MOLECULAR SURVEILLANCE OF CYTOCHROME B MUTATIONS IN PLASMODIUM FALCIPARUM CONFERRING RESISTANCE TO MALARONE: A STUDY IN BORDEAUX.

Supervisor: **Prof. Pascal Millet**
Centre René Labusquière
Université Victor Segalen Bordeaux 2
146, Rue Leo Saignat
33076 Bordeaux
France

Candidate: **Praveen K. Thadigiri**

Thesis towards partial completion of the Erasmus Mundus European Master of Science in International Health (2006-07) under the auspices of the tropED network, Europe.

Thesis Institution: **Centre René Labusquière, Université Victor Segalen Bordeaux 2, Bordeaux, France.**

Parent Institution: **Institute of Tropical Medicine, Charite Medical School, Humboldt University, Berlin, Germany**

Partner Institution: **Centre for International Health, University of Bergen, Bergen, Norway.**

MOLECULAR SURVEILLANCE OF CYTOCHROME B MUTATIONS IN PLASMODIUM FALCIPARUM CONFERRING RESISTANCE TO MALARONE: A STUDY IN BORDEAUX.

Candidate:

Praveen K. Thadigiri
Track 1(Tropical Medicine and Disease control
Erasmus Mundus M. Sc. (International Health)

Supervisor:

Prof. Pascal Millet
Centre Rene Labusquiere
Universite Victor Segalen, Bordeaux 2
Bordeaux, FRANCE

TABLE OF CONTENTS

FOREWORD

This thesis is the written documentation of research done towards partial completion of the Erasmus Mundus European Master of Science in International Health organized by the tropED network. Research was conducted at the molecular biology laboratories of the Centre Hospitalier Universite de Bordeaux (CHU). With the primary objective of identifying mutations in the cytochrome b gene of Plasmodium falciparum implicated in therapeutic failure related to Malarone® administration, this project is designed to strengthen the drive for the establishment of a global open access database of resistance conferring molecular markers in Plasmodia.

The smooth conduct of the research project was made possible under the able guidance of Prof. Pascal Millet, Universite Victor Segalen, Bordeaux2, France.

Praveen K. Thadigiri
Track 1(Tropical Medicine and Disease Control)
Erasmus Mundus M. Sc. (International Health)

ACKNOWLEDGEMENTS

To the year that was…….. every step towards the finish line of this grand masters' programme has been reminiscent of the steadfast support from friends and loved ones.

Special mention of Prof. Pascal Millet beyond the lines customary for supervisors for the way he guided me, a new-comer to the field of molecular biology, making it a learning experience I am sure will stand me in good stead in future.

A note for the staff at the CHU laboratories in Bordeaux for their smiling faces and willingness to teach, making my stint in the lab a memorable one.

And can I deny the hand of THE ALMIGHTY over me as I embarked on this endeavor right until the finish?

Merci a tous!

I. ABSTRACT

With the passing years malaria seemed to have moved closer to joining the list of 'neglected' diseases. But with the appearance on the horizon of Plasmodial resistance to all known first line antimalarials, research using molecular biological tools got a fresh lease of life. But for sheer numbers that distinguish the endemic from non-endemic, whose burden of disease is from imported malaria, the key issues for both the categories of countries were similar.

In this respect, P. falciparum with its dubious association with complications assumes the prime position needing immediate attention. This report encompasses a review of literature in this direction to trace out evidence and the patterns in the development of drug resistance. The core though, is the study using the FRET RT-PCR(Fluorescence Resonance Energy Transfer Real Time-Polymerase Chain Reaction) which was instrumental in the detection of a substitution, A803G designated as mutation Y268S in the P. falciparum cytochrome b gene, that led to therapeutic failure in a traveler returning from Africa, who had been on *Malarone*® prophylaxis. Though mutations conferring resistance to *Malarone*® whose major component is *Atovaquone* are a known entity, they assume special significance due to the chemotherapeutic profile of *Malarone*®, which figures in the therapeutic regimen for multidrug resistant P. falciparum malaria.

