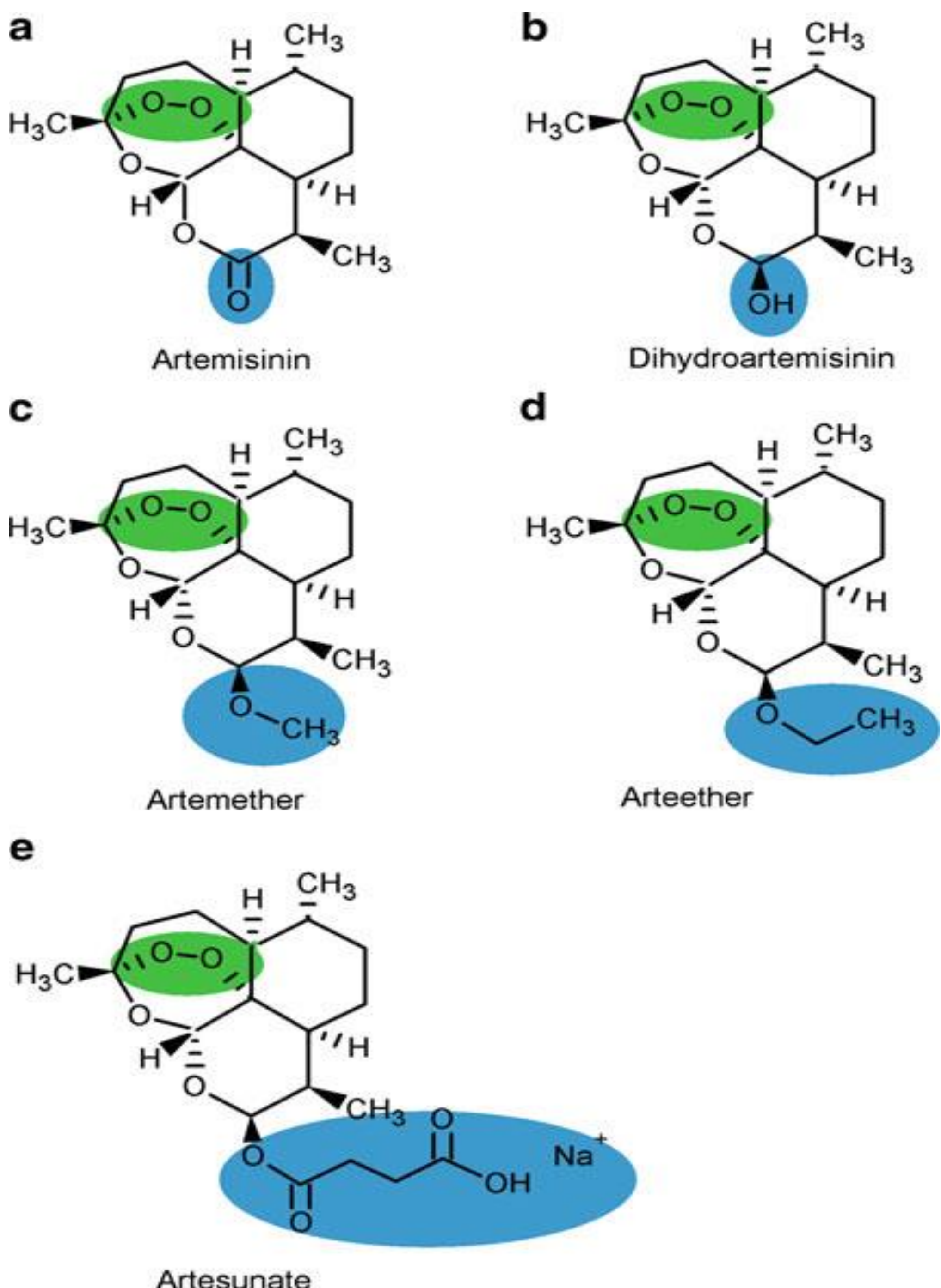


Figure 10. Chemical structure of Artemisinin and its derivatives (Staines and Krishna, 2012)

2.2. Antimalarial drug resistance

Drug resistance was defined in 1967, by the WHO as "the ability of a parasite strain to survive or multiply despite the administration and absorption of a drug given in

doses equal to or higher than those usually recommended but within the tolerance of the subject" (WHO,1967).

However, once treatment fails it does not necessarily mean that there is drug resistance, as treatment failure may be due to incorrect dosing, not finishing the recommended regimen within the prescribed time, poor quality drugs, drug interactions, poor or erratic absorption of the drug, and misdiagnosis (Sisowath, 2009). These factors nevertheless could lead to the development of resistant parasites to the drug they were inadequately exposed to (WHO, 2010).

The emergence of resistance in *Plasmodium* parasites depends on several factors, namely, the rate of mutation of the parasite, the fitness costs associated with the resistance mutations, the parasite load and the strength of the drug used (Petersen, Eastman and Lanzer, 2011).

2.3. Development and Spread of Drug Resistance

In order to successfully control and eliminate malaria, the use of effective drug therapy is important and the increasing levels of resistant *P. falciparum* parasites to commonly used drugs is hampering this goal (Nsanzabana *et al.*, 2018). The development of resistant parasites has been said to be caused by several different factors which include; the development of de novo resistant mutations of the *plasmodium* parasite which can be single or multiple, these mutations allows the parasite to survive the administered treatment, and in turn multiplies and is transmitted by the vector (WHO, 2010). These mutations give the parasite a survival advantage over the susceptible parasites, allowing them to be transmitted to the next host leading to the spread of resistant parasites (Goswami *et al.*, 2014).

2.4. Mechanisms of Antimalarial drug resistance

2.4.1. Resistance mediated by transporter mutations

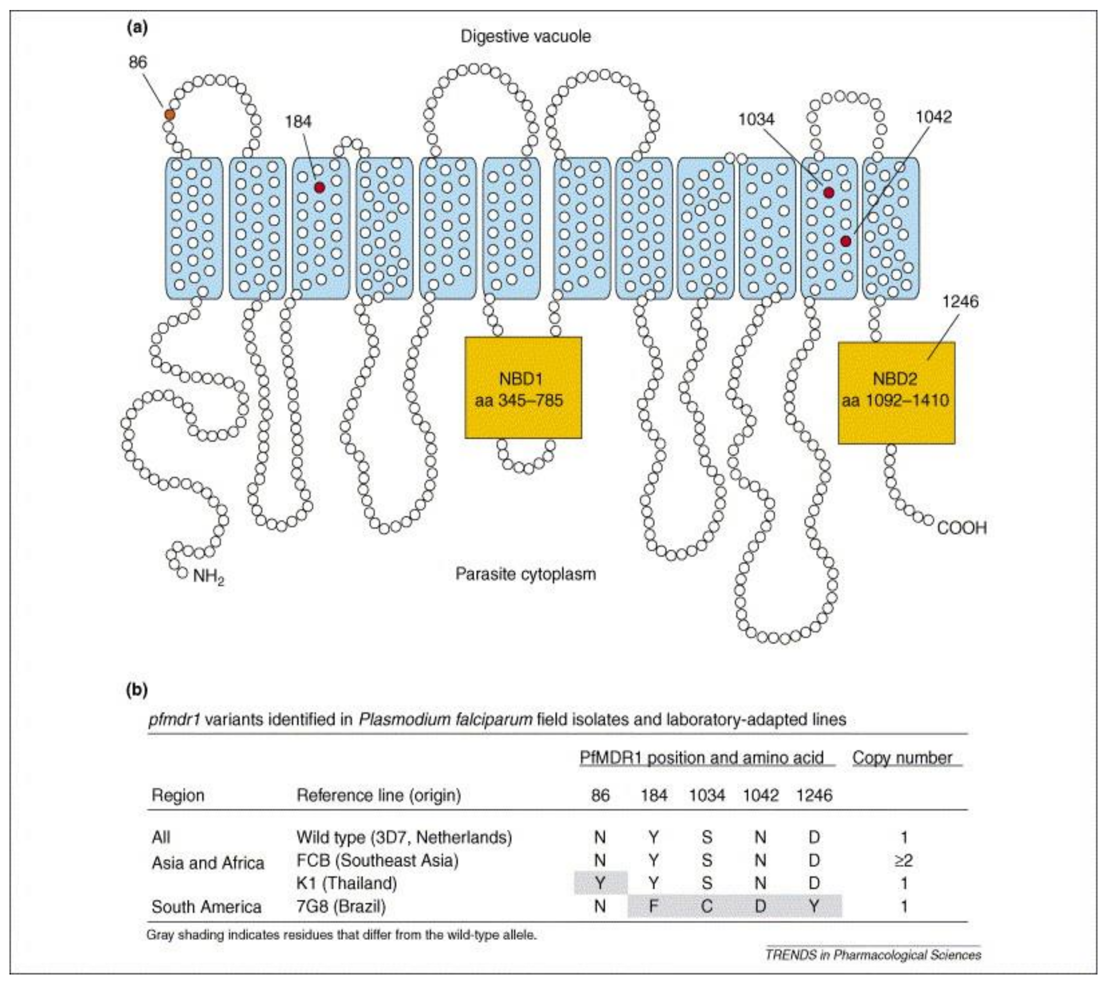
From previous studies it has been established that there are several genes which encode the parasite transporter proteins, *P. falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance1 (*pfmdr1*), they are located in membranes of digestive vacuoles, and are proposed to play a role as key contributors of resistance of *P. falciparum* to antimalarial drugs (Muhamad *et al.*, 2011). The parasite digestive vacuole is where many antimalarial drugs act, and amino acid substitutions at these sites may lead to antimalarial drug resistance by causing efflux of the drugs from the cells in the digestive vacuoles out of the parasites (Rosenthal, 2013).

2.4.1.1. The pfmrd1 gene

The *pfmrd1* gene encodes a 162 kDa protein, *P. falciparum* homologue of the P-glycoprotein (Pgh1), and it is located on chromosome 5, Pgh1 is in turn located in the parasite food vacuole (Figure 11) (Wurtz *et al.*, 2012). Pgh1 is believed to play a role in resistance to several antimalarial drugs (Inoue *et al.*, 2014). In many studies *pfmdr1* point mutations have been observed in both CQ resistant and sensitive strains of *P. falciparum* and are said to be responsible for the movement of antimalarial drugs from the cytosol into the digestive vacuole of the parasite (Ibraheem *et al.*, 2014). Studies showed that the substitution of amino acid, asparagine (N) by tyrosine (Y) at codon N86Y was linked to chloroquine and amodiaquine resistance, and it is also associated with increased sensitivity to MQ and artemisinin derivatives (Inoue *et al.*, 2014).

Furthermore, four other single nucleotide polymorphisms (SNP's) have been identified from field isolates in the *pfmdr1* gene (Y184F, S1034C, N1042D and

D1246Y). The mutant alleles at codons 1034C, 1042D and 1246Y have been linked to resistance in quinine and increased susceptibilities to artemisinin, mefloquine and halofantrine (Kavishe *et al.*, 2014). The mutant alleles at codons N86Y and D1246Y has been associated with the decrease in drug sensitivity in artesunate-amodiaquine (AS-AQ), whereas the wild type forms at these codons were shown to cause resistance in artemether-lumefantrine (AL) which is responsible for the movement of Al from the its site of action the cytoplasm into the food vacuole of the parasite (Kavishe *et al.*, 2014). According to Wurtz et al (2012), increased copy number of *pfmdr1* is the main cause of resistance to MQ in *P. falciparum*. Additionally, *pfmdr1* amplification has been linked to reduced sensitivity in artemisinin derivatives (Inoue et al.2014).



Region	Reference line (origin)	PfMDR1 position and amino acid 86	184	1034	1042	1246	Copy number
All	Wild type (3D7, Netherlands)	N	Y	S	N	D	1
Asia and Africa	FCB (Southeast Asia)	N	Y	S	N	D	≥2
	K1 (Thailand)	Y	Y	S	N	D	1
South America	7G8 (Brazil)	N	F	C	D	Y	1

Figure 11. Structure of the the P-glycoprotein and amino acid positions (Ibraheem *et al.*, 2014)

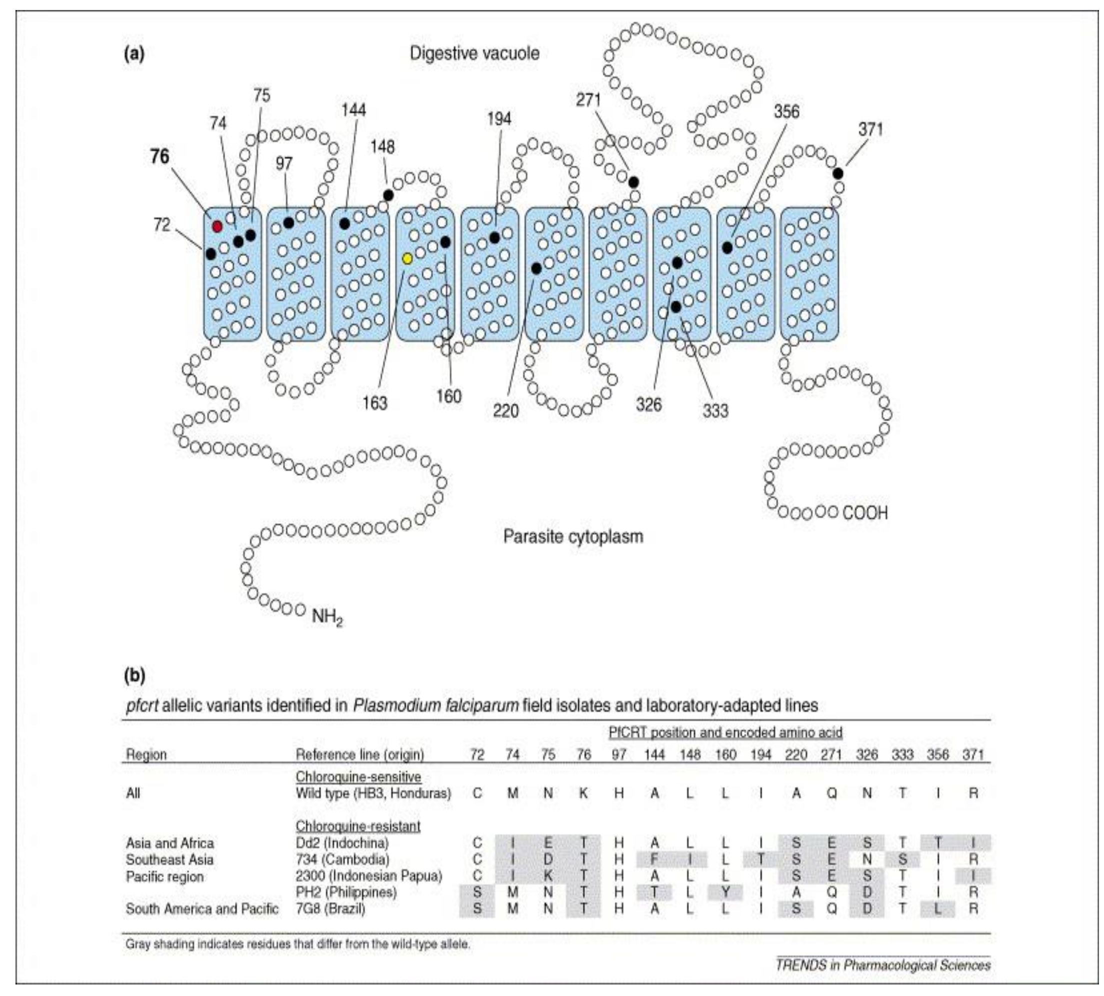
2.4.1.2. The pfcrt gene

The *pfcrt* gene encodes a transporter protein of 424 amino acids and 48.6 kDa which is located in the digestive vacuole of the parasite, and is located on chromosome 7 (Figure 12) (Saleh, Handayani and Anwar, 2014). Previous research indicate that change in K76T on the *pfcrt* gene results in the resistance phenotype, and is said to be the most reliable molecular marker of resistance (Olasehinde et al., 2014).

