Molecular Surveillance of Antimalarial Drug Resistance Related Genes
in *Plasmodium Falciparum* Isolates from Eritrea (2014-2016)

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in Medical Laboratory Sciences in Parasitology

Department of Parasitology
Faculty of Medical Laboratory Sciences
University of Gezira

*July 2017*

Abduselam Mohammed Nurahmed Said

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Date: July 2016
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Date of Examination: July 2016
Declaration

I hereby declare that this dissertation entitled, “Molecular surveillance of antimalarial drug resistance related genes in Plasmodium falciparum isolates from Eritrea” submitted by me under supervisions and guidances of Prof. Bakri YM. Nour, dean Faculty of Medical Laboratory Sciences, and Dr. Albadawi Ali Talha, Assistant Professor, Faculty of Medical Laboratory Sciences, Department of Medical Parasitology, University of Gezira for partial fulfillment for the award of MASTERS DEGREE IN MEDICAL PARASITOLOGY at University of Gezira, Wad Medani, Sudan is original and done by my own effort and with the collaboration and assistance of Istituto Superiore di Sanità (ISS), Rome, Italy and that it was not submitted in part or full for any degree and that all the sources that we have used have been indicated and acknowledged by means of complete references.
Dedication

All praise and glory be to ALLAH the almighty for honoring and blessing me and for allowing me to finish my M.Sc. study successfully. I would like to dedicate this work to my father, Mohammed Nurahmed Said to my mother, Siedi Kelifa Nurbussien, to my wife, Husnia Abdu Hussien and my daughter, Hurya Abduselam Mohammed for their patience, understanding and motivation. I would also like to dedicate it to my brother, my sisters, my friends, to my supervisors and instructors Prof. Bakri Y.M. Nour and Dr. Albadawi Talha, for their support, cooperation and sharing knowledge and to all staff of Medical Laboratory Sciences, University of Gezira, to Dr. Carlo Severini and Michela Menegon, from Istituto Superiore di Sanità, Rome, Italy.
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*Abduselam Mohammed Nurahmed Said*

Abstract

The introduction of Artemisinin-based combination therapy has led to extraordinary results in malaria control; however the recent emergence of partial resistance to Artemisinin therapy in Southeast Asia jeopardizes these successes. This study aimed at investigating resistance to the antimalarial drugs by evaluating the polymorphisms in the Kelch 13 propeller (PfK13), Pfcrt and Pfmdr1 genes in Plasmodium falciparum isolates obtained from patients in Eritrea. The molecular based study is the first of its kind in the country. For the study a total of 209 malaria infected blood samples were collected from the two most malarious regions in Eritrea; in three study sites: The age of the study participants ranged from 1.5 to 70 years, but the majority of them were young adults. Out of the total 209 blood samples tested, 160 were infected with *P. falciparum*, 24 with *P. vivax*, 20 were *P. falciparum/P. vivax* mixed infections and 5 were negative. On the 180 samples positive for *P. falciparum* infection, the polymorphism analysis of K13 propeller gene, the frequency of single nucleotide polymorphisms (SNPs) at pfcrt 72-76 codons and at pfmdr1 86-184 codons was performed by PCR amplification and sequencing. No mutations were detected in the K13 propeller region in the falciparum isolates, 84.5% showed the mutant alleles Pfcrt 74I-75E-76T and 85.5% of isolates carried out the Pfmdr1 184F mutation. The wild type codon Pfmdr1 N86 was identified in 88.8% of the isolates. Additionally, 41.6% of isolates displayed a novel point mutation in Pfmdr1 at codon 153, resulting in a change of a Serine (TCT) to Phenylalanine (TTT). In conclusion, we can only speculate that the 7% of treatment failure reported in the country recently is probably related to the loss of efficacy of the AQ, hence to the Pfcrt and Pfmdr1 polymorphisms rather than the Artemisinin derivative component of the ACT.
الرصد الجزيئي للجينات ذات الصلة لمقاومة العقاقير المضادة لل الملاريا في عزلات المتصور اله من إريتريا

عبد السلام محمد نور أحمد سيد

ملخص البحث

إن إدخال العلاج المزدوج القائم على مادة الأرتيميسينين أدى إلى نتائج غير عادية في مكافحة الملاريا؛ إلا أن ظهور مقاومة لعقار الأرتيميسينين في جنوب شرق آسيا يعد من هذه النجاحات. هدفت هذه الدراسة إلى تقييم العقاير المضادة للملاريا من خلال تقييم الطفرات الجينية Pfmdr1 و PfK13 في عزلات المتصور اله المتصور اله من إريتريا. تم الحصول عليها من المرضى في إريتريا. هذه الدراسة الجزيئية هي الأولى من نوعها في البلاد. لهذه الدراسة تم جمع 209 عينات دم مصابة بالملاريا من ثلاث مواقع في منطقة أكثر وانتشارية باريترا: نراح عمر المشاركون في هذه الدراسة من 1.5 إلى 70 عاما ومعظمهم من الشباب، بينما في مجموع 209 عينات الدم التي تم اختبارها، كان 160 مصابة بالمرض، و24 بالتصور اله والبالي، و20 كانت خليط بالتصور اله والبالي، وكانت سلبية. على 180 عينة إيجابية للمرض، تم تحصيل تعداد الأشكال للطفرات الجينية لK13، وتكرار الكودونات في Pfmdr1: 76-86 الكودونات وفي 184-213 الكودونات، و86 الكودونات. وظلت هذه الدراسة بأنه لم يتم الكشف عن أي طفرات جينية ل K13 في عزلات المتصور اله وأظهرت 84.5% الأليلات المحولة بكتر 74 T6T-75-76T أو 85% من العزلات نفذت الطفرات هل Pfmdr1 في 88.8% من العزلات، بالإضافة إلى ذلك، أظهرت 41.4% من العزلات طفرة جزئية جديدة في البري N86 Pfmdr1. في كودون 153، في الختام، تخلص هذه الدراسة بأن 7% من عينة المعالجة التي تطورت في إريتريا مؤخرا قد يكون مرتبطاً بفقدان فعالية الأموداكونين، وبالتالي إلى تعدد الأشكال بفرت و Pfmdr1 و P74T.
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<td>WHO</td>
<td>World health Organization</td>
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<tr>
<td>MOH</td>
<td>Ministry of Health</td>
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<tr>
<td>OSMDM</td>
<td>Orotta Schools of Medicine and Dental Medicine</td>
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<td>NMCP</td>
<td>National Malaria Control Program</td>
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<tr>
<td>HMIS</td>
<td>Health Management Information System</td>
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<tr>
<td>ACT</td>
<td>Artemisinin (ART) based combination therapy</td>
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<tr>
<td>AQ</td>
<td>Amidiaquine</td>
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<td>AS</td>
<td>Artesunate</td>
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<td>CQ</td>
<td>Chloroquine</td>
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<td>S/P</td>
<td>Sulphadoxine/Pyrimethamine (Fansidar)</td>
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<td>ITN</td>
<td>Insecticide – Treated Net</td>
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<td>Roll Back Malaria</td>
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CHAPTER ONE