This report is an effort to drive home the point that there is an immediate need to identify drug resistant strains of P. falciparum as also the other species of Plasmodia using molecular biological tools and to record and make available on an easily accessible international database these details along with their geographic correlates. Rigorous surveillance and monitoring in this direction is required to provide credible information that would serve to enable governments/health departments to formulate their antimalarial chemotherapeutic strategies to the best effect. Not done now we would be facing a potential disaster as there are not very many antimalarials in the developmental pipeline. The magnitude of mortality and morbidity would be unprecedented. On a different note it 'pays' to take this path now rather than face the financial ramifications of more elaborate drug resistance patterns in the Plasmodia.

II. ABBREVIATIONS

A = Adenine

ACT = Artemesinin based Combination Therapy

asp = Aspartic acid

ATP = Adenosine Tri-Phosphate

C = Cytosine

C = Carbon; **H** = Hydrogen; **O** = Oxygen; **Cl** = Chlorine; **N** = Nitrogen

CT = Combination Therapy

cys = Cysteine

DNA = Deoxyribo-nucleic acid

DALY's = Disease Adjusted Life Years

Emax = Maximum Effect

EC_{50} = drug concentration required to produce 50% of its maximum effect

EDTA = Ethylene Diamine Tetra Acetic Acid.

ETC = Electron Transport Chain

FRET RT-PCR = Fluorescence Resonance Energy Transfer Real Time Polymerase Chain Reaction

G = Guanine

H_2O = Water

ICD = International Classification of Diseases.

LC = Light Cycler

$MgCl_2$ = Magnesium Chloride

MUT = Mutant

Non- ACT = non-Artemesinin based Combination Therapy

P = Plasmodium

pfcrt = Plasmodium falciparum Chloroquine Resistance Transporter protein.

pfdhfr = Plasmodium falciparum Dihydrofolate Reductase gene

pfdhps = Plasmodium falciparum Dihydropteorate Synthetase gene.

pfmdr = Plasmodium falciparum Multidrug Resistance gene

phe = Phenylalanine

RFLP = Restriction Fragment Length Polymorphism

rt-PCR = Reverse Transcriptase-Polymerase Chain Reaction

T = Thymine

TDR = Tropical Disease Research

tyr = Tyrosine

thr = Threonine

WHO = World Health Organisation

Wt = Wild Type

III. INTRODUCTION

III.1 MALARIA

III.1.1 The History of Malaria, the History of Mankind?

The history of malaria parallels the history of mankind on this planet. Some researchers even vouch for the possibility that the disease is older than man himself. The history of the Plasmodium species, the causative organism is a story of survival, a story of the ability of this protozoan parasite to evolve over the ages as it moved across the continents with man [1], cited from as far back as the Neolithic Period. This said, it probably wouldn't be as much a success story for this parasite had it not been for the concomitant breeding potential and dexterity of its vector [2], the Anopheles species of mosquitoes to adapt to newer pastures as well. The survival ability of this agent is best demonstrated by the preservation of its virulence inspite of the migration into geographical areas with different a topography and thus different weather conditions that it entailed. Our knowledge of the adaptability of this agent culminates today in the discovery of drug resistant strains of this parasite.

Since times immemorial malaria has found a mention in works of literature and the annals of medicine alike the world over with a description of disease symptoms that would concur with today's clinician's perception of malaria. While mention of the characteristic paroxysms of fever have been mentioned by literary geniuses like Shakespeare who called them 'augues', the first written description of symptoms representative of malaria have been found in an ancient Chinese medical treatise, the *Nei Ching* [3]. Yet another medical treatise from the Orient, the *Sushruta* [4, 3] implicates the cause of a fever like illness that would correspond with malaria to insect bites. An idea that is paralleled by the Italians' [5] notion of this illness originating

from the air from marshy areas, popular in the medical circles then as the Miasmatic Theory of Disease Causation [6] until the late nineteenth century. From here was born the name 'malaria', from the words 'malo' and 'aria' bad and air respectively [3]. The Greek historian Thucydides [7] mentions that it was this know-how that enabled the Syracusans in 413 BC to defeat the Athenians by forcing them to camp on the marshy ground around Syracuse. The Athenians awaiting a coup from within the ranks of the Syracusans were not prepared for the terrible epidemic of malaria that was to strike down their army like nine pins. Biological warfare in the form of malaria thus seems to have made its way into the arsenal of many armies unknowingly as can be seen from similar historical accounts from all over the world.