Mutations in the *pfcrt* gene which cause amino acid substitutions (Threonine (T), Asparagine (N) or Isoleucine (I)) changes the electric charge of the membrane, leading to the efflux of CQ and AQ from the digestive vacuole (Inoue *et al.*, 2014). Mutations

in the *pfcrt* gene has been associated with artemisinin, quinine and AQ susceptibility (Heuchert *et al.*, 2015). AQ and quinine were found to show cross-resistance with CQ, mediated by 76T, whereas lumefantrine displays an inverse cross-resistance, with the wild type K76 which in turn leads to reduced susceptibility to lumefantrine (Petersen, Eastman and Lanzer, 2011).

Previous studies show that 76T is usually found together with additional SNPs which create specific *pfcrt* 72–76 and the 271 and 371 haplotypes that indicate the origin of CQ resistance (Pathak *et al.*, 2014).



pfcrt allelic variants identified in *Plasmodium falciparum* field isolates and laboratory-adapted lines

Region	Reference line (origin)	72	74	75	76	97	144	148	160	194	220	271	326	333	356	371
	Chloroquine-sensitive															
All	Wild type (HB3, Honduras)	C	M	N	K	H	A	L	L	I	A	Q	N	T	I	R
	Chloroquine-resistant															
Asia and Africa	Dd2 (Indochina)	C	I	E	T	H	A	L	L	I	S	E	S	T	T	I
Southeast Asia	734 (Cambodia)	C	I	D	T	H	F	I	L	T	S	E	N	S	I	R
Pacific region	2300 (Indonesian Papua)	C	I	K	T	H	A	L	L	I	S	E	S	T	I	I
	PH2 (Philippines)	S	M	N	T	H	T	L	Y	I	A	Q	D	T	I	R
South America and Pacific	7G8 (Brazil)	S	M	N	T	H	A	L	L	I	S	Q	D	T	L	R

Gray shading indicates residues that differ from the wild-type allele.

TRENDS in Pharmacological Sciences

Figure 12. Structure of the transporter protein and amino acid positions (Pulcini *et al.*, 2015)

2.4.2. Resistance to Antifolates

Antifolates, target parasite dihydrofolate reductase (*DHFR*) and dihydropteroate synthase (*DHPS*), they bind to enzymes necessary for parasite folate biosynthesis and are subdivided into two classes: Type 1 (Pyrimethamine, chlorproguanil, trimethoprim) and Type 2 (sulphonamides: Sulfadoxine and dapsone) , they inhibit the enzymes DHFR, and DHPS respectively (Agomo *et al.*, 2016). According to Sharma et al, (2015) the aforementioned drugs inhibit *P. falciparum* dihydrofolate reductase (*pfdhfr*) thus indirectly blocks the synthesis of nucleic acids in the malaria parasite.

Molecular studies show that resistance to pyrimethamine has been associated with parasites that carry mutations at codons 51, 59, 108, and 164 in the *pfdhfr* gene, and resistance to Sulfadoxine with mutations at codons 436, 437, 540, 581, and 613 in the *P. falciparum* dihydropteroate synthase (*pfdhps*) gene (Ako *et al.*, 2012).

2.4.3. Artemisinin and Derivatives

Partial artemisinin resistance is observed when the parasite clearance rate is slow after treatment with ACT (Ashley *et al.*, 2014). However this definition is said to be affected by multiple factors such as patient immunity, blood drug concentration or partner drug activity (WHO, 2014). From treatment failures which were observed in South East Asia, studies showed that resistance was correlated either with lower efficacy of the partner drug and with slower parasite clearance rate due to artemisinin partial resistance (Escobar *et al.*, 2015).

Artemisinin resistance was previously linked with the sarco-/endoplasmic reticulum Ca 2+ -ATPase ortholog of *P. falciparum* (*PfATP6*), suggesting that mutations in *pfATP6* was involved in the mechanism that cause resistance in artemisinin (Muhamad *et al.*, 2011). This assumption was made because it was found that artemisinin decreases the ATPase activity in *Xenopus oocytes* that expresses the *PfATP6*, which has a similar potency to thapsigargin that is also another SERCA inhibitor (Winzeler and Manary, 2014). However recent studies state that resistance in artemisinin is caused by mutations on the Kelch 13 propeller protein K13 which is encoded in the *PF3D7_1343700* gene (Escobar *et al.*, 2015). Artemisinin resistance was first described in Cambodia, and the presence of K13 mutants (mainly Y493H, R539T, I543T and C580Y) are associated with *in vitro* parasite survival rates and *in vivo* parasite clearance rates (Taylor *et al.*, 2015).

Table 2; Summary of molecular markers associated with antimalarial drug resistance for the different drugs

Drug	**Gene**		
	Pfcrt	***Pfmdr1***	**Other**
Chloroquine	76T	86Y, Y184, 1034C, 1042D, 1246Y	
Quinine	76T	86Y, 1042D	*pfnhe1* ms4760
Amodiaquine	76T. 72-76 SVMNT	86Y, Y184, 1246Y	
Mefloquine		N86, Amplification	
Lumefantrine	K76	N86, 184F, D1246, Amplification	
Sulphadoxine-pyrimethamine			*Pfdhps* 437G, 540E *Pfdhr* 51I, 59R, 108N
Artemisinin	K76	N86, D1246, Amplification	K13 propeller C580Y, M476I, Y439H, R539T, I543T

Antimalarial drug resistance can be assessed in three ways; 1) The use of *in vitro* and *ex vivo* assays that involve the culturing of malaria parasites and exposing them to a

particular drug at different levels for the assessment of sensitivities of cultured parasites. 2) The use of *in vivo* test which involve the testing of malaria positive individuals with a known dose of a particular drug, during this test the participants are followed up and monitored for parasitological and clinical response for a specified period of time depending on the drug in use. 3) The evaluation of genetic polymorphisms associated with antimalarial drug resistance in specific genes (Eyasu, 2015).

Although the use of *in vitro* and *in vivo* tests provide data on clinical treatment failures, these test can be expensive to conduct in some endemic countries as they require highly trained personnel and well equipped laboratories (Nsanzabana *et al.*, 2018). Additionally using *in vivo* tests in a low transmission setting such as Namibia can become challenging because it gets difficult to get the required sample size to conduct efficacy studies (Nsanzabana *et al.*, 2018).

This study will focus on the evaluation of genetic polymorphisms associated with drug resistance, where the prevalence of these antimalarial drug resistance polymorphisms will be investigated. Thus, antimalarial drug resistance polymorphisms used as molecular markers may provide information on the emergence of drug resistance patterns in the field and hence can be used to design malaria control strategies particularly during case management studies (Olasehinde et al., 2014).

3. Research Methods

3.1. Research Design

This study was a qualitative study that employed the interpretation of results from Quantitative PCR, nested PCR and allele specific restriction enzyme digestion strategy. Malaria positive samples from the Zambezi region were investigated for the prevalence of mutations in the *pfmdr1, pfcrt* and Kelch 13 genes.

3.2. Methods

3.2.1. Study Population and Study Site

The target population was individuals from all households that were reported at the different randomly selected health facility catchment areas as well as the population from households surrounding the index case. The study was conducted in the Zambezi region (Figure 13) of Namibia. The Zambezi region is in the north-east of the country. The region shares borders with Angola, Botswana and Zambia (Kamwi *et al.*, 2015). Two rivers cross the region, namely the Kavango river which is in the western part and the Kwando river, both crossing into Botswana The Linyanti and Chobe rivers are found at the border, while the Zambezi river forms its north-eastern border.

A total of 143 samples from study participants that tested malaria positive with Rapid Diagnostic Test's (RDTs) were included in this study. All patients either provided an informed consent if over 18 and if under, the consent was provided by their parents or guardians.

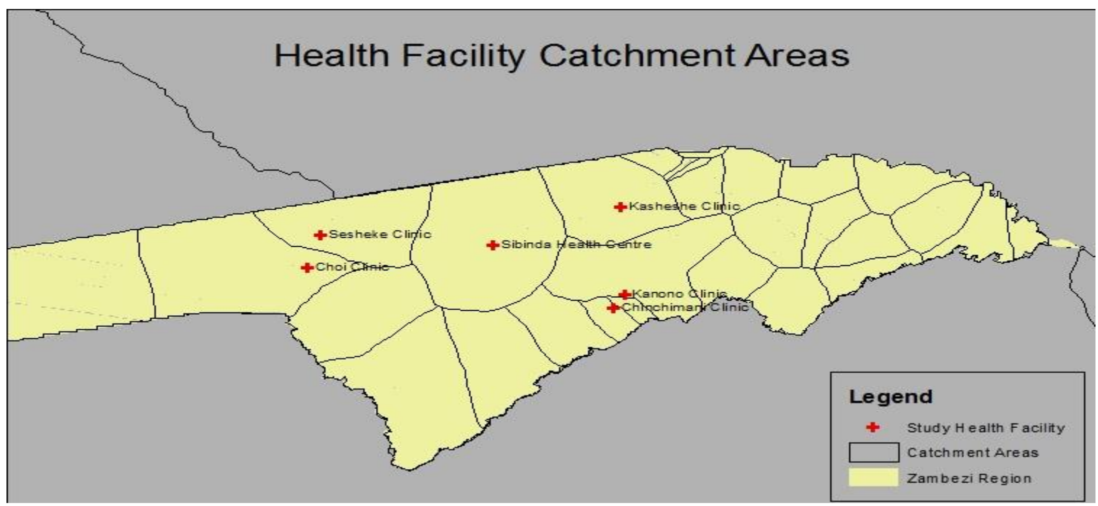


Figure 13. Showing the Zambezi region of Namibia where this study was conducted

3.2.2. Sample Collection

Blood samples were collected by trained nurses. Finger prick blood samples from malaria patients were collected and then a Rapid Diagnostic test (RDT) (Figure 14) was performed by adding 5µl of blood into the buffer of the RDT, at the same time blood were collected from the same individual on Whatman (3M) filter paper (Krackeler Scientific Inc., New York) and left to air dry to create dried blood spots (DBS) (Figure 14). Both the RDT and DBS were labelled with a unique patient ID and stored at -20°C for further analysis. This study is part of a larger study that detected malaria in the population of Zambezi region using serological and molecular tools and samples were collected from November 2016 to June 2017 in Zambezi region, Namibia.

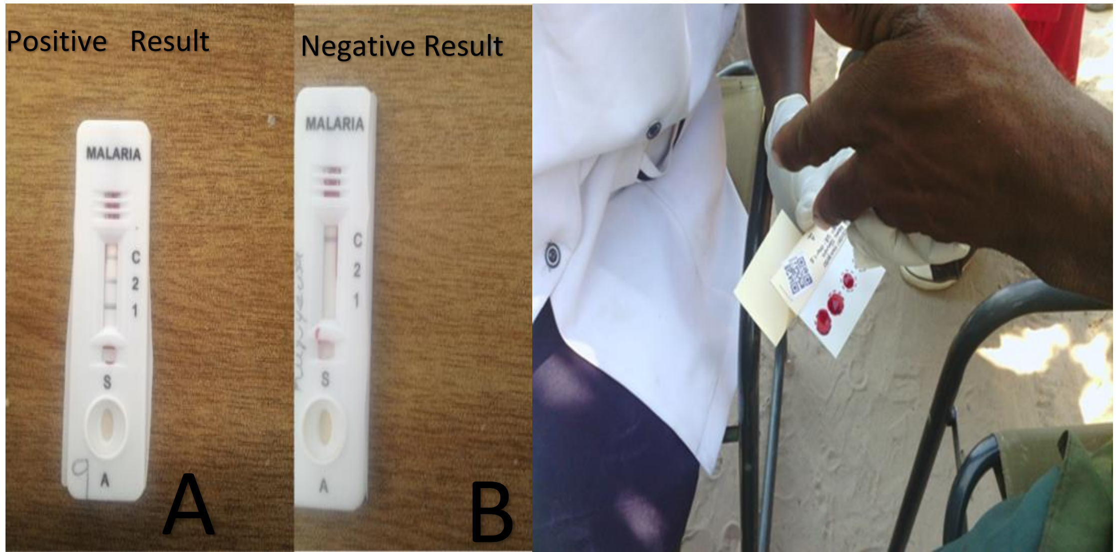


Figure 14. Representative pictures of RDTs (left) showing examples of a positive (A) and a negative (B) result. An example of a DBS (right) showing the collection of blood from a finger prick on filter paper.