1. Introduction

1.1. General Introduction:
Malaria is the most common parasitic disease in developing countries. According to the WHO, the vast majority of malaria deaths occur in Sub-Saharan Africa, where malaria also presents major obstacles to social and economic development. (1,2) About 75% of malaria deaths are in African children less than five years old and infected with plasmodium falciparum (P.falciparum). In fact, malaria accounts for one in five of all childhood death in Africa (1). Every 30 seconds a child dies in Africa from malaria. Pregnant women are the main adult risk group for malaria in most endemic areas of the world. In areas of Africa with infrequent or episodic (seasonal) malaria transmission (where most adult women have not developed significant immunity to malaria), pregnant women are at a two to three fold greater risk than non pregnant women of developing severe disease, increasing their risk of maternal and infant complications or death. HIV infected people are also considered particularly vulnerable to malaria. In areas with endemic malaria, HIV increases the risk of malaria infection and clinical malaria in adults, especially in those with advanced immunosuppression (2). The success in malaria eradication achieved in Europe and North America during the 19th and 20th centuries has not been replicated anywhere in Sub Saharan Africa and most tropical countries. This is despite better scientific understanding the biology of the vector, treatment methods and other means of malaria prevention and control. Malaria causes one million deaths annually in Africa especially among vulnerable groups of pregnant women and children under five years of age (3). This can be attributed to a number of factors including inadequate preventive measures for the groups at the highest risk of contracting malaria such as pregnant women and children under five years of age and drug resistance (4). Drug resistance in malaria is the most formidable obstacle in the fight against the disease since it jeopardizes the most elementary objective of malaria control reducing suffering and eliminating mortality. Although several mechanisms have been described; an important cause of drug resistance appears to be point mutations in the malaria parasite protein target genes. Clinically significant resistance to
antimalarial agent seems to require the accumulation of multiple mutations. Efforts to circumvent antimalarial drug resistance range from the use of combination therapy with existing agents to studies directed toward discovering novel targets and therapies. However, the combination of host genetic factors, particularly those associated with antimalarial drug metabolism, has not yet been explored. Artemisinin combination therapies (ACT) such as Artemether-Lumefantrine, Artesunate-Mefloquine, Artesunate-SP or Artesunate-Amodiaquine are now the recommended first line treatment for falciparum malaria throughout the world. Unfortunately, the future efficacy of ACTs has been overshadowed by reports of the emergence of Artemisinin resistant P.falciparum in Pailin, West Cambodia, near the border with Thailand (5). In Sudan a study conducted in 2007 shows that 93.4% of P.falciparum carried a mutant allele of at least one gene associated with resistance to antimalarial drugs Chloroquine and Sulphadoxine Pyrimethamine. Moreover, the isolates carried out mutant genes at pfdhfrI51 (75.3%), pfdhfr N108 (72.7%), while pfdhfr R 59 (7.6%), with respect to pfdhps gene. Pfdhps A 436 (8.1%), pfdhps G (20.7%), and pfdhps E (16.7%). Gene polymorphism for pfATPase 6 two mutated codons (431K and 402V) were found at low frequencies (17.7% and 4% respectively) (42). In Eritrea 67% of the population lives in malaria endemic areas, in 41 out of the 58 sub Zones; of this population 18% are children five years and below and 22% are women aged between 15 and 45 years. Plasmodium falciparum is the most prevalent (94%) parasite species (6), and mainly transmitted by Anopheles arabiensis (7). Eritrea is inhabited by more than 13 different species of Anopheline mosquitoes all capable of spreading the disease and with varying geophysical habitats (8). Also, inoculation rates have a high seasonal variability, with peak inoculation rates during the rainy season and minimal or no transmission during the dry season (9).

Currently, in Eritrea the first line of treatment for malaria is Artesunate and Amodiaquine. The treatment failure was 7.6% in 2011 (10) and it is very close to the WHO cut-off recommendation to change treatment which is at 10%. The failure’s exact cause is unknown; it could be due to re-infection, due to parasite drug resistance or other reasons.