Mankind has battled and won over the scourge of many diseases in the past but with malaria, given the present status of the disease, the bitter truth is that it will continue to plague humans into the future. The fact remains that the only way to get over this apparently primitive yet intelligent parasite that has beleaguered man over the ages is to out-smart it. Even in the face of the vast medical knowledge we have accumulated it seems an insurmountable task. How else can we explain the fact that after about 5000 years of knowing the disease and 3 Nobel Prizes conferred to scientists who worked on this disease we still find ourselves at sea when faced with this illness! What we need today is coordinated efforts between different sectors, the importance of which we seem to have grasped but to put to work has been wrought with difficulties. What we need is an amalgamation of knowledge from different fields, a union of technologies and the realization that it is but a shame that we boast of achievements of having made fearless forages into the realms of space and having set foot on the moon but yet on our very own earth we remain fearful of the threat of fatal attacks from a

minute parasite that has in essence dictated the history of mankind. It is time to take the most viable steps to win the battle against this agent.

III.1.2 Epidemiology of Malaria

Even though our knowledge of the disease spans over 5000 years, a comprehensive understanding of the disease came only in 1898-99 with the discovery of the transmission of the parasites by the Anopheles species of mosquitoes [3]. This discovery completed the classical epidemiological triad [8] of disease causation with the details of the agent and host having been elucidated before. This is one disease which so clearly demonstrates the flux between these three facets (agent, environment and host) of disease causation. The disease is caused by the protozoan parasite belonging to the genus *Plasmodium* transmitted to man by the bite of the some species of infected mosquitoes belonging to the genus *Anopheles*. The following four are implicated in causing human disease:

1. Plasmodium falciparum (ICD* 084.0)
2. Plasmodium malariae (ICD* 084.2)
3. Plasmodium ovale (ICD* 084.3) and
4. Plasmodium vivax (ICD* 084.1)

*ICD = International Classification of Diseases. WHO.

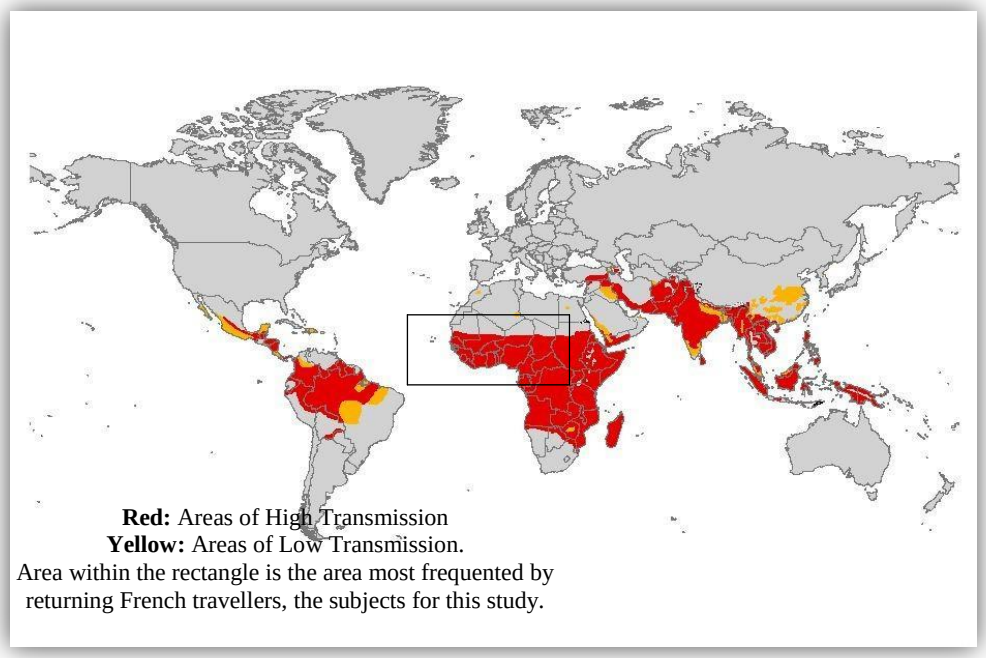
Table 1. Human Plasmodia species with the ICD identification tags.