3.3. Molecular Methods

3.3.1. Extraction of *plasmodium* parasite DNA using the QIAamp DNA mini-kit (Qiagen Germany)

P. falciparum DNA was extracted from blood samples collected on Whatman (3M) filter paper using the QIAamp DNA mini-kit (Qiagen Germany) as described in the standard procedures (Appendix 4).

3.3.2. *Plasmodium* Species Identification

In order to identify the different species in the *plasmodium* parasites from the Zambezi region, 143 malaria positive samples were amplified by Multiplex PCR. The samples were run in duplicates, to each reaction four positive controls (*P. falciparum, P. vivax*, *P. malariae* and *P. ovale)* were included as well as a negative control. The identification of the different *plasmodium* species involved primers as described in (Table 3).

This procedure targets the conserved 18S rRNA genes of the four *Plasmodium* species. During the amplification of each sample, a single reverse primer was included in the master mix for all four species with four species-specific forward primers to produce products of different sized amplicons. To each 0.2ml PCR tube 5µl of sample DNA was added with 12.5µl of 2G fast multiple mix (2x) Master Mix and 5.5µl of Nuclease free water with 1.0µl of forward primer mix and 1.0µl multi reverse primer. The final amplification was carried out in a total of 25ul. The cycling conditions of the PCR are as follows: the primary denaturing at 94°C for 5 minutes followed by 35 cycles of final denaturing at 94°C for 15 seconds, annealing at 60°C for 30 seconds, an extension step at 68°C for 15 seconds and a final extension step at 68°C for 2 minutes with the holding step at 4°C.

Table 3. Summary of Primer names and sequences used to amplify the different plasmodium species

Primer Name	Primer Sequence
P. falciparum	5'- -AACAGACGGGTAGTCATGATTGAG-3’
P. vivax	5'-AACAGACGGGTAGTCATGATTGAG-3’
P. ovale	5' -CTGTTCTTTGCATTCCTTATGC-3’
P. malariae	5'-CGTTAAGAATAAACGCCAAGCG-3’
Reverse Primer	5' -GTATCTGATCGTCTTCACTCCC-3’

3.3.3. SNP Analysis

To analyse the SNPs, the extracted DNA sample was amplified in a nested PCR process. The PCR process was carried out using mutant specific primers to the SNPs

3.3.3.1. Plasmodium falciparum multidrug resistance gene (pfmdr1) codon N86Y amplification

Amplification of *pfmdr1* gene by outer PCR for *pfmdr1* codon N86Y, involved primers as described in (Table 4). For *pfmdr1* codon N86Y amplification outer primers MDR-A and MDR-B were used. To each of the 0.2ml PCR tubes 5µl of sample DNA was added to 12.5µl One*Taq*® 2X Master Mix with Standard Buffer, 6.5µl Nuclease free water and 0.5 µl of each of the two primers. The cycling conditions for the reaction was programmed on PCR machine as one cycle of primary denaturing at 94°C for 30seconds followed by 30 cycles of final denaturing at 94°C for 30 seconds, annealing at 65°C 30 seconds, an extension step at 68°C for 1 minute and a final extension step at 68°C for 5 minutes with the holding step at 4°C.

Nested PCR of the *pfmdr1* gene at codon N86Y was performed in order to increase the sensitivity of low parasitaemia samples (Atroosh *et al.*, 2012). The product of the outer PCR was used as a template for the nested PCR (Table 4).

Inner primers MDR-D1 and MDR-D2 were used for codon 86, 5µl of the outer PCR products 12.5µl of Master Mix 6.5µl of Nucleus free water with 0.5 µl of each primer was added to the 0.2ml PCR tubes. The cycling conditions of the nested PCR was allowed to proceed for one cycle of primary denaturing at 94°C for 30 seconds followed by 30 cycles of final denaturing 92°C for 30 seconds, annealing at 53°C for 1 minute, an extension step at 68°C for 1 minute and a final extension step at 68°C for 5 minutes with the holding step at 4°C.

Table 4. Summary of the Primer names and sequences used for the amplification of the pfmdr1 gene at codon 86

Codon 86		
Primer Name		
Primary	Primer Sequences	Product bp
Pfmdr 86 sense primer MDR-A	5'**GCGCGCG**TTGAACAAAAAG AGTACCGCTG3'	
Pfmdr 86 antisense primer MDR-B	5'**GGGCCC**TCGTACCAATTCCT GAACTCAC3'	450
Secondary		
Pfmdr 86 sense primer MDR-D1	TTTACCGTTTAAATGTTTACCT GC	
Pfmdr 86 antisense primer MDR-D2	CCATCTTGATAAAAAACACTT CTT	291

3.3.4. Restriction Digest

3.3.4.1. Detection of pfmdr1 codon N86Y

Following amplification of the targeted fragments, polymorphisms in the *pfmdr1* gene were assessed as follows. Mutation in codon N86Y was detected by incubation of nested PCR fragments with *Af111*, 5µL of nested PCR product was added to 0.5µL of buffer, 0.5µL of enzyme and 17µL of water in total volume of 25µL. These aliquots were done according to enzyme manufacturer's specifications and instructions. The final volume was incubated for 5-15 minutes at 37°C. The product of the digest was

run on a 2.5% gel stained with ethidium bromide and bands were visualized using Gel documentation system.

The endonuclease *AflII* was purchased from Inqaba Biotechnical Industries (Pty) Ltd South Africa and incubations were setup following the manufacturer's instructions. Appropriate control DNA of samples with known *pfmdr1*sequences was used in parallel with field-collected parasite isolates in every PCR-RFLP protocol; these were Dd2 (genotype *pfmdr1* 86Y).

A 2% agarose gel was prepared by boiling 4g of LE agarose powder in 200ml of 1X TAE buffer (40mM Tris, 20mM Borate, and 1mM EDTA, pH=8). A total of 6µl of Ethidium Bromide (EtBr) was added to stain the agarose gel. After casting the gel using a gel casting apparatus, 3µl of each nested PCR product of either *mdr1* or *crt* genes were mixed with 2µl of 6X gel loading dye that contained bromophenol blue on a plastic sheet (para film). Then, each mixed nested product was loaded into the wells of the solidified gel, 3µl molecular weight marker with 100bp-ladder were loaded on to the gel as well.

3.3.6. Quantitative Real Time PCR for the Detection Of crt72-76 Haplotypes

To detect the two *Plasmodium falciparum crt* alleles, multiplex Real Time quantitative PCR (RT-PCR) (CFX Real Time Machine) was performed. Parasite DNA was extracted from dried bloodspots using the Qiagen extraction kit extracted DNA was stored at -20°C. For this experiment, labelled probes, CVMNK and CVIET were used to detect three common haplotypes at codons 72 to 76 of the *pfcrt* gene. Each probe was labelled with a reporter dye at the 5' end and a quencher moiety at the 3' end. Primers which are specific to the conserved region of *pfcrt* and the two probes were combined in one reaction, the oligonucleotide and probe sequences are described

in the tables below (Table 5). To each 96 well plate 2µl of extracted DNA was added to 12.5µl IQSuperMix, 7.9µl Nuclease free water and 1.0 µl of each *crt* forward and crt reverse primer as well as 0.3µl of the CVMNK probe (FAM) and CVIET probe (HEX), reactions were carried out in a total volume of 25µl. The cycling conditions for the reactions were 95°C for 10 minutes; (95°C for 15 seconds, 58°C for 1minute) x39 cycles. The different fluorescent molecules allowed for the detection of the three haplotypes that are present in each sample. Each sample was considered positive if a reporter dye was observed above the baseline threshold cycle, only samples with CT values between 16 and 35 cycles were considered successful. Samples with the FAM amplification curve were reported to carry the wild type (CVMNK) haplotype and samples with the HEX amplification were reported to carry the mutant (CVIET) haplotype. Furthermore, samples with both FAM and Hex amplifications were considered as a mixed infection carrying both the wild and mutant haplotypes. Two positive controls with known , 3D7 (CVMNK) and Dd2 (CVIET) as well as a no template control and an extraction negative control was included in all the experiments that were performed, samples were ran in 96 well plates (Inoue *et al.*, 2014).

Table 5. Primers used in the Multiplex real-time PCR for the detection of two P, falciparum crt alleles

Primer Name	Primer Sequences
crt forward primer	5’-TGG TAA ATG TGC TCA TGT GTT T-3’
crt1 Reverse primer	5’- AGT TTC GGA TGT TAC AAA ACT ATA GT-3’

Table 5.1. Probes used in the Multiplex real-time PCR for the detection of two P, falciparum crt alleles

Probe Name	Probe Sequences	Quencher
CVMNK	5'FAM-TGT GTA ATG AAT AAA ATT TTT GCT	AA-BHQ1 3'
CVIET	5'HEX- TGT GTA ATT GAA ACA ATT TTT GCT	AA-BHQ1-3'

3.3.7. Amplification and Sequencing of the K13 Propeller Gene

In order to amplify the K13-propeller domain of the parasite primers: (K13-1 5′-cggagtgaccaaatctggga-3′ and K13-4 5′-gggaatctggtggtaacagc-3′) were used for outer PCR amplification and for nested PCR the following primers were used: (K13-2 5′-gccaagctgccattcatttg-3′ and K13-3 5′-gccttgttgaaagaagcaga-3′) (Ariey *et al.*, 2014).

For primary PCR, 1 µl of DNA was amplified with 1 µM of each primer, 0.2 mM dNTP (New England biolabs), 3 mM $MgCl_2$ and 2U *Taq* DNA polymerase (New England biolabs) in a total reaction of 25ul. The following cycling conditions were used: 5 min at 94 °C, then 40 cycles of 30 s at 94 °C, 90 s at 60 °C, 90 s at 72 °C and final extension 10 min at 72 °C.

For the nested PCR, 2 µl of primary PCR products were amplified with 1 µM of each primer, 0.2 mM dNTP (New England biolabs), 2.5 mM $MgCl_2$ and 2U *Taq* DNA polymerase (New England biolabs) in a total reaction of 25ul. The following cycling conditions were used: 5 min at 94 °C, then 40 cycles of 30 s at 94 °C, 90 s at 60 °C, 90 s at 72 °C and final extension 10 min at 72 °C.

3.3.7.1. Gel Electrophoresis and Sequence Analysis

PCR products were detected using 2% agarose gel electrophoresis and ethidium bromide staining. Double-strand sequencing of PCR products were performed by Inqaba biotech Sanger DNA BI 3500XL sequencer. The sequence alignments and analysis were carried out using the Bio Edit software. Twenty-five amino acid sequences which were previously reported to be associated with drug resistance in Artemisinin were analysed by comparing them to wild-type sequences. The sequences of the amplicons were aligned with protein XP_001350158.1 (3D7 Kelch 13 sequences) from the NCBI database by BLAST analysis to identify specific SNP combinations.

Table 6. Primer names and sequences used during the amplification of the Kelch 13 gene

K13 Propeller		
Primer Name		
Primary	Primer Sequences	Product bp
K13-Propeller F	5′-cggagtgaccaaatctggga-3	
K13-Propeller R	5′-gggaatctggtggtaacagc-3′	2096
Secondary		
K13-Propeller F	5′-gccaagctgccattcatttg-3′	
K13-Propeller R	5′-gccttgttgaaagaagcaga-3′	848

3.4. Data Analysis

Base-line frequencies of *P. falciparum* point mutations associated with artemisinin resistance in Northern Namibia will be calculated, using data that has been entered and validated with Microsoft Office Excel® 2013 (Microsoft Corporation

4. Research Ethics

Approval for this study was obtained from the medical directors and administrators of health facilities before samples were collected. The study was explained to the participants in their own language. A written informed consent of the participants or their parents /guardians /representatives, has been received before samples were collected. The names of the participants were kept anonymous. Samples were collected and handled by trained nurses. There was no risk to participants except for mild pain during blood collection. Sterile techniques were enforced at all times during the study.

This study was conducted within larger parent studies conducted in the Zambezi region. Ethical approval for the parent study has been obtained from the University of Namibia and the Ministry of Health and Social Services Biomedical Research Ethics Committee. Research Ethical Clearance has been obtained from the University of Namibia Research Ethics Committee.

5. Results

A total of 143 RDT positive malaria samples were speciated using multiplex PCR. They were also analysed for polymorphisms at codon N86Y of the *pfmdr1* gene and crt 72-76 haplotypes of the *pfcrt* gene and sequenced of the Kelch 13 codons. Additionally, mutations at codons crt 72-76 were analysed using quantitative real-time PCR.