1.1.1. Country Background:

Eritrea is situated in the horn of Africa and lies between 16°30¢ and 43°20¢ east longitude and between 12°42¢ and 18°2´ north latitude. It is bordered by Sudan to the north and north west,
Ethiopia to the south, Djibouti to the south east and the Red Sea to the east. Its area is approximately 124,000 square kilometers, including the Dahlak Archipelago and the islands in the Red Sea. Rain fall is scanty and highly seasonal; the annual average ranges from 400-650 mm in the highlands and from 200-300mm in the lowlands. Since 1996, the country has been divided in to six administrative Zobas (Zones). It is further divided in to 58 Sub Zobas, Kebabis (group of villages) and Adis (villages), with an estimated 1500 villages overall (See figure 1). The total population is about 4 million (World Bank 1999) with an annual growth rate of 3% and it remains largely rural (more than 90%). Eritrea is one of the poorest nations in Africa with a GNP of about $200 per capita (World Bank 1998). Eritrea regained independence in 1991 (ratified by a referendum in 1993) following a 30 year war with Ethiopia. This extended war, combined with the recent border conflict, which caused the temporary dispersal of tens of thousands of people, continues to imperil the development of the economy, health and other social sectors, and the overall quality of life.

There are nine major ethnic groups. The most recent Demographic and Health Survey (DHS 2002) produced the following estimates: Crude birth rate to be 32/1000; infant mortality ratio to be 48/1000, and under five mortality rate to be 93/1000. These place the country in the pre-health transition stage, with a life expectancy of 51 years (World Bank 1999). Literacy is estimated to be only 10% for women and 20% for men.

Figure 1.1 Administrative zones in Eritrea
1.1.2. Eritrea’s Health Care System:
In 2006 there were a total of 358 health facilities of various types in the country, and the number has increased since then. Hospitals, mini hospitals, health centers, and health stations managed by the Ministry of Health, and by private sectors. Other specialized facilities such as maternal and child health clinics and eye clinics. There is a hospital in each Zoba and at least one mini hospital or health center in each Sub Zoba. Several health stations operate under the direction of each health center or mini hospital. In addition to the formal clinics, many people obtain treatment from licensed pharmacies or rural drug vendors in the cities and towns. Community workers (Village health agents) work from their own houses to treat fever cases during the peak malaria transmission months. The National Malaria Control Program (NMCP) gives annual training to the village health agents, and the MoH provides drugs. All health facilities at health center level and above have diagnostic laboratories. A WHO/Roll Back Malaria (RBM) survey showed that in 2001 more than half of all health facilities had malaria diagnostic capacity. The NMCP is currently providing the optimal rapid test to health facilities in malarious areas that do not have microscopists.

1.1.3. Malaria Transmission in Eritrea:
Eritrea is at the northern limit of malaria transmission in Sub Saharan Africa, and the lengthy dry periods limit the intensity of transmission. Large differences in altitude across the country contribute to the complex transmission picture. Transmission is usually described as highly seasonal and unstable, although this generalization masks a high variability. There are three distinct climate systems affecting the rainfall and hence malaria in Eritrea: the Azmara (March-May) short rains, which fall mainly in the Eritrean high land, the Kremti (July-October) rains, which usually bring heavy rains to the south-west of the country including the western escarpments, and the Bahri (December-February) rains that occur mainly in the eastern lowlands and escarpments. Following these rainfall patterns, transmission in the south and west areas of the country usually peak in the months of September through November, while in the eastern coastal Zones transmission is highest in the months of January through April. The March to May Azmara rains do not have a large influence on malaria in the central highlands because these
rains are generally light and fall mostly at altitudes too high for transmission. However, they don’t affect the transmission pattern in some escarpments such as Sub Zoba Ghindae. Anopheles arabiensis and Anopheles gambiae both belonging to the Anopheles gambiae complex, form the main vectoral system in Eritrea; despite lengthy dry periods even in the south and west, year-round transmission nevertheless occurs in some areas. This may partly be due to persistence of manmade water sources (8). In 1999, malaria accounted for one-fifth of all hospitalizations and one–tenth of all outpatient visits and until now it is accounted as the leading cause of mortality for those aged 5 and older. At the end of 2009 a decline of malaria mortality and morbidity was 90% (HMIS). The maternal mortality rate is high much of this is attributed to malaria anemia during and after pregnancy. Nevertheless, malaria incidence in Eritrea is relatively low compared to countries further south in Africa (see Fig. 2.)

In Eritrea, incidence peaked in 1998 and has been declining since. Now once again it is on the increase since 2010. The majority of confirmed cases seen at health facilities are due to P.falciparum 89% in 2009, 93% in 2001, 83% in 2006, and 88% in 2007. In the past, the proportion of P.vivax case was higher, but it is unclear whether this change has occurred because of decline in P.vivax incidence, increase of P.falciparum incidence, or a combination of the two.

![Malaria distribution in Eritrea](image)

Figure 1.2 Malaria distribution in Eritrea

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1.1.4. Malaria Control Program:
The National Malaria Control Program working under the umbrella of the MoH of the state of Eritrea has plans, policies and guidelines based on five year plan. The NMCP sets out ambitious goals for percentage reduction of malaria proportional morbidity and mortality rates and incidence of epidemics. The NMCP does different activities including case management, larval control, free insecticide treated bed net (INT) distribution strategies, prompt and effective epidemic response, and indoor residual spray (IRS). The overall goal of the NMCP is to reduce the burden of malaria considerably so that it is no longer a major public health concern to the Eritrean people.

1.1.4.1. National anti malarial treatment policy:
The objective of the national anti malarial treatment policy is to enable the population at risk of malaria infection to have access to safe, good quality, effective and affordable anti malarial drugs/ medicines in order to:

☐ Ensure a rapid and long lasting clinical cure for individual malaria patients
☐ Prevent the progress of uncomplicated malaria to severe diseases and death
☐ Shorten clinical episodes of malaria and reduce the occurrence of malaria associated anemia in population residing in areas of high malaria transmission
☐ Delay the development and spread of resistance to anti malarial drugs/medicines
☐ Enable health professionals in having specific and uniform treatment protocols in different categories of health services.