Each of these species is associated with a characteristic paroxysm of fever. Plasmodium ovale and vivax cause ‘tertian’ malaria and P. ovale ‘quartan’ malaria [9]. The clinical condition caused by P. falciparum goes by names of aestivo-autumnal fever, malignant tertian malaria and cerebral malaria [10]. Both tertian and quartan malaria could lead to severe illness but unlike malignant tertian malaria they are rarely fatal.

The phenomenon of relapse and recrudescence make this disease entity a bigger challenge to grapple with. While relapse may be defined as a return of the disease after its apparent cessation, recrudescence refers to a recurrence of symptoms after temporary abatement [11]. Though the two look similar, good clinical acumen can help distinguish one from the other based on the time lag and demographic correlates like the country of origin of the infection and to a certain extent even the character of the fever peaks in the patient. Arguably the most important disease in the tropical setting, malaria occurs in some temperate zones too. A broad over-view of its geographical distribution according to the data from the Special Programme for Research and Training in Tropical Diseases (TDR) is as follows:

Plasmodium falciparum: Predominates in Africa and much of SE Asia
Plasmodium vivax: Southern Asia and Central America
Plasmodium ovale: Africa
Plasmodium malariae: Similar to P. falciparum but much less common.

Table 2. Plasmodia and their geographic attributes



Red: Areas of High Transmission
Yellow: Areas of Low Transmission.
Area within the rectangle is the area most frequented by returning French travellers, the subjects for this study.

Figure 1[12]. Global Distribution of Malaria.

III.1.3 Problem Statement:

With a distribution as widespread and with a predominance amongst the poorer countries in the tropics, the mortality and morbidity due to malaria can be at best, an estimate. But the statistics are nevertheless staggering. With over 40% [13], or numbers in excess of 2 billion living in over a hundred countries exposed to the risk of this disease, the morbidity stands at 273 million and the mortality at 1.09 million [14]. Over 90% [14] of these deaths occur in Sub-Saharan Africa. In terms of Plasmodium falciparum malaria, which can lead to severe malaria, the figures for the same region are about 500 million cases and a million deaths [15]. From these numbers it becomes clear that the vicious circle of poverty that most of these countries have been battling with perennially continues with DALYs from malaria alone at 44.99 million [14]. Malaria thus remains the first in the list of parasitic diseases as far as deaths are concerned [16] and Plasmodium falciparum stands out as the species that is the cause for the maximum mortality [17].

III.1.4 Malaria in Non-Endemic Countries

Notwithstanding the grim scenario portrayed by the numbers stated above for malaria endemic countries, previously non-endemic countries are now faced with the novel phenomenon of dealing with malaria as an imported [18] disease. With globalization has come the unavoidable consequence of extensive international travel. This movement of people from malaria endemic regions to the more protected environs of the non-endemic countries has posed a serious threat to the health systems in these countries. This has spurred an interest in research activities into a disease which previously occupied a status close to that of the other neglected diseases. The

less fortunate, resource poor countries with malaria endemicity would benefit from such research as long as there is an external validity in these studies. This exchange of knowledge and qualified manpower could help lay new protocols in the process of disease control in the endemic countries, strengthened by new knowledge emanating from research activities spearheaded by the more non-endemic resource-rich countries. This phenomenon of imported malaria and its implication for Europe with special attention to France has been dealt with in greater detail in this document specifically in the chapter dealing with the study itself. This coupled with the rapid emergence of resistance to first line antimalarials and reports of therapeutic failure has brought about a refocus into a disease that was erstwhile considered a bane of the malaria endemic zones.