5.1. Identification of *Plasmodium* species by Multiplex PCR

To identify the different plasmodium species in the Zambezi region 143 samples were amplified by multiplex PCR with species specific primers. Only 84/143 (58.7%) samples were successfully amplified. The species distribution was as follows 64/84 (76.2%) *P. falciparum*, 6/84 (7.1%) *P. vivax* and 5/84 (6.0%) *P. malariae* parasites. Additionally, 9 mixed infections were found of which 8/84 (9.5%) were *P. falciparum* mixed with *P. vivax* mixed and 1/84 (1.2%) was *P. falciparum* mixed with *P. malariae* and. No *P. ovale* parasites were found. Figure 15 below shows a representative of the results obtained during the multiplex PCR. Additionally, frequency of the different parasites found are summarized below in (figure. 15.1)

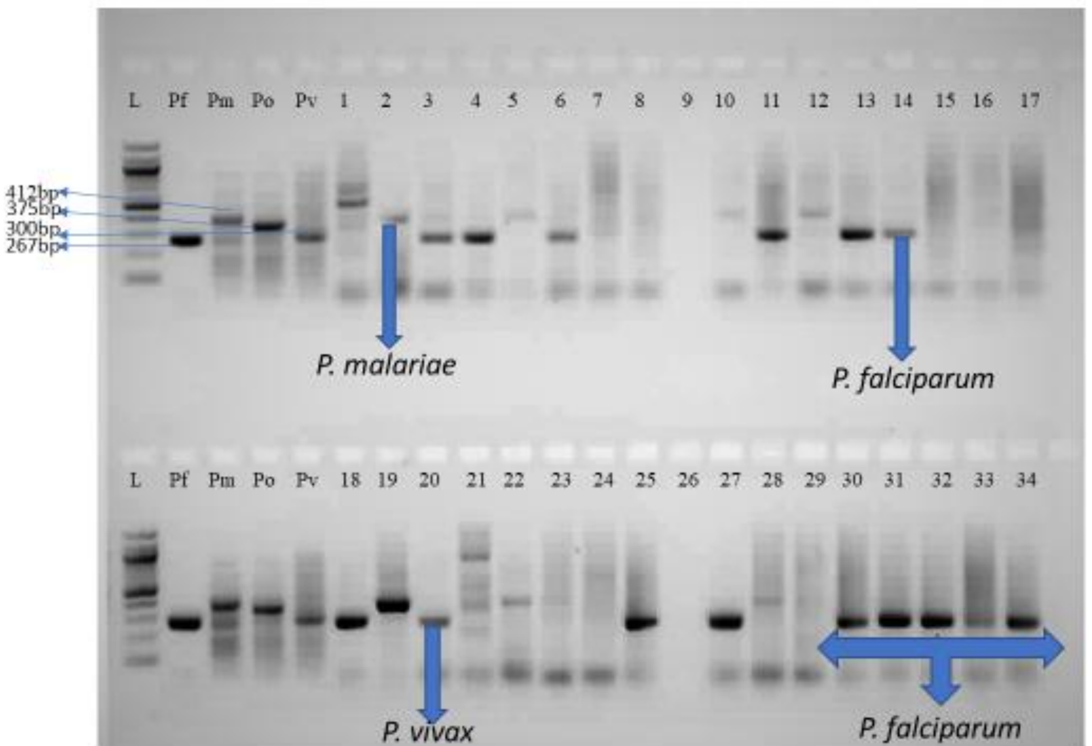


Figure 15; Gel representative showing the identification of the four different Plasmodium species. Four positive controls (P. falciparum, P. malariae, P. ovale and P. vivax) were included during each run as well as a negative control. Sample 2 shows an example of a P. malariae infection, Sample 20 shows an example of a P. vivax infection whereas samples 30-34 shows examples of P. falciparum infections. Additionally, lanes 9 and 26 contain no template samples.

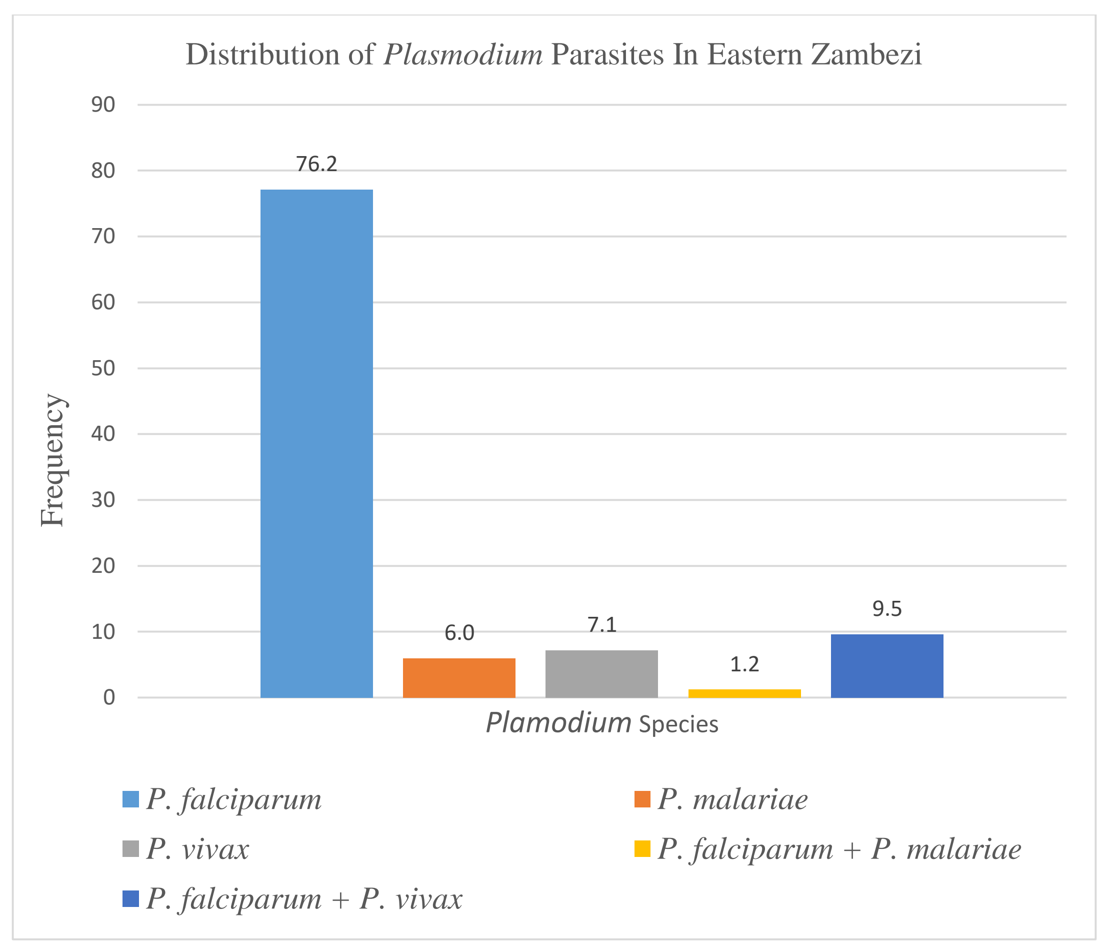


Figure 15.1. Prevalence of the Plasmodium species in western Zambezi Region

5.2. Prevalence of the *pfcrt* 72-76 haplotypes

The *pfcrt* 72-76 haplotype was determined in 73 samples from western Zambezi region which were either *P. falciparum* positive or mixed with *P. falciparum* and either *P. vivax* or *P. malariae*. Only 53/73 (72.6%) samples were successfully amplified by q-PCR. The results show that 49/53 (92.5%) samples contained wild-type (CVMNK) haplotype, 2/53 (3.8%) contained resistant (CVIET) haplotype while 2/53 (3.8%) samples had mixed (CVMNK/CVIET) infections (Table. 7).

Table 7; Prevalence of wild type mutant and mixed haplotypes found in the pfcrt gene 72-76 in gene from the Zambezi Region of Namibia.

Haplotypes	Frequency	Percentage %
CVMNK Wild Type	49	92.5
CVIET Mutant	2	3.8
MIXED	2	3.8

5.3. Prevalence of the *pfmdr1* N86Y mutation

At position N86Y, 27/73 (37.0%) samples were amplified with primers with an artificially introduced restriction site for the enzyme and digested following amplification with the *AflII* restriction enzyme. Results showed 5/27 (18.5%) mutants and 16/27 (59.3%) wild and type alleles. Figure 16 below shows a representative of the results obtained for wild type and mutant type at position N86Y.

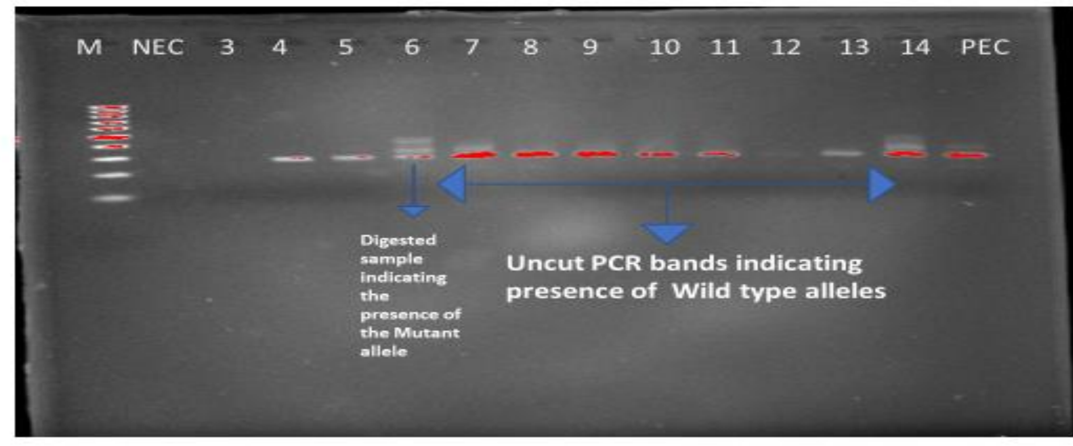


Figure 16; Gel representative showing restriction digest PCR product from the pfmdr1 gene. Lane M=Molecular weight; NEC= negative control, Lane 3=no template control, Lane 4,5,7,8,9,10,11,12 and 13=P. falciparum malaria positive

isolates carrying the wild type allele, Lane 6 and 14= P. falciparum malaria positive isolates carrying the mutant allele, PEC= Positive control

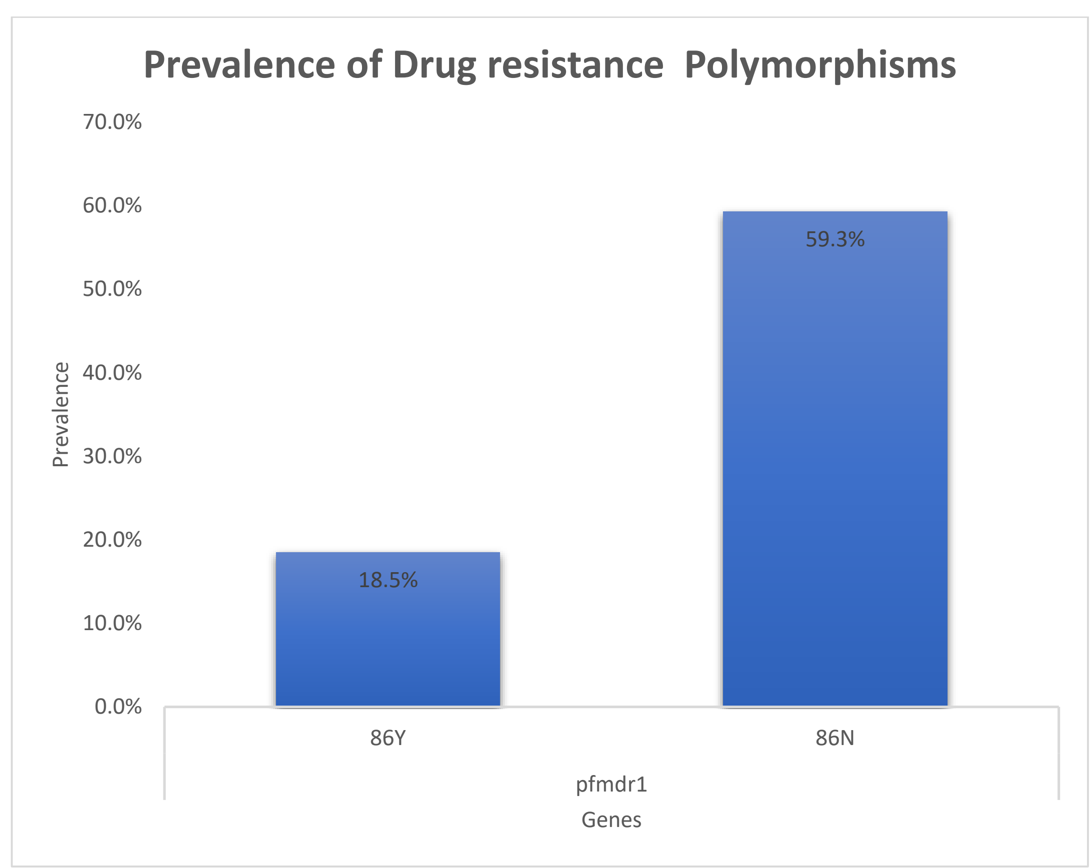


Figure 17. Prevalence of Drug resistance Polymorphisms, wild type and mutant alleles at codon N86Y in the pfmdr1 gene

5.4. Prevalence of SNPs in the Kelch 13 propeller gene

In order to analyse the previously validated markers as well as candidate markers in the Kelch 13 gene associated with delayed parasite clearance, 25-point mutations that are linked to reduced artemisinin efficacy were investigated. A full-length sequencing of *P. falciparum* Kelch 13 gene was evaluated in 64 *P. falciparum* malaria positive samples. Only 49/64 (76.6%) isolates were successfully amplified (nested PCR

products are shown in figure 18 below) and sequenced by comparing them to the 3D7 (XP_001350158.1) reference strain which cover codons 441–675. All samples evaluated carried the wild type allele for all the markers assessed during this study 49/49 (100%).