1.1.4.2. National anti malarial drugs/medicines resistance pattern:
The MoH has been conducting routine antimalarial treatment efficacy studies at specific sentinel sites since 1998. In 1998, the drug efficacy studies were conducted for Chloroquine at 12 sentinel sites. Clinical failure for Chloroquine was 35.6% for patients above five years and adults; and 51.9% for children under five years of age. In 1999, studies were conducted in 9 sentinel sites, in 2000 at 5 sentinel sites and in 2002 at 4 sentinel sites. Though the efficacy studies have shown declining clinical and parasitological response rates, the sample size have been very small.

Eritrea changed antimalarial treatment policy in 2002 from Chloroquine alone to Chloroquine + Sulphadoxine/Pyrimethamine (S/P) as Chloroquine failure rate was above
40% in some sentinel sites. The therapeutic efficacy of Chloroquine + S/P has been on the decline in the recent past. Tokombia which had studied Chloroquine + S/P regimen showed an efficacy rate of 96% and 84% in 2002 and 2003 respectively, while Tessney conducted efficacy study for Artesunate (AS) + Amodiaquine (AQ) showed an efficacy of 100% in 2002, 2003 and 2004.

In 2005, the program piloted AS+AQ for confirmed malaria cases in 4 highly malarious Zones. This was piloted in 16 pilot health facilities in the country. The results were very promising. Out of 3977 malaria confirmed patients treated with AS+AQ, the treatment success rate showed 99.9%. Only one patient did not respond to the treatment and six had minor side effects.

The NMCP with the support of WHO conducted efficacy studies in 2006 to report policy makers as to whether to change AS+AQ or continue with CQ+SP. Artesunate + Amodiaquine were studied in Tokombia, Engela and Goluj; and Chloroquine + S/P in Tessney, Sawa, and Debarewa. Therapeutic efficacies for the combination of AS+AQ and CQ+S/P were 96.6% and 50% respectively after PCR correction of late clinical and parasitological failures.

1.1.4.3. Diagnosis of Malaria:

Diagnosis of malaria is base on clinical criteria (clinical diagnosis) supplemented by the detection of parasites in the blood (parasitological or confirmatory diagnosis). Clinical diagnosis has low specificity as many illnesses present with “malaria like” sign and symptoms. The two most commonly used methods for parasitological diagnosis are light microscopy and rapid diagnostic tests (RDTs).

1.1.4.4. Treatment of Malaria:

- The first line of uncomplicated falciparum malaria is Artesunate + Amodiaquine (AS+AQ)
- The second line of treatment failures is oral Quinine
- The treatment for severe malaria is parenteral Quinine
- Treatment for malaria during pregnancy is Quinine in all trimesters Coartem (Arttmether 20mg + Lumefantrine 120mg) is indicated as a reserve.
1.2. Problem identification and Justification:
Between 2000 and 2013, in Eritrea, malaria admission and malaria mortality rates decreased by 75% and by 83%, respectively (9). This progress can be attributed to the implementation of adequate control strategies including prompt treatment with ACT (10). However, in the last 3 years, despite these control measures, there has been an alarming increase of incidence of malaria cases and the occurrence of sporadic outbreaks in many areas of the country (10). In this scenario, the possible occurrence of resistance to artemisinin derivatives would compromise the situation furtherly.

Artesunate-Amodiaquine (AS-AQ) was introduced in Eritrea in 2007 as first-line treatment for uncomplicated malaria. In 2010, a study, conducted in five sentinel sites in Gash Barka by the National Malaria Control Programme to evaluate the therapeutic efficacy of AS-AQ in this area, showed a treatment failure in 7.6% of patients treated with AS-AQ (5). This ratio is close to the cut-off limit suggested by WHO, i.e. 10%, to take into consideration a change in the local malaria treatment policy. With the reports of Artemisinin resistance, which is the one of the ACT used in the treatment of malaria in Eritrea, investigating the root cause of the recent treatment failure and monitoring the Artemisinin resistance was urgently required and vital.

1.3. Objectives of the study:

1.3.1 General Objectives:
The objectives of the present study were to investigate the presence of mutations in the \textit{P. falciparum} \textit{K13} propeller gene to monitor the possible emergence of resistance to the artemisinin derivatives in Eritrea and mutations in \textit{pfmdr1} and \textit{pfcrt} genes as markers of resistance to AQ, the partner drug in the ACT in Eritrea and investigate the root cause of the recent increase in malaria treatment failure.

1.3.2 Specific Objectives:
To investigate the polymorphisms in the PfK13 propeller gene in plasmodium falciparum isolates from Eritrea.

- To investigate the polymorphisms in the Pfcr gene in Plasmodium falciparum isolates from Eritrea.
- To investigate the polymorphisms in the Pfmdr1 gene in the Plasmodium falciparum isolates from Eritrea.
• To detect any resistance to Artemisinin or the partner drugs in ACT in Plasmodium falciparum isolates from Eritrea.

• To investigate the cause of the recent increase in the malaria treatment failure in Eritrea.
CHAPTER TWO

2. Literature Review

2.1 Literature Review:

Globally, the transmission of the Plasmodium species responsible for human malaria, by Anopheles mosquitoes, is affected not only by a mix of natural determinants, such as temperature, rainfall, flood, wind speed and altitude, (17) but also by several demographic, socio-economic and genetic determinates. For any at risk individual, his or her ethnic group, level of education and occupation (for those of his or her parents/guardians), his or her genotype, level of acquired immunity, housing conditions, household’s use of preventive measures (such as the home use of insecticide and/or bed nets) and knowledge and attitude towards malaria, and (following treatment) the level of resistance to anti malarial drugs in the local parasites, may all affect his or her chances of developing symptomatic malaria (13). In 2006, the WHO officially recommended that Artemisinin (ART) based combination therapies (ACTs) be adopted as first line of treatment for uncomplicated malaria caused by Falciparum (14). This recommendation came in response to the global spread of resistance to the former first line anti malarials Chloroquine and Sulfadoxine-Pyrimethamine (15,16). The global implementation of ACTs including insecticide treated bed nets and indoor residual spraying has helped to considerably reduce the disease burden in many endemic countries. In the past decade, over 40 countries have reported a greater than two fold reduction in malaria cases (2). Despite these achievements, malaria continues to have a devastating impact, with estimated deaths and over 225 million cases in 2009. The greatest burden falls on young children residing in Sub Saharan Africa (17). An outstanding clinical review that covers epidemiology and disease burden, clinical presentation, diagnosis, treatment, prevention through prophylaxis, vector control, and approaches to developing a malaria vaccine (18).