IV. STUDY CONDUCTED AT BORDEAUX

IV.1 OBJECTIVES

IV.1.1 Primary objective

- To identify the presence of mutations of the cytochrome b gene using the RT-PCR technique and to ascertain its relation to resistance to Malarone in hospital patients in Bordeaux.

IV.1.2 Secondary Objectives

- To evaluate the use of existing molecular markers in Plasmodium falciparum to monitor resistance to first line antimalarials.
- To establish the grounds for the creation of an elaborate database of molecular markers that confer resistance to antimalarials.

IV.2 BACKGROUND TO THE STUDY IN BORDEAUX

The conduct of the present study is in the light of the importance imported malaria has acquired in non-endemic countries. One cannot over state the importance of this form of the disease as it poses a special challenge to physicians in these countries who aren't always conversant with malaria as a cause of pyrexia of unknown origin in routine day to day practice. Every missed diagnosis could allow for the progression of the disease onto the complicated stages endangering the life of the patient.

Another interesting point to note here is the issue of immunity with respect to malarial infection. Acquired immunity to malaria seen in persons living in endemic regions is a result of a delicate interplay involving positive and negative modulation of host's cell-mediated and humoral immunity [19]. This immunity acquired from repeated infections with the parasite could be short-lived should the person move to an area with a parasite population of a different strain, or to a non-endemic area. Suffice to say here that immunity acquired to malaria is species specific as also it is stage specific [20]. Acquired immunity here doesn't provide protection against infections but what it does protect against is the severe effects of the disease [21]. For this 'non-sterile' [20] immune status to last, the patient needs to be in a state of constant exposure to infections [22] and should also possess a functional spleen. As a previously immune individual is no longer exposed to repeated infections, in other words when the intensity of transmission falls, so do levels of acquired immunity against the malarial parasite.

This was of tantamount importance in this study as the subjects recruited in this study comprised either patients who were non-immune travellers or previously immune persons who were returning from vacations to their native countries in Sub-

Saharan Africa. The point made regarding acquired immunity applied to the latter group of patients. Though it is beyond the scope of this report it is important to note here that it has been hypothesized that transmission intensity through the very same epidemiological mediator of immunity along with clonal multiplicity of the parasite and the threat of infection to the host [22] essentially controls the evolution of resistance to a drug.

The above factors give an interesting edge to this study which is underpinned firstly by the phenomena of imported malaria and then by that of drug resistance in Plasmodium falciparum to first line antimalarials. The emphasis of this study has been to work on an already established base of previous research (from a literature review) showing credibly the evolution of drug resistance in Plasmodium falciparum and the entity of imported malaria specific to France. Imported malaria bears a special significance in the health care system in France as we can see from data collected during this study and from other records that the vast majority of returning travellers who visited the hospital with complaints of fever had visited malaria endemic countries and an overwhelming number had been to malarious Sub-Saharan African countries.

The following sections of this document will address the rationale behind this study which is based primarily on the issues of imported malaria and evidence of Plasmodium falciparum having developed resistance to first line antimalarials.

IV.2.1 The Phenomenon of Imported Malaria

By definition, it refers to an episode of malaria wherein the infection is diagnosed in a country that isn't endemic for malaria and the origin of infection can be traced back to a second country where malaria is endemic [23]. Imported malaria poses a special challenge as health systems in countries which are not frankly endemic

for malaria now have to deal with this novel form of the disease. With an increase in international travel as a fall-out of globalization, non-endemic countries are now faced with a unique public health challenge in the form of imported cases some of which are drug-resistant. Another dimension to this problem is the lack of familiarity of physicians in non-endemic countries with tropical entities like malaria [24]. It thus creates a diagnostic dilemma wherein a lack of accuracy in the assessment of the patients could be fatal. Inspite of the threat that this form of the disease carries with it, it is often ignored and doesn't get its due as an important health problem. Evidence to this is the fact that little information is available today concerning the incidence of imported malaria with relation to exposure [25].