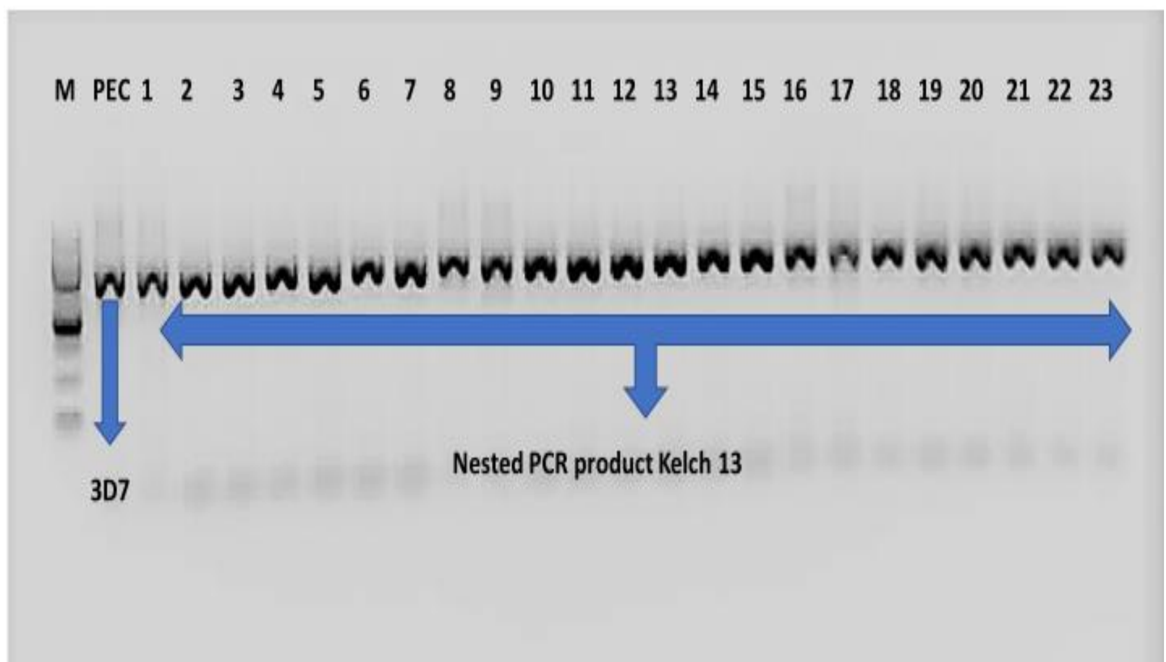


Figure 18. Gel representative showing nested PCR product from the Kelch 13 propeller gene. Lane M=Molecular weight lane PEC= Positive control, Lane 1- 23= P. falciparum malaria positive isolates

6. Discussion

Using Single Nucleotide Polymorphisms (SNPs) as surveillance tools is an effective way to monitor early emergence of antimalarial drug resistance before *in vivo* efficacy studies are conducted as these studies can be time consuming and in some instances can take long to get ethical approval (WHO 2009). To date there are no reports on the prevalence of drug resistance markers in any part of Namibia although there are other reports in the Southern African region such as in Angola, Mozambique and Zambia (Foumane-Ngane *et al.*, 2015) (Escobar *et al.*, 2015) (Mwanza *et al.*, 2016). This is the first such study conducted in Namibia reporting on antimalarial drug resistance markers in *P. falciparum* isolates. The study also investigated the distribution of *plasmodium* species in malaria positive participants in the Zambezi region from the different catchment areas. One hundred and forty-three samples collected from the Zambezi region in the North eastern part of Namibia which were confirmed malaria positive by RDT and LAMP were used to extract DNA to assess the presence of molecular markers associated with antimalarial drug resistance. In this study three genes were investigated namely *pfcrt, pfmdr1* and the Kelch13 propeller gene.

The study revealed that the majority of the infections were due to *P. falciparum* (76.2%), these findings were consistent with previous reports stating that *P. falciparum* accounts for majority of the malaria infections in Namibia (Smith-Gueye *et al.*, 2014)(Noor *et al.*, 2013). These results also support findings that show that the majority of malaria cases are caused by *P. falciparum* in Africa (Noor *et al.*, 2013) (Balogun *et al.*, 2016). This number however was found to be much lower than what was reported previously by Noor et al 2013, of which showed that 90.97% of cases were caused by *P. falciparum* in Namibia. The decrease could be due to the decrease in the number of malaria cases since 2013 (Smith-Gueye *et al.*, 2014). Studies have

shown that as malaria cases decrease, the parasite density also decreases, which makes the identification of parasites using PCR more difficult (Vallejo *et al.*, 2015). This may have been the case during this study which resulted in the detection of the lower prevalence of *P. falciparum* cases as previously compared and could also explain why only 58.7% of the samples were successfully amplified during species identification essay. Additionally, previous studies have shown that PCR is not as sensitive in detecting the presence of parasites in a low transmission setting such as Namibia with a detection limit of 6 parasites/µl of blood from dried spots (Laufer *et al.*, 2010) thus could explain the lower prevalence of 76.2% *P. falciparum* parasites as compared to the 90% previously report by Noor et al, since Multiplex PCR was used for species identification during this study.

Although mixed infections and other *plasmodium* species were not expected as previously reported, during this study other species were found with *P. vivax* accounting for 7.1% of the total malaria cases followed by 6.0% of *P. malariae* infections, which could be as a result of imported malaria cases from other endemic countries. The findings were in contrast with what was reported by WHO that stated that 100% of all malaria infections were due to *P. falciparum* (WHO, 2016) and thus calls for molecular species identification studies to detect and treat all malaria cases effectively. It is however suspected that these *plasmodium* species may have been missed during the previous studies, for the reason that firstly *P. vivax* is known to remain dormant for weeks to years after infection (Lindblade *et al.*, 2013). Additionally *P. vivax* cannot be easily detected during asymptomatic patients and thus can have been missed during the time these studies were conducted and may have remerged during the present study (Lindblade *et al.*, 2013). Secondly because Namibia is a low transmission setting the tools used such as light microscopy which

is the gold standard for species detection may have missed these parasites at the time of previous studies (Hopkins *et al.*, 2013). Thirdly studies have found that *P. malariae* is found at a lower prevalence and density in endemic areas (Kwenti *et al.*, 2017). Since Namibia is a low transmission setting, the conventional methods used previously could have missed the presence of this particular parasite in Namibia (Perera *et al.*, 2017). Finally, because most Sub-Saharan African countries report that *P. falciparum* is the predominant species, diagnostics tools such as RDTs and Loop-mediated isothermal amplification (LAMP) kits are developed with the focus on *P. falciparum* sensitivity only and with limited effectiveness directed towards the sensitivity of other species thus could be missing these species during diagnosis (Hayashida *et al.*, 2017).

Nevertheless, the results observed during this study were similar to the findings reported in Luanda, Angola. It was reported that *P. falciparum* (92%) accounted for the majority of infections followed by low prevalence of infections caused by *P. vivax* (7.0%) and *P. malariae* (3.0%) (Pembele, Rivero and Fraga, 2015). Furthermore, a study done in Zambia also observed that 88% of their cases were *P. falciparum* making up majority of the cases and 10.6% were mixed infections (Sitali *et al.*, 2015).

These similarities in parasite distributions may be a result of the number of travellers from these countries to Namibia and as previously reported, the majority of malaria cases that occur in Northern Namibia are from Angola (Njovu *et al.*, 2013) (Smith Gueye *et al.*, 2014). Thus, to eliminate malaria, countries sharing borders are proposed to work together to detect and treat all cases of malaria as malaria parasites are known to move freely between countries. Additionally, mono and mixed infections with *P. ovale* species (*P. ovale curtisi* and *P. ovale wallikeri*) were observed in Angola and Zambia, whereas during this study, 0% *P. ovale* infections were found (Pembele,

Rivero and Fraga, 2015)(Hayashida *et al.*, 2017). Similarly, no *P. ovale* infections have been reported in previous studies that were conducted in Namibia (Noor *et al.*, 2013).

Although a low prevalence of *P. malariae* cases and no *P. ovale* cases were found in the present study, a study conducted in Jiangsu Province, China has found that most of their imported *P. malariae* and *P. ovale* cases came from Africa and stated that the majority of the *P. malariae* cases were from Sub-Saharan Africa (Cao *et al.*, 2016). These findings by Cao et al have been confirmed by a study done in the Democratic Republic of the Congo where they found that 26.3 % of the parasites tested positive for *P. malariae* and 17.2 % were positive for *P. ovale* (Doctor *et al.*, 2016). Furthermore, a low frequency of mixed infections with *P. falciparum* was observed in this study of which 1.2% were *P. malariae* mixed with *P. falciparum* and 9.5% were *P. vivax* mixed with *P. falciparum.* These findings are however in contrast with what was reported in Zambia, where they found that majority of their mixed infections were a combination of *P. falciparum* and *P. malariae* (Sitali *et al.*, 2015).

This study has shown that *P. falciparum* is not the only *plasmodium* species present in Namibia that cause malaria infections as previously reported by WHO (WHO, 2016). Thus, in order to detect and treat all malaria cases with the appropriate antimalarial drugs and to avoid the misuse of antimalarial medicine. Species identification should be done after mixed infections are detected by RDTs using both molecular methods and the recommended gold standard, as the gold standard has been previously reported to not be as accurate in the successful differentiation between different species (Stanis *et al.*, 2016)(Dafalla *et al.*, 2017). This will allow for the accurate administration of correct antimalarial medicines once an individual is tested positive for malaria.

Following the identification of the distinct species, samples that tested positive for *P. falciparum* as well as mixed samples with *P. falciparum* were included to determine the prevalence of antimalarial drug resistance makers in the *pfcrt* gene. The occurrence of the CVIET (mutant), CVMNK (wild type) and the Mixed (CVIET/CVMNK) haplotypes were studied at codons 72-76. Many studies have linked point mutations in this gene with drug resistance in previously used antimalarial drugs such as Chloroquine and Amodiaquine (Rosenthal, 2013)(Golassa *et al.*, 2014). Whereas the wild type form at codon K76T has been linked to the reduced sensitivity in Lumefantrine which is currently being used as a partner drug with artemisinin in Namibia (Rosenthal, 2013). Additionally, the presence of the CVMNK haplotype has been linked with reduced sensitivity in Artemisinin's which are currently in use with partner drugs as a first line treatment in many African countries.

The CVMNK and SVMNT are known as the "mother" haplotypes in the *pfcrt* gene. However derivatives for SVMNT such as CVIET which has been previously reported to be present in Africa is being used as the mutant haplotype in different parts of the world (Zhou *et al.*, 2016). During this study the CVIET derivative was used as the mutant haplotype, because the SVMNK haplotype is said to be dominant in regions outside Africa (South America, Indonesia, Papua New Guinea, and the Philippines in Southeast Asia) (Reteng *et al.*, 2017).

In order to test for the prevalence of resistant, sensitive and mixed haplotypes in the *pfcrt* gene 73 samples found to be either *P. falciparum* positive or mixed with *P. falciparum* and either *P. vivax* or *P. malariae* were genotyped. The 3D7 strain that harbours the CVMNK haplotype and the Dd2 harbouring the CVIET haplotype were

included as controls during the amplification along with a no template and a PCR negative control.

During this study a high prevalence of isolates carrying the wild type haplotype (92.5%) and low prevalence of (3.8%) isolates carrying the mutant haplotype was observed. Additionally, only 3.8% of the samples carried the mixed haplotype in the isolates from Zambezi region. Similar results were found in studies done in Nigeria (Salissou *et al.*, 2014), Zambia (Mwanza *et al.*, 2016) and Malawi (Laufer *et al.*, 2010). Studies conducted in Ethiopia (Golassa *et al.*, 2014), North Cameroon (Ali *et al.*, 2013) , Gabon (Salissou *et al.*, 2014) and Angola (Fancony, Brito and Gil, 2016) presented contrasting findings to the present study where a high prevalence of the CVIET haplotype was observed and low prevalence of the CVMNK haplotype. The results from this study has shown that the withdrawal of Chloroquine as treatment for *P. falciparum* malaria after CQR strains have been encountered has led to the increase in wildtype haplotypes in the *pfcrt* gene, thus showing the revival of chloroquine sensitive (CQS) populations of *P. falciparum* in Namibia. These findings support the possible reintroduction of chloroquine as a partner drug with ACTs in the future with continual observation of antimalarial drug resistance. Additionally, studies have shown that the increase in the wild type allele at codon K76T can lead to the reduced sensitivity in Lumefantrine as parasites have selected for the wild type allele during *ex vivo* studies (Taylor *et al.*, 2016). However, this selection alone does not lead to a resistance phenotype in Lumefantrine therefore calling for studies on *pfmdr1* copy number which has been said to cause resistance in the presence of the K76 allele to support these findings (Shah *et al.*, 2015).

Furthermore, this study also aimed to observe the presence of point mutations in the *pfmdr1* gene at codon N86Y. Mutations in this gene have also been linked to

antimalarial drug resistance in Chloroquine, Amodiaquine and Quinoline (QN) (Ibraheem *et al.*, 2014). Whereas the wild type alleles have been linked to reduced susceptibility in Halofantrine and Lumefantrine. Additionally the amplification of this gene has been linked to reduced susceptibility in Mefloquine (MQ) and Artemisinin (Heuchert *et al.*, 2015).

At position N86Y, 37.0% samples were amplified and digested with the *AflII* restriction enzyme. There was a low prevalence of (18.5%) mutant alleles and a high prevalence of 59.3% wild type alleles. The low prevalence observed at this position is consistent with the low prevalence found in the *pfcrt* CVIET haplotype also observed in this study. These results do show that the withdrawal of chloroquine did indeed lead to the selection of the wild type allele over the parasites with the mutant allele which could no longer survive due to the better fitness of parasites which selects for the wild type allele. Furthermore, these findings are in agreement with reports that stated that the CQR phenotype is a result of both the presence of the N86Y mutation and the K76T mutation (Norahmad *et al.*, 2016). Additionally, Amodiaquine resistance is also linked to the mutant haplotype in the *pfcrt* gene as well as to the mutant allele in the *pfmdr1* gene at codon N86Y (Fröberg *et al.*, 2012) (Gadalla *et al.*, 2015). Although some studies found a low prevalence of mutations in both the *pfcrt* gene and *pfmdr1* gene at codon N86Y, like the study done in Nigeria (Agomo *et al.*, 2016), studies such as the one done in Jimma, Ethiopia observed a low prevalence of mutations at position N86Y and a high prevalence of the *pfcrt* mutant haplotype (Heuchert *et al.*, 2015). Moreover, a high prevalence of wild type alleles were observed at codon N86Y and this may be due to the introduction of artemether lumefantrine as the first line treatment in Namibia, studies reported that this

antimalarial drug selects for the wild type allele which can explain this observation (Nguetse *et al.*, 2017).