Ever since the discovery of the first case of Chloroquine resistance along the Thai-Cambodian border in the late 1950s, South East Asia has played an important role as a focus for the development of drug resistance in Plasmodium falciparum. Although the first case of quinine resistance had been reported much earlier from South America, the onset of
Chloroquine resistance finally had to be replaced by the combination of Sulphadoxine and Pyrimethamine (SP) as first line for treatment of uncomplicated malaria in Thailand and more than 10 African countries have also switched their first line drug to SP. In 1985 eventually SP was replaced by Mefloquine. The rapid development of resistance to this new drug leads to the introduction of Artemisinin as a combination drug in the mid 1990s. It is mandatory to mention here that therapeutic regimens for prevention and treatment of Chloroquine resistant P. falciparum are associated with higher costs and side effects compared to Chloroquine. Additionally, some of these alternative treatments are associated with more side effects, take longer time for cure and are more difficult to comply with than Chloroquine. Urgent efforts are needed to identify effective, affordable, and alternative anti malarial regimens. Molecular markers for anti malarial resistance have been identified, including pfmdr-1 and pfcr polymorphisms associated with Chloroquine resistance and dhfr and dhps polymorphism associated with SP resistance. Polymorphisms in pfmdr-1 may also be associated with resistance to Chloroquine, Mefloquine, and Artemisinin. Use of such genetic information for the early detection or resistance foci and future monitoring of drug resistant malaria is a potential useful epidemiological tool, in conjunction with the conventional in vitro and in vivo drug sensitivity assessments. Drug resistance is a recurrent theme in the history of infectious disease control.

In the case of malaria, resistance to all but one of the major classes of drugs is wide spread (19). Such resistance occurs because of the strong selection pressure associated with giving patients anti malarial drugs. The most effective way to stall resistance would therefore be to eliminate selection by halting drug treatment, (20) but this is rarely feasible option. Hence alternative approaches to managing resistance are needed. First of all, we need to better understand manner in which resistance evolves and spreads with in population. Molecular methods provide the tools needed for investigating the evolution and the spread of resistance genes. These methods can be used to answer a multitude of pertinent question: Do resistance alleles have few or many origins? Do they spread locally or globally? Do parasites form a single pan-African population, or are there barriers to gene flow? In turn, the answers these questions can make rational decisions about drug treatment policy (21).
Reasons for the development and spread of drug resistance involve the interaction of drug use patterns, characteristics of the drug itself, human host factors, parasite characteristics, and vector and environmental factors (22). Vector and environmental factors may influence the proliferation of resistant parasites. For example, Chloroquine resistant parasites may be more fit for reproduction in certain anopheline mosquitoes than non-resistant strains (23). Polymorphism in the tumor necrosis factor (TNF) gene have been associated increased susceptibility to severe malaria (24,25) Furthermore, genetic analysis of sibling pairs evidenced linkage of mild malaria to the MHC region, with a peak close to the TNF locus (26,27). The study conducted in Burkinafoso, showed that several TNF variants were found to be associated with phenotypes related to malaria infection and mild disease, and may be part of the genetic determinant for human malaria resistance/susceptibility (28).

There has been one example of the effect of the removal of drug pressure on the prevalence of drug resistant parasites. In 1993, Malawi was the first country to replace Chloroquine with SP for the oral treatment of malaria, due to high rates of Chloroquine resistance. Immediately after Chloroquine use was stopped, there was a decrease in the prevalence of the pfcr7T polymorphism that is associated with Chloroquine resistance (20). From 2001 until today, no Chloroquine resistant parasite have been identified in the major cities where surveillance has occurred (29). A clinical trial demonstrated that Chloroquine is now highly effective in the treatment of malaria, after high rates of failure documented just 12 years prior (30). As Chloroquine is withdrawn from use throughout the country, it is possible that Chloroquine susceptible parasites will return and Chloroquine may once again play a role in the treatment or prevention of malaria in the future.

2.1. Plasmodium falciparum resistant gene:

The gene encoding pfmdr is an ortholog of P-glycoproteins found in mammals that mediate multi drug resistance in cancer cells . The protein is found on the digestive vacuole membrane and seems to play a role in regulating traffic across the membrane, including a variety of antimalarial drugs (31, 32).
2.2. PfATP6:

Another transporter, pfATP6, is ortholog of the mammalian sarcoplasmic reticulum Ca2 ATPase (SERCA). There is evidence that this pump is inhibited by Artemisinin, suggesting that mutations may alter Artemisinin susceptibility. One molecular marker for Artemisinin resistance has been proposed, PfATP6 S769N, based on an ecological study in Senegal, French Guiana, and Cambodia and supported by differences in IC values (33). Genotypic prediction of drug resistance, using molecular techniques that apply radio isotopic methods, holds a significant advantage in that it is faster and many samples can be analyzed together (34). Artemisinin based combination therapies (ACTs) have been deployed globally with remarkable success for more than 10 years without having lost their malaria treatment efficacy. However, recent reports from the Thai-Cambodian border reveal evidence of emerging resistance to Artemisinin. Preliminary indications of clinical treatment failures with ACTs came from observational studies in the early 2000s of Artesunate-Mefloquine use near the Thai Cambodian border (35, 36, 37). It was unclear, however, whether this resulted from resistance to Artesunate or its partner drug, or other host or pharmacological factors. Compelling evidence supporting the emergence of ART resistance came from the study published mid 2009 by White et al., 2009 (38) showing delayed parasite clearance rates in Pailin, West Cambodia, compared with Wang Pha, north western Thailand, following Artesunate monotherapy or Artesunate-Mefloquine combination therapy.