Travel medicine clinics work hard to offset the burden of acquiring a disease in an endemic, nevertheless, the number of imported cases of malaria stands at 30,000[26] cases. France tops the list with 7000[27] cases annually (while the aggregate for Europe per annum is 12, 000 cases [26]). About 75% of these being native Africans who have been residents in France coming back from their home countries in Africa after a vacation [27]. And of these Plasmodium falciparum is the species identified most commonly [28]. It's a triangle of factors that make it an important public health consideration for France:

1. Over 80% of the cases of imported cases come from Africa, especially Sub-Saharan Africa [28].
2. This is the zone that is endemic for Plasmodium, which is paralleled by studies that show figures upwards of 80% for imported cases being Plasmodium falciparum infestations [29].
3. Drug resistance to first line antimalarials has been reported from these areas in Africa.

Table 3. The triangle of facts encompassing malaria in France.

IV.2.2 The Phenomenon of Drug Resistance

IV.2.2.1 Definition and History

Resistance has been defined as the ability of the parasite to survive and multiple inspite of the recommended treatment being fully enforced [30]. It was in 1950s that the first reports of drug resistance to antimalarials were made from the Thai-Cambodian border [31]. The 1980s saw reports of resistance to Sulfadoxine-Pyremethamine from the Thai-Cambodian border and the Thai-Myanmar border [32]. Since then there have been multitudes of studies that brought out the frightening scenario of drug resistance to Amiodaquine[33], Mefloquine[34], Quinine[34], and Antifolates[34], these include all but the artemesinin[35] derivatives, which together include literally every single drug we have today in our arsenal against malaria. Once again it seems like the Plasmodium falciparum reigns supreme amongst the others of its genus with evidence of resistance to all antimalarials in use today [36]. A variety of reasons starting with the indiscriminate use of drugs to high parasite loads can be attributed to the development of resistance in the parasite. But all of this centres on the selection of parasites with mutations in their genomes that render them tolerant to drug levels they were previously susceptible to [36].

IV.2.2.2 The Evolution of Resistance

The evolution of resistance can be studied via the classical ‘epidemiological triad’ of the agent, host and the environment. The agent being Plasmodium falciparum, the host man and the pharmacological interventions here would be considered under the environmental factors which come in to complete the triad. Resistance evolves via what some scientists call ‘epidemiological or clinical mediators’ and ‘effectors’ [22]. All of these factors can no doubt be fitted into and well explained by the three entities of the epidemiological triad. Clonal multiplicity

and the degree of sexual recombination in the agent in conjunction with host factors like innate dynamics like the level of acquired immunity and the level of parasitemia in the environs of the level of drug use in the community and the proportion of treated cases, all factor in when it comes to the dynamics of resistance development [22]. Other environmental factors like the pharmacological characteristics of the drugs in use and also the policies that govern their use play a vital role in the development of drug resistance. A complex web of connections between these factors under the influence of the transmission intensity dictate the dynamics of the evolution of resistance to a particular drug [22]. Development of resistance follows the following pattern [37]:

Phase 1: Emergence (parasite mutations and spread to second host)
Phase 2: Spread (Spread within a host population)
Phase 3: Post-Emergence (Slowing down as it reaches a fixed level)

Table 4. Phases in the evolution of drug resistance.

IV.2.2.3 Dose-Response Curves with Emerging Resistance

A dose-response curve can be used to study the varying responses of the parasite to a drug along the path to developing resistance to the drug. The parameters of threshold concentration, EC_{50}, the maximal response and the slope of the curve can be used to understand the evolution of the fully resistant strain from the susceptible one with the sigmoid curves on this graph. This signifies the development of resistance to the drug as can be evidenced by the curves as we move from left to right.