Mutations observed at codon D1246Y together with S1034C and N1042D has been linked to resistance in QN and correspondingly to increased susceptibility in MQ, halofantrine (HF) and artemisinin (Mbaye *et al.*, 2016). Additionally the presence of the wild type alleles at codons N86Y, F184Y and D1246Y have been linked to reduced artemether-lumefantrine (AL) susceptibility, whereas the presence of mutant alleles at these codons have been linked to amodiaquine and chloroquine reduced susceptibility (Kavishe *et al.*, 2014). Thus, the low prevalence of the wild type allele found at codon N86Y in this study could be an indication that the *P. falciparum* parasites in Namibia are still susceptible to AL which is currently used as first line treatment for malaria. However, although it has been shown that N86Y is an important marker for antimalarial drug resistance in Al it has been suggested that studies look at the DFN and YYY haplotypes in the *pfmdr1* gene at codons (86,187 and 1256) to give a more conclusive report (Dhorda *et al.*, 2015)(Sondo *et al.*, 2016). Furthermore, it is suggested that in order to make a definite claim on the status of AL in a country, *in vitro* and *in vivo* studies should be done to confirm the susceptibility of AL (Apinjoh *et al.*, 2016).

ACTs are currently used as first line treatment for *falciparum* malaria in majority of malaria endemic countries in Africa including Namibia, with artemether-lumefantrine and artesunate-Amodiaquine being the most commonly used antimalarial drugs (Li *et al.*, 2016). Namibia adopted artemether-lumefantrine as first treatment in 2006 and since then the number of malaria cases have reduced (WHO, 2015c). However, recently, delayed parasite clearance in ACTs were reported in the Greater Mekong Sub region after *in vivo* and *in vitro* studies presented delayed parasite clearance in

the presence of non-synonymous single nucleotide polymorphisms (SNPs) found in the Kelch 13 propeller gene (Lin and Zaw, 2015)(Huang *et al.*, 2015)(Sondo *et al.*, 2016).

In order to determine the prevalence of Kelch 13 SNPs in the Zambezi region of Namibia, 49 *P. falciparum* malaria positive samples were successfully amplified and sequenced which covered codons 441–675 including the three mutations (C580Y, R539T and Y493H) that were said to be strongly associated with delayed parasite clearance in South Asia as well as codon M476I found in Tanzania (Huang *et al.*, 2015). During this study we found zero mutations at any of the sequenced codons, with a high prevalence of wild type alleles. These findings were consistent with the results observed in The Gambia and in Bioko Island, Equatorial Guinea which observed no mutations at any of the codons that has been associated with delayed parasite clearance (Amambua-Ngwa *et al.*, 2017) (Li *et al.*, 2016). Even though some studies found a low prevalence of mutations in the Kelch 13 gene, studies done in Angola and Mozambique observed synonymous mutations at low frequencies in codons R471R, V494I, and R575R which are believed to play a role in artemisinin resistance. This calls for further i*n vivo* and *in vitro* studies to determine whether their occurrence leads to delayed parasite clearance (Escobar *et al.*, 2015) (Fancony, Brito and Gil, 2016).

Nevertheless, another study done in Angola did however find lower than the expected drug efficacy in the Zaire Province in northern Angola. This report showed that the AL efficacy was less than 90% which is the lower limit recommended for ACTs by WHO. However, they found that this reduced drug efficacy was not linked to any K13 mutations (Plucinski *et al.*, 2017). This raises concerns as it may show that artermisinin reduced drug efficacy may not be linked to K13 mutations in some

endemic regions, suggesting that antimalarial drug resistance should be routinely monitored using both molecular markers which were previously linked to antimalarial drug resistance, as well as clinical and parasitological response to antimalarial drugs in patients as recommended by WHO (WHO, 2017a).

Additionally, majority of the studies done in Africa presented that none of the isolates carried the mutant allele in any of the four candidate markers described in Asia that are strongly linked to Artemisinin failure (Huang *et al.*, 2015). These findings suggest that ACTs in Africa including Namibia are still highly effective. Nonetheless, continued surveillance is needed to detect early emergence of drug resistance as some countries in East Africa and Angola have observed frequencies of synonymous mutations (Lin and Zaw, 2015).

During this study the *pfmdr1* copy number was also not evaluated which has been linked to artemisinin resistance. Therefore, more studies need to be done as presence of point mutations alone does not lead to artermisinin resistance. Additionally, due to the smaller sample size used and the limited geographic region that was covered by the samples, this study may have not have detected all the cases of artermisinin-resistant malaria and the findings may be limited to the north eastern region of Namibia where the study was conducted.

7. Conclusion

During this study a low prevalence of point mutations in both the *pfmrd1* gene at codon N86Y and at the codons 72-76 in the *pfcrt* gene was observed. Additionally, no mutations were found in all 25 markers in the Kelch 13 gene associated with drug resistance in artemisinin. These observations show that the use of artemether-lumefantrine is still effective in western Namibia. Furthermore, the study showed that the prevalence of *P. falciparum* malaria cases is not as high as previously reported and that a higher prevalence of *P. vivax* malaria cases was found which was more than expected. Additionally, the presence of *P. malariae* cases were also detected, nonetheless no *P. ovale* cases were observed.

This study also showed that using multiplex PCR for *plasmodium* species identification is an effective and faster way to identify different species as many studies have found that the gold standard of using conventional light microscopy for the examination and identification of *plasmodium* have some draw backs. Thus, it is recommended to use multiplex PCR along with the gold standard for the accurate identification of *plasmodium* species.

Moreover, this study showed that using molecular markers as surveillance tools for antimalarial drug resistance may be an easier and faster way to detect early emergence of drug resistance. However, studies have found that not all mutations lead to a resistance phenotype as some antimalarial drugs may select for the wild type allele such as with AL, thus it is recommended that in *in vivo* and *in vitro* drug efficacy studies are done to see if the presence of these polymorphisms lead to treatment failure.

8. Recommendations

During this study a low prevalence of point mutations was found, and this may suggest that AL efficacy is still high in Namibia. However, a small sample size was used from only one geographical area therefore, it is recommended that further studies be done including other malaria endemic areas in Namibia with a larger sample size to give a better representation of the country.

Findings from this study also suggest a need for continued monitoring of non-*falciparum* infection prevalence to decide when species-specific RDTs should be introduced for diagnostic purposes, because species-specific diagnosis will be important when the prevalence of malaria in this population reduces, as the country moves towards malaria elimination. Additionally while it should not necessarily be the primary diagnostic tool, diagnostic approaches that have the capacity to investigate and detect non-*falciparum* malaria may become important in future. In health facilities where microscopy services are present, there is need to conduct refresher courses for laboratory staff and microscopists with emphasis on *Plasmodium* species-wide microscopy to strengthen non-*falciparum* malaria diagnosis

Although multiplex PCR is a much faster way compared to light microscopy to identify different *plasmodium* parasites, this assay has some drawbacks, which include that it is qualitative assay and it is not as sensitive as qPCR assays. Thus, it is recommended that a further study be done using quantitative assays to supplement these findings.

Finally, it is recommended that other studies should be done in different malaria endemic areas to give a clear representation of the current antimalarial drug resistance status in Namibia.

9. References

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10. Appendices

Appendix 1: Carestart RDT Manufactures guideline

CareStart™ Malaria HRP2/pLDH (Pf/PAN) Combo Test

Rapid One Step Malaria HRP2/pLDH Combo Test

A rapid test for the detection of HRP2 and parasite LDH in human blood

Intended Use

For the rapid qualitative determination of Malaria Histidine-rich Protein 2 (HRP2) and parasite lactate dehydrogenase (pLDH) in human blood as an aid in the diagnosis of Malaria infection.

Summary

Malaria is a serious parasitic disease characterized by fever, chills, and anemia and is caused by a parasite that is transmitted from one human to another by the bite of infected Anopheles mosquitoes. There are four kinds of malaria that can infect humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. In humans, the parasites (called sporozoites) migrate to the liver where they mature and release another form, the merozoites. The disease is now occurs in more than 90 countries worldwide, and it is estimated that there are over 500 million clinical cases and 2.7 million malaria-caused deaths per year. At the present, malaria is diagnosed by looking for the parasites in a drop of blood. Blood will be put onto a microscope slide and stained so that the parasites will be visible under a microscope.

The *CareStart*™ Malaria pLDH/HRP2 combo Test contains a membrane strip, which is pre-coated with two monoclonal antibodies as two separate lines across a test strip. One monoclonal antibody (test line 2) is pan specific to lactate dehydrogenase (pLDH) of the *Plasmodium* species (*P. falciparum, vivax, malariae, ovale*) and the other line (test line 1) consists of a monoclonal antibody specific to Histidine-Rich Protein 2 (HRP2) of the *Plasmodium falciparum* species. The conjugate pad is dispensed with monoclonal antibodies, which are pan specific to pLDH and *P. falciparum* specific to HRP2.

So, the ***CareStart***™ Malaria pLDH/HRP2 Antigen Test is designed for the differential diagnosis between *Plasmodium falciparum* and the other *Plasmodium* species.

Materials Provided

CareStart™ Malaria Antigen Test Kit contains following items to perform the assay:

- Test Device
- Package Insert
- Assay Buffer
- Sample Pipette (Optional)
- Lancet (Optional)
- Alcohol Swab (Optional)

Precautions

In order to obtain reproducible results, the following rules must be observed:

1) For *in vitro* diagnostic use only.
2) Use disposable gloves while handling potentially infectious material and performing the assay. Wash hands thoroughly afterwards.
3) Do not use it beyond the expiration date.
4) Do not eat or smoke while handling specimens.
5) Clean up spills thoroughly using an appropriate disinfectant.

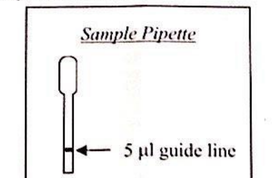
Specimen Collection and Storage

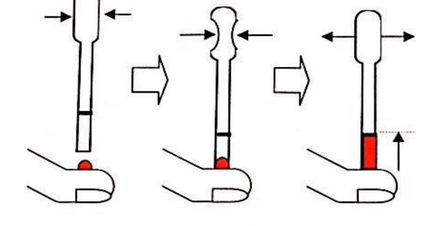
[Collection by venipuncture]

1) Collect the whole blood into the collection tube (containing EDTA, citrate, or heparin) by venipuncture.
2) If specimens are not immediately tested, they should be refrigerated at 2 ~ 8°C. For storage periods greater than three days, freezing is recommended. They should be brought to room temperature prior to use. Using the specimen in the long-term keeping more than three days can cause non-specific reaction.
3) When storage at 2 ~ 8°C, the whole blood sample should be used within three days.

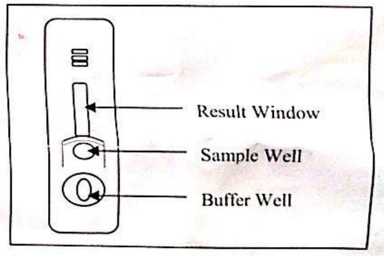
[Collection using a lancet]

1) Clean the area to be lanced with an alcohol swab.
2) Squeeze the end of the fingertip and pierce with a sterile lancet provided.
3) Wipe away the first drop of blood with sterile gauze or cotton.
4) Take a sample pipette provided, and while gently squeezing the tube, immerse the open end in the blood drop and then gently release the pressure to draw blood into the sample pipette up to the black line.

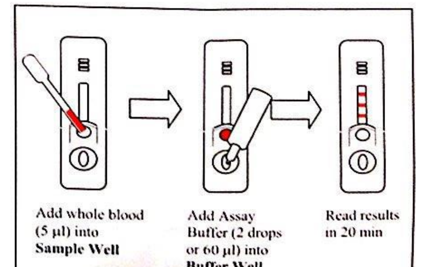




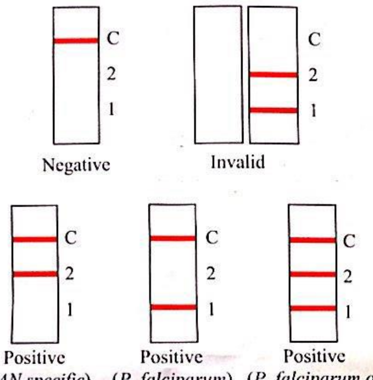
Test Procedure



1) Add 5 µl of whole blood into the Sample Well (small well).
2) Add two drops (60 µl) of assay buffer into the buffer well.
3) Read the test result in 20 min.



Interpretation of the test



1) **Negative reaction**
   The presence of only one band in the Control Area within the result window indicates a negative result.

2) **Invalid**
   The test is invalid if the line in the Control Area does not appear. If this occurs, the test should be repeated using a new strip.

3) ***P. vivax, P. Malariae, or P. Ovale* Positive reaction**
   The presence of two color bands (one band in the Control Area and another band in the "2" area) indicates a positive result for *P. vivax, P. malariae,* or *P. ovale*. The pLDH present in the sample reacts with the pan anti-pLDH conjugate and move through the test strip where the pLDH is captured by pan specific anti-pLDH.

4) ***P. falciparum* Positive reaction**

   The presence of three color bands (three bands in the Control, "2" and "1" areas) or two bands (one band in the Control Area and another band in the "1" area) indicates a positive result for *P. falciparum*.

5) **Mixed infection of *P. falciparum* and other species Positive reaction**

   The presence of three color bands (three bands in the Control, "2" and "1" areas) indicates a positive result for *P. falciparum* or Mixed infection of ***P. falciparum*** and other species.

Limitations and Interferences

1) The test procedure, precautions and interpretation of results for this test must be followed when testing.
2) Anti-coagulants such as heparin, EDTA, and citrate do not affect the test result.
3) Do not mix reagent of different lots.
4) The test is limited to the detection of antigen to Malaria *Plasmodium* sp. Although the test is very accurate in detecting HRP2 and pLDH, a low incidence of false results can occur. Other clinically available tests are required if questionable results are obtained. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the results of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.

Performance Characteristics

The ***CareStart™* Malaria HRP2/pLDH** combo kit has tested with positive and negative clinical samples tested by microscopic examination of whole blood.