Drug resistance in P.falciparum parasites has evolved and spread rapidly, leading to the loss of Chloroquine (CQ) and Sulphadoxine-Pyrimethamine (SP) as first line treatment in most endemic areas. Mutations in MAL7 P1.27 (also known as pfart, the gene encoding the Pf ATPase of the P.falciparum CQ resistance transporter) and in the genes encoding P.falciparum dihydrofolate reductase (pfhdfr) and P.falciparum dihydropteroate syntase (pfdrps) have been shown to confer resistance to CQ and SP, respectively. Additionally, copy number and/or point mutations at pfmdrl (which encodes a homolog of human P- glycoprotein) on chromosome 5 have been associated with parasite response to Mefloquine (MQ), Quinine (QN), ART and other antimalarial drugs, although other unknown genes may also have roles in the responses.

P.falciparum resistance to antimalarial drugs emerged after wide spread deployment of these drugs (with in the past 60 years). This may not have been enough time for recombination to
completely break down linkages between causal alleles and nearby genetic markers. Indeed, by scanning for regions of high linkage disequilibrium (LD), the chromosome segment carrying the pfcrt locus was correctly identified using 342 genome-wide micro satellite markers and 92 parasites isolates collected from different parts of the world (39). Here they report the first genome-wide P.falciparum maps of population recombination events, signatures of recent positive selection and GWAS of multiple drug resistance phenotypes using a custom SNP typing micro array. The host immune response to malaria infection likely influences the speed of drug resistance and the extent to which drug resistance translates in to clinical drug failure (40). Immunity to malaria is acquired through repeated exposure to infection and is maintained through boosting by infectious bites throughout an individual’s life time. In high transmission settings, children are susceptible to symptomatic and severe malaria infection, whereas adults are considered semi-immune because they can acquire infection, but are not at risk for severe disease and often experience asymptomatic infection. Malaria parasites in these semi-immune adults are not under drug pressure because infection is not usually recognized and treated. In contrast, individuals in low and sporadic transmission settings, such as South East Asia, are not exposed to malaria with enough frequency to develop immunity. As a result, all infected individuals develop symptomatic infection, and the infections always prompt treatment with an anti malarial drug. It is possible that the difference in the extent of drug pressure on the parasite population drives the spread of most parasites in human hosts are under selective drug pressure, and later spread to high transmission settings, where many parasites survive without drug pressure (41).

2.3. Pfcrt:

Plasmodium falciparum Chloroquine resistance transporter gene (Pfcrt) has been used to detect and determine chloroquine resistance in malaria parasites.

2.4. Kelch 13 propeller gene:

Resistance to Artemisinin in Plasmodium falciparum has been associated with the parasites’s Kelch 13 propeller gene polymorphisms (43). This gene is very vital in tackling Artemisinin resistance in malaria parasites. There has been progressive loss of wild type parasites in western Cambodia during the decade of emerging ART resistance in this region. Mutations in
the PF3D7_1343700 Kelch propeller domain (‘K-13 propeller’), is located 5.9 kilobases upstream of the 35-kb is associated with Artemisinin resistance in vivo and in vitro (44).

In nutshell, after the emergence and spread of Plasmodium falciparum multi-drug resistant isolates insensitive to most of the available antimalarials, the introduction of artemisinin-based combination therapy (ACT) as first-line drug treatment for non-complicated malaria has opened a new horizon in the fight against malaria, with extraordinary results: in practical terms, over the last decade, a dramatic reduction of mortality due to malaria in children, especially in sub-Saharan Africa, has been achieved and the total malaria cases dropped by 40% worldwide (45). Unfortunately, a study carried out in 2009 in Cambodia showed unequivocally that P. falciparum was developing resistance against artemisinin, (46) and to date, Artemisinin resistance is spreading across mainland Southeast Asia (47). The possible extent of resistance to artemisinin in Africa would have a devastating effect on child mortality and could wipe out the successes achieved in this decade in the fight against malaria. The recent identification of Kelch 13 propeller gene as determinant of artemisinin resistance (48) provided the international scientific community of a molecular marker to track in real time the emergence of resistant falciparum isolates before the artemisinin resistance spread in Africa.
CHAPTER THREE

3. Materials and Methods

3.1. Study Site:
Between November 2013 and November 2014, a total of 209 blood samples were collected in Eritrea from three study sites: 200 samples from Barentu and Agordat, in the Gash Barka region, and 9 samples from Medefera, in the Debub region, Fig.3. Below shows the study sites for the study. Both regions are in a stable endemic malaria zone and represent the two areas with the highest malaria burden in Eritrea. Following informed consent of the patient or their guardian, a blood sample from malaria-infected individuals was spotted in triplicate on a filter paper, dried and stored at room temperature until use. Ethical approval for sample collection was obtained from the Ethics Committee of the Eritrean Ministry of Health. The majority of patients were young adults (135 men and 74 women) with a median age of 24.2 years (range 1.5-70 years).

Figure3.1 The study sites for the study in Eritrea
3.2. Materials and Methods:

P. falciparum and P. vivax infections were confirmed by Real-Time PCR method as previously described by Veron et al. (6). Of 209 blood samples tested, 160 were infected with P. falciparum, 24 with P. vivax, 20 were mixed infections P. falciparum/P. vivax and 5 were negative. On the 180 samples positive for P. falciparum infection, the polymorphism analysis of K13 propeller gene was performed by PCR amplification and sequencing using the following primers: Artinner For - 5’ GCCTTGTTGAAAGAAGCAGAA 3’ and Artouter Rev -5’ CGCCATTATTCCTCCTGTA 3’, with PCR conditions described by Taylor et al. (50). In addition, the frequency of single nucleotide polymorphisms (SNPs) at pfcrt 72-76 codons and at pfmdr1 86-184 codons was assessed as described by Menegon et al. (51). All PCR products were sent to Eurofins Genomics Company (Germany) for sequencing. The obtained sequences were compiled and analyzed by Accelrys DS Gene software.

3.2.1. DNA Extraction:

Parasite DNA was extracted from dried blood spots by PureLink™ Genomic DNA Kit (Invitrogen, USA) following the manufacturer’s instructions. Briefly, punches of dried blood spot were placed on a sterile micro centrifuge tube and 20 µl of Proteinase K and 180 µl of PureLink™ Genomic Digestion Buffer were added and were mixed well by vortexing. The solution was incubated at 55°C for 30 minutes to promote protein digestion. After incubating, 200 µl of PureLink™ Genomic Lysis/Binding Buffer was added and was mixed well. DNA was precipitated with 200 µl 96-100% ethanol. The mixture was applied to a PureLink™ Spin Column and after centrifugation at 10,000 × g for 1 minute, the collection tube was discarded and the spin column was placed into a clean PureLink™ Collection Tube. The column was washed twice with Wash Buffer 1 and 2. DNA was eluted with 150 µl of PureLink™ Genomic Elution Buffer. The purified DNA was stored at -4°C for immediate use. (See fig.4. below).
Figur 3.4. Flow chart for purifying genomic DNA using the purelink genomic DNA mini kits
3.2.2. Amplification of PfK13 (Kelch 13) gene:
The PfK13 gene was amplified as previously described in Taylor et al. 2014 J Infect Dis.pii: jiu467.
PCR reaction was carried out in a total volume of 25 µl containing 5 µl of extracted DNA, 1X buffer, 2.5 mM MgCl2, 400 µM of each of dNTPs, 10 picomoles of each primer and 2.5 U of FastStart Taq DNA Polymerase (Roche, Germany). The PCR reaction was performed using the following cycling conditions: 94°C for 1 min, 58°C for 1 min and 72°C for 2 min for 40 cycles. PCR products were separated by electrophoresis through a 1.8% agarose gel and their positions were marked by a 100 base pair marker (Nzytech, Portugal). The agarose gel was then stained with ethidium bromide and bands were visualized by UV transillumination and the DNA concentration estimated. About 80 ng of PCR products were sequenced as described below.

3.2.3. Amplification of Pfcrt gene:
The PvMCA1 gene was amplified by using couples of specific primers designed on the genomic sequence from the NCBI GenBank AF030694 Plasmodium falciparum strain Dd2. Primers were selected by using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, USA; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).
PCR reaction was carried out in a total volume of 25 µl containing 5 µl of extracted DNA, 1X buffer, 2.5 mM MgCl2, 400 µM of each of dNTPs, 10 picomoles of each primer and 2.5 U of FastStart Taq DNA Polymerase (Roche, Germany). The PCR reaction was performed using the following cycling conditions: 94°C for 30 sec, 60°C for 1 min and 72°C for 1 min for 40 cycles. PCR products were separated by electrophoresis through a 1.8% agarose gel and their positions were marked by a 100 base pair marker (Nzytech, Portugal). The agarose gel was then stained with ethidium bromide and bands were visualized by UV transillumination and the DNA concentration estimated. About 60 ng of PCR products were sequenced as described below.

3.2.4. Amplification of Pfmdr1 Gene:
The PvMCA1 gene was amplified as previously described in Duraisingh et al. 2000 (Molec Bioc Paras 108, 13-23).
PCR reaction was carried out in a total volume of 25 µl containing 5 µl of extracted DNA, 1X buffer, 2.5 mM MgCl2, 400 µM of each of dNTPs, 10 picomoles of each primer and 2.5 U of FastStart Taq DNA Polymerase (Roche, Germany). The PCR reaction was performed using the following cycling conditions: 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min for 40 cycles. PCR products were separated by electrophoresis through a 1.8% agarose gel and their positions were marked by a 100 base pair marker (Nzytech, Portugal). The agarose gel was then stained with ethidium bromide and bands were visualized by UV transillumination and the DNA concentration estimated. About 60 ng of PCR products were sequenced as described below.

3.2.5. Sequencing:
All the PCR products were purified and concentrated by NZYGelpure (Nzytech, Portugal), following the manufacturer's instructions. The purified PCR products were sequenced with reverse primers at BIOFAB (Rome, Italy), and analyzed by means of Accelrys DS Gene 2.5 software (ACCELERYS, Cambridge).
4. Results and Discussion:

P. falciparum and P. vivax infections were confirmed by Real-Time PCR method as previously described by Veron et al. (49). Of 209 blood samples tested, 160 were infected with P. falciparum, 24 with P. vivax, 20 were mixed infections P. falciparum/P. vivax and 5 were negative. (See Fig.5).

Compared to the reference 3D7 strain (PF3D7_1343700), no mutations were detected in the K13 propeller region examined in the Eritrean falciparum isolates. Notably, a synonymous polymorphism, G538G (GGT instead of GGA), was identified in all 180 falciparum isolates analyzed.