1) Malaria *P. vivax* evaluation results

	Pv-positive confirmed specimen		Sensitivity
	Positive	Negative	
CareStart™ Malaria pLDH/HRP2	96	4	96/100 x 100% = 96%

2) Malaria *P. falciparum* evaluation results

	Pf-positive confirmed specimen		Sensitivity
	Positive	Negative	
CareStart™ Malaria pLDH/HRP2	98	2	98/100 x 100% = 98%

3) Malaria-negative normal human specimen evaluation results

	Random normal human specimen		Specificity
	Positive	Negative	
CareStart™ Malaria pLDH/HRP2	5	195	195/200 x 100%=97.5%

Precision

Within-run and between-run precisions have been determined by the testing 10 replicates of three specimens: a negative, a low positive and a strong positive. The agreement between the test results and the expected results were 100%.

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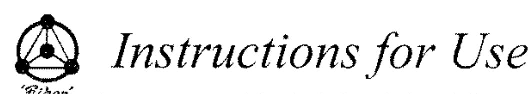
G0131-C21

September 2009

Appendix 2: Eiken Japan PAN/Pf LAMP guidelines



380201-A

For performance evaluation

REF LMP561

Loopamp™ MALARIA Pan Detection Kit

INTENDED USE

The Loopamp™ MALARIA Pan Detection Kit is for qualitative detection of *Plasmodium* DNA extracted from human blood samples.

TEST PRINCIPLES

This product is based on the nucleic acid amplification method, LAMP (Loop-mediated Isothermal Amplification), developed by Eiken Chemical Co., Ltd.

The LAMP method has the following characteristics: (1) Only one polymerase enzyme is required and the amplification reaction proceeds under isothermal conditions;[1,2] (2) it has extremely high specificity because of the use of four primers recognizing six distinct regions on the target; (3) it has high amplification efficiency and can produce a high concentration of amplified product in a short time, which makes visual or automated detection possible.[3,4]

The Malaria Pan (genus)-specific primers provided with this product have been designed to detect the mitochondrial DNA of the four most widespread *Plasmodium* species causing malaria (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*). The targeted DNA sequences have been confirmed by alignment analysis to have well-conserved base sequence in all these *Plasmodium* species.

The test DNA solution extracted from blood samples is dispensed into a reaction tube. The strand displacement DNA polymerase, deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), calcein, reaction buffers and Malaria Pan (genus)-specific primers are stored in dried form in the cap of the reaction tube. These dried LAMP reagents (Malaria Pan detection reagents (dMAL Pan)) dissolve when the DNA solution is added. The reaction tube is then incubated at 65.0°C and the DNA is amplified by the strand displacement DNA polymerase in accordance with LAMP reaction.

The detection of amplified products is based on turbidimetric measurement of magnesium pyrophosphate (a white precipitate produced as a by-product of DNA amplification).[3] Alternatively, visual detection under ultraviolet light may be used. Before DNA amplification, calcein contained in the reagent is in the quenched state as it is bound to manganese ions. At the start of DNA amplification, the pyrophosphate ions that are generated out-compete the manganese ions for binding sites, and thus the calcein becomes fluorescent.[4]

CONTENTS OF THE KIT

Reagents are stable until the date on the label assuming the container remains unopened within a storage temperature of 1 – 30°C.

Malaria Pan detection reagent (dMAL Pan) ·············· 10 X 48 tubes

The following reagents in dried form are contained in each reaction tube:

- *Bst* DNA polymerase*1
- Deoxynucleotide triphosphates
- Magnesium sulfate
- Calcein
- Manganese chloride
- Primers*2

Positive control Mal (PC Mal)*3 ·························· 5 X 1.0 mL

Negative control Mal (NC Mal) ························ 20 X 0.5 mL

*1: *Bst* DNA polymerase derived from *Bacillus stearothermophilus* is a strand displacement DNA polymerase that lacks 5'→3' exonuclease activity.

*2: Primers designed for the mitochondria DNA of *Plasmodium* parasites, purified from synthesized oligonucleotides by HPLC.

*3: PC Mal contains a product resulting from *in vitro* amplification of an artificial gene designed from the mitochondrial DNA of *Plasmodium falciparum* (GenBank No.M76611).

The abbreviations of names of the following reagents, their Lot No. and the manufacturer (EKN), are printed on the containers.

Reagents	Labelling on the tube	Code on the cap
Positive control Mal	PC Mal Lot No., EKN	PC Mal
Negative control Mal	NC Mal Lot No., EKN	NC Mal

WARNINGS AND PRECAUTIONS

(1) For performance evaluation only. Do not use for other purposes including for any medical purpose such as patient diagnosis or patient care decisions.
(2) This product is designed only for detection of DNA of *P. falciparum*, *P. vivax*, *P. malariae and P. ovale* parasites in blood samples of human origin. Do not use for other purposes.
(3) When using this product, always follow this package insert.
(4) Do not freeze the reagents.
(5) Do not use any expired reagent.
(6) Do not mix reagents from different lots.
(7) Do not replenish any reagent.
(8) Performance of the Loopamp™ MALARIA Pan Detection Kit is dependent on operator proficiency and adherence to procedural directions. Testing should be performed by properly trained personnel strictly according to the instructions provided.
(9) Exposure to heat, humidity and light may cause deterioration of the dMAL Pan. Remove only the required number of reaction tubes (number of samples + number of controls) and re-seal the aluminium pouch immediately.
(10) Do not remove the desiccant from the aluminium pouch. High humidity can cause deterioration of the dried LAMP reagent in the reaction tubes.
(11) Read the instruction manual and ensure required equipment (turbidimeter or incubator) is available before commencing the procedure.
(12) Blood samples pose a potential risk for infection. Use universal precautions to minimize biohazard.[5]
(13) PC Mal and NC Mal both contain a small amount of sodium azide as preservative. Sodium azide is classified as toxic. Avoid any contact with eyes, mouth, or skin.
(14) In case of accidental contact of any reagent with eyes, mouth, or skin, immediately rinse the affected site with running water and seek medical advice.
(15) Do not dilute or add the PC Mal to the samples. Use the PC Mal only as described in this package insert in order to avoid DNA contamination.
(16) Store the PC Mal and any positive blood samples separately from the other kit reagents.
(17) The cap of each reaction tube contains dMAL Pan in dried form. Do not touch the inside of the cap.
(18) Before using the reaction tubes, check carefully to see if they have any cracks or scratches. Damaged tubes might give false results and lead to DNA contamination of the incubator and work area.
(19) Do not expose reaction tubes to UV light before the end of the LAMP reaction. Prolonged exposure to UV light might damage the tubes and lead to false results.
(20) When a UV lamp is used for visual fluorescence judgment, do not stare directly at UV light. Since UV light is harmful to the eyes, even watching for a short period may irritate eyes and cause symptoms similar to conjunctivitis. Use a glass screen or wear protective goggles/glasses/face shield whenever looking directly at the UV lamp.
(21) Refer to the manual of the incubator. When the LF-160 or the real-time turbidimeter is used, be careful when removing the reaction tubes from the incubator to avoid burns.

WASTE DISPOSAL

(1) Do not open the tubes after DNA amplification. Leave the cap closed and dispose of the used tubes as medical waste by incineration or after double bagging with sealable plastic bags.
(2) Never autoclave or re-use the reaction tubes. Amplified products will disperse and cause contamination.

(3) The main material for the reaction tubes and reagent tubes is polypropylene (PP); for the reaction tube tray, polyethylene (PET); for the aluminium pouch, aluminium; and for the kit case, paper.
(4) Dispose of any other reagent, container, or lab ware in accordance with regulations.

SPECIMEN COLLECTION

(1) Blood samples should be used immediately after collection.
(2) Collect blood in a separate room from the LAMP amplification room. Aerosols containing *Plasmodium* DNA can be generated during blood collection and may cause contamination.
(3) DO NOT USE EDTA and Citrate as anticoagulant for blood collection if the result is to be read by fluorescence. The use of heparin as anticoagulant is recommended.

MATERIALS REQUIRED BUT NOT PROVIDED

- Loopamp™ PURE DNA Extraction Kit (REF LMC802) (optional)

For Visual fluorescence detection

(For LF-160 incubator)

- LF-160 (REF MVKM17)

(For other incubator using UV lamp)

- Incubator (temperature accuracy: ±0.5°C: with hot bonnet)
- Heating block
- UV lamp (wavelength: 240 to 260 nm, and 350 to 370 nm)
- Goggles/glasses or other UV-blocking eye mask

For real-time turbidity detection

- Real-time turbidimeter LA-500 (REF MVL300) (only for use in the LAMP method; wavelength: 600 to 700 nm; amplification temperature: 65.0°C)

For reagent and sample mixing

- Micropipettes (10 to 100μL, and 20 to 200μL) and pipette tips with filter
- Centrifuge for micro-tubes (optional)
- Centrifuge for eight connected tubes (optional)

PREPARATION OF SAMPLE DNA SOLUTION

The following DNA extraction methods are recommended.

(For boil and spin)

To extract the DNA from blood sample, follow the standard operating procedures published at http://www. finddiagnostics.org/programs/malaria-afs/lamp/standard_procedures/index.html.

(For PURE)

To extract the DNA from blood sample, follow the instruction for the Loopamp™ PURE DNA Extraction Kit® (sold separately). Use the DNA solution obtained for LAMP amplification.

PREPARATION OF REAGENTS

(1) Malaria Pan detection reagent

Remove the required number of tubes from the aluminium pouch and put them in the rack provided. (number of samples + number of controls).

Note: After removing the required tubes, seal the aluminium pouch with any unused tubes immediately.

(2) Negative control Mal (NC Mal)

Flick (or spin) down the tube before using, in order to collect the content at the bottom of the tube.

Note: NC Mal should be used with every run.

(3) Positive control Mal (PC Mal)

Flick (or spin) down the tube before using, in order to collect the content at the bottom of the tube.

Note: PC Mal should be used with every run.

MEASUREMENT PROCEDURE

Reagent and sample mixing

(1) Turn on the incubator or the real-time turbidimeter.
(2) Dispense 30μL of extracted DNA solution into a reaction tube using the pipette, and close the cap.
(3) Dispense 30 μL of NC Mal into a reaction tube using the pipette, and close the cap.
(4) Dispense 30μL of PC Mal into a reaction tube using the pipette, and close the cap.
(5) Flick (or spin) down all tubes to collect the solution at the bottom of the tubes.

Note: When using the PURE device make sure the liquid level is closer to the upper line of the two lines on a reaction tube to ensure that the correct volume has been dispensed.

(6) Reconstitute the dried reagent in the cap by inverting the reaction tubes and collecting the DNA solution in the cap. Leave the tubes standing upside down for 2 minutes to reconstitute the dried reagent.
(7) Invert the reaction tubes five times to mix the contents. Make sure that the dried reagent in the cap is fully dissolved (the solution should have a slight orange colour).
(8) Flick (or spin) down all tubes to collect the solution at the bottom of the tubes.

Amplification

For visual fluorescence detection

(for LF-160)

(1) Check that the temperature showing on the incubator is 65.0°C.
(2) Load the reaction tubes into the LF-160 incubator and press the green button to start the LAMP reaction (40 minutes at 65.0°C). See the LF-160 instruction manual for details on how to operate the incubator.
(3) Confirm the completion of polymerase inactivation (automatically completed by the LF-160). Take all reaction tubes from the LF-160.

(for other incubator using UV lamp)

(1) Set the temperature of the incubator at 65.0°C (with hot bonnet temperature set to 10 degrees above the reaction temperature or as near to this figure as possible – temperature accuracy: ±0.5°C). Wait until the temperature displayed reaches the set value.
(2) Load the reaction tubes, and then start amplification reaction (for 40 minutes at 65.0°C).
(3) Forty minutes later, inactivate the polymerase using the heating block (for 5 minutes at 80°C, or for 2 minutes at 95°C) to terminate the reaction.

For real-time turbidity detection (see Flow chart of the procedure)

(1) If not already correctly configured, configure the real-time turbidimeter for detection with this product.
(2) Check if the temperature displayed reaches 65.0°C (allow the turbidimeter to warm up for 20 minutes before use).
(3) Load the reaction tubes, and start measurement.
(4) Watch the display of the turbidimeter to check the positive and negative controls for any increase in turbidity. If the turbidity increases in the positive but not in the negative control solution, amplification reaction is proceeding properly (Fig1). If this is not the case, amplification reaction may be proceeding in a wrong way. In such a case, restart testing from reagent preparation.
(5) Confirm the completion of polymerase inactivation (automatically completed by the turbidimeter). Take all reaction tubes from the turbidimeter and discard them without opening.

Amplification plots by Malaria Pan detection reagent

(Analyzer: real-time turbidimeter LA-500 (two different reaction blocks are shown here))

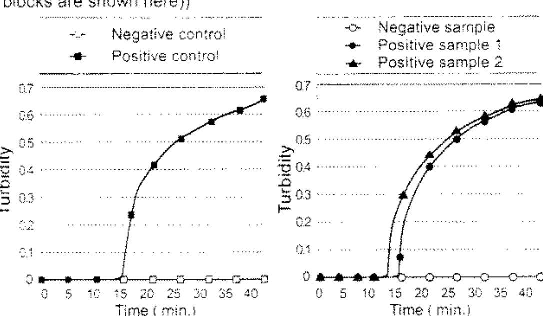


Fig1 : Amplification plots for controls   Fig2 : Amplification plots for samples

PROCEDURAL NOTES

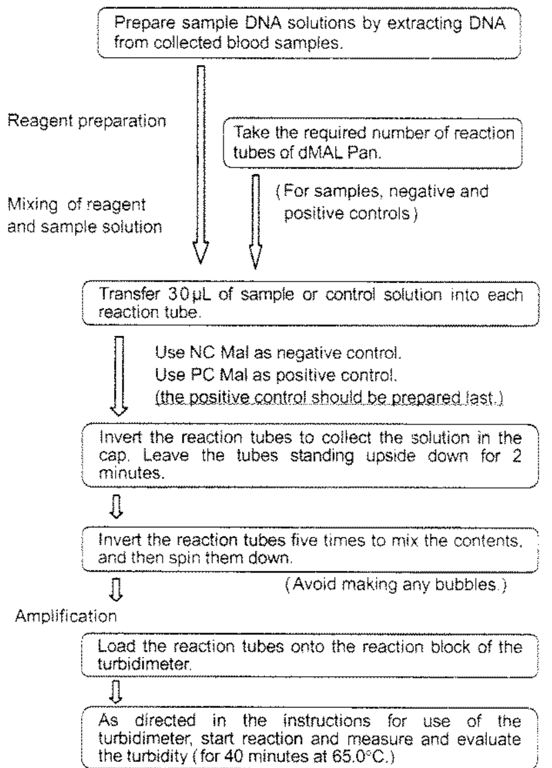
(1) The LAMP reaction is very sensitive, and contamination with small amounts of amplified product might lead to false positive results.
(2) Separate the sample preparation and the amplification areas.
(3) Clean benches with over 0.5% sodium hypochlorite before and after performing the test.