Otherwise, 84.5% of P. falciparum isolates showed the mutant alleles pfcr7 74I-75E-76T and 85.5 % of isolates carried out the pfmdr1 184F mutation. The wild type codon pfmdr1 N86 was identified in 88.8% of the isolates. In addition, 41.6 % of isolates displayed a novel point mutation in pfmdr1 at codon 153, resulting in a change of a Serine (TCT) to Phenylalanine (TTT). The frequencies of SNPs in pfcr7 and pfmdr1 genes are shown in Figure 6. Overall, taking in consideration the main three SNPs linked to AS-AQ susceptibility, eight haplotypes were detected among the 180 analyzed isolates, and among these the haplotype pfcr7 76T /pfmdr1 N86-184F was the predominant (66.7%) (Table1).
**Figure 4.1** Confirmation of *Plasmodium* spp. by nested PCR.

*Plasmodium falciparum* (205-bp fragment). Lane 1, molecular mass marker; lane 2, positive (+) control; lane 3, negative (-) control; lanes 5,9,12,14,20,22,23,25, from the top row and 6,7,14,16-19,21,23-26,28-30 negative samples; All the rest of the lanes positive samples for Plamsodium falciparum.

**Figure 4.2.** Frequencies of SNPs in *pfcrt* codons 72-76 and in *pfmdr1* codons 86,153,184 in *P. falciparum* isolates from Eritrea
Table 4.1. Prevalence of haplotypes of *pfcrt* and *pfmdr1* genes detected in analyzed *P. falciparum* isolates from Eritrea.

<table>
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<tr>
<th>Haplotype</th>
<th>Pfcrt</th>
<th>Pfmdr1</th>
<th>No. isolates</th>
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<tr>
<td>3 mutations</td>
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CHAPTER FIVE

5. Conclusions and Recommendations

5.1. Conclusions:

Between 2000 and 2013, in Eritrea, malaria admission and malaria mortality rates decreased by 75% and by 83%, respectively (52). This progress can be attributed to the implementation of adequate control strategies including prompt treatment with ACT (53). However, in the last 3 years, despite these control measures, there has been an alarming increase of incidence of malaria cases and the occurrence of sporadic outbreaks in many areas of the country. (53) In this scenario, the possible occurrence of resistance to artemisinin derivatives would compromise the situation furtherly.

In order to monitor the AS resistance, we carried out the analysis of the Pf K13 gene that showed a very limited polymorphism, involving only the synonymous G538G substitution in all analyzed P. falciparum isolates. A recent similar study carried out in the neighboring country Ethiopia (54) did not reporting any polymorphism in the Pf K13 gene suggesting that the wild type genotype is by far the most prevalent genotype in this area of Eastern Africa. This result is consistent with the possible full susceptibility to AS of the parasite population of this area. Noteworthy, the G538G was previously identified in 3 isolates from Kenia (55) and a mutant version of this codon, G538V, was found in Myanmar. (56)

Concerning the pfmdr1 polymorphism linked to the AQ susceptibility, a high frequency (88.8%) of the wild type codon N86 was observed in Eritrean falciparum isolates. In a recent study (56), this allele seem to be significantly associated with a decreased susceptibility to AQ and dihydroartemisinin, the active metabolite of all artemisinin compounds. However the role of the Pfmdr1 is still to be well elucidated, since, conversely, others studies have reported a link between the mutation 86Y, mainly in association with the mutation pfcr 76Tand a reduced AQ efficacy. (57)

A limitation of our study was the impossibility to associate our molecular findings with treatment outcome and to test in vitro the sensitivity to ASAQ of the falciparum isolates.
This lack of information does not allow us to make a correct evaluation about the level of sensitivity of the Eritrean falciparum isolates to ASAQ, including the possible role of the novel pfmdr1 S153F mutation detected in almost half of the analyzed isolates.

Our data do not suggest the artemisinin resistance in Eritrea in 2013–2014; we only can speculate that the 7% of treatment failure reported in this country is probably related to the loss of efficacy of the AQ, hence to Pfcr1 and Pfmdr1 polymorphisms, rather than the artemisinin derivative component of the ACT.

In conclusion, the present study represents an investigation focused on the molecular surveillance of falciparum drug resistance carried out for the first time in Eritrea, an African country where this kind of information are largely lacking.

5.2. Recommendations:

Based on our findings we recommend the following:

- Further study should be conducted that would be able to associate the molecular finding with treatment outcomes.
- In vitro the sensitivity to ASAQ of the *falciparum* isolates should be carried out.
- There should be molecular surveillance along with the routine antimalarial treatment efficacy studies that are done at sentinel sites because it is difficult to predict how soon resistance mutations could appear and spread.
- Early detection of resistant genes through molecular surveillance would provide a framework to rapidly monitor for the emergence or importation of resistant alleles.
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Spread of artemisinin-resistant Plasmodium falciparum in Myanmar: a cross-sectional

Clearance From the Ethical Committee Ministry Of Health, Eritrea

Health Research Proposal Review and Ethical Clearance Result

Name of researcher: Abduselam Mohammed Nurahmed

Address: OSM & OSDM

Title of Research: Research Proposal on Genetic basis of malaria drug resistance in Eritrea

Sponsor: OSM & OSDM

Letter of Reference: Ref, OSM 29/377/13

The Health Research Proposal Review and Ethical Committees have reviewed your paper for its research relevance and ethical soundness and come up with the following conclusion. Based on their deliberations

1. The research proposal is accepted
2. The research proposal is not accepted

Signed and approved on date 07/11/2013 by:

1. Prof. Andemariam Ghebremichael
2. Dr. Negussi Leuke
3. Dr. Berhane Debru
4. Asmerom Siyum
5. Mr. Mehari Woldu

Ministry of Health, Eritrea
| Date of collection: __________________________ | Date of collection: __________________________ |
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<td><strong>A research on genetic basis of malaria drug resistance in Eritrea:</strong></td>
</tr>
<tr>
<td>Date of collection: ____________________</td>
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<tr>
<td>Zoba: _______ Hospital: ___________</td>
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<td>Name (Optional): ____________________</td>
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<td>ID: ___________</td>
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**Some Pictures from ISS; the Lab in Rome, Italy**