REFERENCES

1) Notomi T., et al.: Nucleic Acids Research 28, No. 12, e63 (2000)
2) Nagamine K., et al.: Clin. Chem. 47, No. 9, 1742–1743 (2001)
3) Mori Y., et al.: Biochem. Biophys. Res. Commun. 289, No. 1, 150–154 (2001)
4) Tomita N., et al.: Nat. Protoc. 3, No. 5, 877–882 (2008)
5) The guideline for the bio-safety and bio-hazard (by the Japanese Society for Bacteriology): Japanese Journal of Bacteriology 54, No. 3, 667–715 (1999)
6) The package insert of Loopamp™ PURE DNA Extraction Kit.

Flow chart

Operation procedure for real-time turbidity detection



Confirm the completion of polymerase inactivation (for 5 minutes at 80°C, or for 2 minutes at 95°C). Take all reaction tubes from the turbidimeter and discard them without opening. Be careful not to damage the tubes.

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Appendix 3: New England Biolabs Restriction Digest Guidelines

OPEN ACCESS

Restriction Digest

NEB

Abstract
The following is a "typical" restriction endonuclease reaction. Please see the "guidelines" tab below for the NEB tips on optimizing restriction digests.

Citation: NEB: Restriction Digest. **protocols.io**
https://www.protocols.io/view/Restriction-Digest-imst4v
Published: 08 Oct 2014

Protocol

1. Set up the following reaction (total reaction volume **50ul**).

REAGENT

Restriction Digest Reaction
By New England Biolabs View

ANNOTATIONS

NEB 06 Oct 2014
Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol.

NEB 06 Oct 2014
A 50 µl reaction volume is recommended for digestion of 1 µg of substrate.

NEB 08 Oct 2014
The enzyme should be the last component added to reaction

1.1. DNA **1ug**

1.2. 10X NEBuffer **5ul** (1X)

1.3. Restriction Enzyme, 10 units is sufficient, generally **1µl** is used

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3. Quick ("touch") spin-down in a microcentrifuge. **Do not vortex the reaction.**

4. Incubate for 1 hour at the enzyme-specific appropriate temperature.

ANNOTATIONS

NEB 03 Oct 2014
Can be decreased to 5-15 minutes by using a Time-Saver™ Qualified enzyme.

NEB 06 Oct 2014
See the NEB Activity/Performance Chart for the incubation temperatures.

Guidelines

Guidelines for Optimizing Restriction Endonuclease Reactions

If you are using a Master Mix, see "optimizing RE-Mix® reactions".

There are several key factors to consider when setting up a restriction endonuclease digest. Using the proper amounts of DNA, enzyme and buffer components in the correct reaction volume will allow you to achieve optimal digestion. By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes. This enzyme : DNA : reaction volume ratio can be used as a guide when designing reactions. However, most researchers follow the "typical" reaction conditions listed, where a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity. NEB offers the following tips to help you to achieve maximal success in your restriction endonuclease reactions.

A "Typical" Restriction Digest

Restriction Enzyme	10 units is sufficient, generally 1µl is used
DNA	1 µg
10X NEBuffer	5 µl (1X)
Total Reaction Volume	50 µl
Incubation Time	1 hour*
Incubation Temperature	Enzyme dependent

* Can be decreased to 5-15 minutes by using a Time-Saver™ Qualified enzyme.

Enzyme

- Keep on ice when not in the freezer
- Should be the last component added to reaction
- Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.
- Follow with a quick ("touch") spin-down in a microcentrifuge.
- Do not vortex the reaction.
- In general, we recommend 5–10 units of enzyme per µg DNA, and 10–20 units for genomic DNA in a 1 hour digest.
- NEB has introduced a line of High-Fidelity (HF®) enzymes that provide added flexibility to reaction setup.

DNA

- Should be free of contaminants such as phenol, chloroform, alcohol, EDTA, detergents or excessive salts. Extra wash steps during purification are recommended.
- Methylation of DNA can inhibit digestion with certain enzymes. For more information about methylation, Effect of CpG Methylation on Restriction Enzyme Cleavage and Dam and Dcm Methylases of *E.coli*

Buffer

- Use at a 1X concentration
- Supplement with SAM (S-Adenosyl methionine) to the recommended concentration if required.

Reaction Volume

- A 50 µl reaction volume is recommended for digestion of 1 µg of substrate
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- Additives in the restriction enzyme storage buffer (e.g., glycerol, salt) as well as contaminants found in the substrate solution (e.g., salt, EDTA, or alcohol) can be problematic in smaller reaction volumes. The following guidelines can be used for techniques that require smaller reaction volumes.

	Restriction Enzyme*	**DNA**	**10X NEBuffer**
10 µl rxn**	1 unit	0.1 µg	1 µl
25 µl rxn	5 units	0.5 µg	2.5 µl
50 µl rxn	10 units	1 µg	5 µl

* Restriction Enzymes can be diluted using the recommended diluent buffer when smaller amounts are needed.
** 10 µl rxns should not be incubated for longer than 1 hour to avoid evaporation.

Incubation Time

- Incubation time is typically 1 hour
- Can often be decreased by using an excess of enzyme, or by using one of our Time-Saver Qualified enzymes.
- It is possible, with many enzymes, to use fewer units and digest for up to 16 hours. For more information, visit Extended Digests with Restriction Endonucleases.

Stopping a Reaction

If no further manipulation of DNA is required:

- Terminate with a stop solution (10 µl per 50 µl rxn) [1x: 2.5% Ficoll®-400, 10mM EDTA, 3.3mM Tris-Hcl, 0.08% SDS, 0.02% Dye 1, 0.001% Dye 2, pH 8.0@25°C] (e.g., NEB #B7024)

When further manipulation of DNA is required:

- Heat inactivation can be used
- Remove enzyme by using a spin column or phenol/chloroform extraction

Storage

- Storage at -20°C is recommended for most restriction enzymes. For a few enzymes, storage at-70°C is recommended for periods longer than 30 days. Please refer to the enzyme's technical data sheet or catalog entry for storage information.
- 10X NEBuffers should also be stored at -20°C

Stability

All enzymes are assayed for activity every 4 months. The expiration date is found on the label. Exposure to temperatures above -20°C should be minimized whenever possible

Control Reactions

If you are having difficulty cleaving your DNA substrate, we recommend the following control reactions:

- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing.

Appendix 4: Qiagen DNA Extraction Protocol

QIAGEN Supplementary Protocol:

Isolation of genomic DNA from dried blood spots using the QIAamp® 96 DNA Blood Kit

This protocol is designed for the isolation of genomic DNA from dried blood spots using the QIAamp® 96 DNA Blood Kit. Blood, both untreated and treated with anticoagulants, which has been spotted and dried onto filter paper (e.g., S&S 903™, Schleicher and Schuell) is suitable for use with this procedure.

Please be sure to read the *QIAamp 96 DNA Blood Kit Handbook* carefully before beginning this procedure.

Important notes before starting

- Buffer ATL and Proteinase K are not included in the QIAamp 96 DNA Blood Kit, but can be purchased separately (200 ml Buffer ATL, cat. no. 19076; 10 ml QIAGEN Proteinase K, cat. no. 19133).
- Equilibrate Buffer AE to room temperature, for elution in step 18.
- Prepare a 90°C incubator oven for use in step 2, and a 56°C incubator oven for use in steps 4 and 6.
- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to instructions.
- If a precipitate has formed in Buffer AL or Buffer ATL, dissolve by incubating at 70°C.
- Use of a multichannel pipet is recommended.
- All centrifugation steps are carried out at room temperature.

Procedure

1. **Place 3 punched-out circles from a dried blood spot to the bottom of one well of a Round-Well Block (provided), and add 180 µl of Buffer ATL. Use the register card provided to identify the locations of the samples.**

   Cut 3 mm (1/8 inch) diameter punches from a dried blood spot with a single-hole paper puncher (paper punchers may be purchased from Schleicher & Schuell, order no. 10495010).

   It is a good idea to mark the Round-Well Blocks at this stage so that they can be easily identified throughout the protocol.

2. **Seal the wells thoroughly using the caps (for round-well blocks) provided. Incubate at 90°C for 15 min. Centrifuge briefly at 3000 rpm to collect any solution from the caps.**

   Allow centrifuge to reach 3000 rpm, then stop the centrifuge.

3. **Add 20 µl Proteinase K solution. Seal the wells thoroughly using the caps provided. Mix by vigorously shaking the Round-Well Block for 15 s.**

   Note: To avoid cross-contamination when sealing the wells with caps, do not touch the rim of the wells with the pipet tips.

   Note: The addition of Proteinase K is essential.

4. **Centrifuge briefly at 3000 rpm to collect any solution from the caps and incubate at 56°C for 1 h in an incubator oven.**

   Allow centrifuge to reach 3000 rpm, then stop the centrifuge.

   Note: Placing a weight on top of the round-well block will prevent the lids from popping off occasionally during incubation.

5. **Centrifuge briefly at 3000 rpm to collect any solution from the caps. Add 200 µl Buffer AL to each of the samples, taking care not to wet the rims of the wells. Seal the wells thoroughly using the caps provided.**

   Note: Use only the caps provided, since using AirPore™ tape at this stage of the procedure will lead to cross-contamination. Ensure that the wells are sealed thoroughly to avoid spurting during shaking.

6. **Mix thoroughly by shaking vigorously for 15 s and incubate at 56°C for 10 min.**

   In order to ensure efficient lysis, it is essential that the samples and Buffer AL are mixed immediately and thoroughly to yield a homogeneous solution. Hold the Round-Well Block with both hands and shake up and down vigorously.

7. **Centrifuge briefly at 3000 rpm to collect any solution from the caps.**

   Allow centrifuge speed to reach 3000 rpm, then stop the centrifuge.

8. **Remove the caps and add 200 µl ethanol (96-100%) to each of the samples, taking care not to wet the rims of the wells.**

9. **Seal the wells thoroughly using new caps. Shake vigorously for 15 s. Centrifuge briefly at 3000 rpm to collect any solution from the caps.**

   Allow centrifuge speed to reach 3000 rpm, then stop the centrifuge.

10. **Place the QIAamp 96 Plate on top of an S-Block. Mark the QIAamp 96 Plate for later identification.**

11. **Carefully apply the mixture from step 9 (600 µl per well) to the QIAamp 96 Plate.**

    Take care not to wet the rims of the wells to avoid aerosol formation during centrifugation.

    Ensure that blood card punches are not transferred or do not block pipet tips.

    Note: Due to sample volume, lowering pipet tips to the bottoms of the wells may cause overflow if extended, narrow pipet tips (such as Matrix cat. no. 8255) are not used. It is best to remove one strip of caps at a time and begin drawing up samples as soon as pipet tips contact the sample. Repeat until all the samples have been applied to the QIAamp 96 Plate.

12. **Seal the QIAamp 96 Plate with an AirPore tape sheet. Load the S-Block and QIAamp 96 Plate onto the carrier, then place it in the rotor bucket. Centrifuge at 6000 rpm for 4 min.**

13. **Remove the AirPore tape. Carefully add 500 µl Buffer AW1 to each well.**

14. **Seal the QIAamp 96 Plate with a new AirPore tape sheet. Centrifuge at 6000 rpm for 2 min.**
15. **Remove the AirPore tape. Carefully add 500 µl Buffer AW2 to each well.**
16. **Centrifuge at 6000 rpm for 15 min.**

    The heat generated during centrifugation allows for evaporation of any residual ethanol in the sample (from Buffer AW2) that may otherwise inhibit PCR and other downstream reactions.

    Note: In order to ensure efficient ethanol evaporation, do not seal the plate with AirPore tape during this centrifugation step.
17. **Place the QIAamp 96 Plate on top of a rack of elution microtubes (provided).**
18. **To elute the DNA, add 150 µl Buffer AE or distilled water, equilibrated to room temperature, to each well using a multichannel pipet, and seal the QIAamp 96 Plate with a new AirPore tape sheet. Incubate for 1 min at room temperature. Centrifuge at 6000 rpm for 4 min. Use the caps provided to seal the wells of the microtubes for storage.**

    Note: Do not elute the DNA with volumes of less than 100 µl.

    Three punched-out circles (3 mm diameter) typically yield 150 ng and 75 ng of DNA from anticoagulant-treated and untreated blood, respectively. If the yield from untreated blood is not sufficient, use 6 circles per prep instead of 3.

    The volume of the DNA eluate used in a PCR assay should not exceed 10% — for a 50 µl PCR, add no more than 5 µl of eluate.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

QIAGEN handbooks can be requested from QIAGEN Technical Service or your local QIAGEN distributor. Selected handbooks can be downloaded from www.qiagen.com/literature/handbooks/default.aspx. Material safety data sheets (MSDS) for any QIAGEN product can be downloaded from www.qiagen.com/ts/msds.asp.

Trademarks: QIAGEN®, QIAamp®, AirPore™ (QIAGEN); S&S 903™(Schleicher & Schuell, GmbH). The PCR process is covered by US Patents 4,683,195 and 4,683,202 and foreign equivalents issued to Hoffmann-La Roche AG.
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