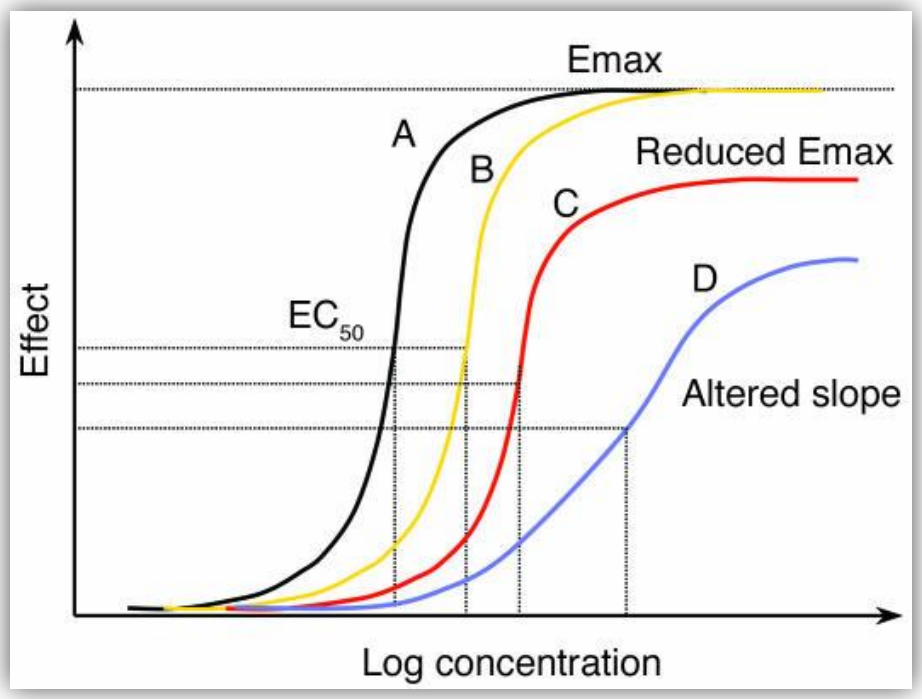


Figure 2[38]. Dose-Response Curves; explained in the following paragraph

X-Axis: Concentration of the drug; **Y-Axis:** Measure of the effect (potency), parasite killing
EC_{50}: Concentration of the drug required to produce 50% of the drugs maximum effect.
Emax: The maximal response at a certain drug comcentration; **Threshold Concentration:** The minimum concentration of the drug required to produce an effect on the parasite; **Slope:** y/x.

Starting with curve A when the parasite was susceptible to the drug, moving on to the right with the passing of the three phases in the development of resistance. Curve A would thus correspond with the wild type genotype of Plasmodium falciparum which is susceptible to the drug. Curves B, C and D represent a falling slope signifying the development of a genotype with increasing levels of tolerance to the drug. This can also be demonstrated by the increasing EC_{50} and falling Emax. With a progression along this path, the slope would decrease to a point where the drug would be no longer effective against the parasite. This signifies the development of the fully resistant form of the parasite.

IV.2.2.4 The Problem and Solution?

In the words of M. J. Mackinnon [37], "Development of drug resistance is inevitable. It is not a question of 'if' but a question of 'when'. With this in mind and the widespread evidence of drug resistance to first line antimalarials from around the world, the call for the formulation and use of drug combinations has become stronger. And at the same time it would be wiser this time around to try to extend the lifespan of a drug/drug combination by monitoring the development and spread of resistance [39]. The wisdom behind the use of combination therapies is the knowledge that the development of resistance can be slowed down with the use of drugs with different mechanisms of action [24].

IV.3 MOLECULAR DIAGNOSTICS

IV.3.1 A Historical Perspective

The evolution of this branch of laboratory sciences can be attributed primarily to the inquisitiveness of some researchers to dwell into the elaboration of the human genome. And to top it up quick coupling of this knowledge with applied technology [40] for its use in diagnostics. Thus, the foundation of modern day molecular diagnostics was undoubtedly laid by the elucidation of the double-helical structure of DNA by Watson and Crick in 1953.

One may cite the invention of the blotting techniques as landmarks in this area but it was not until about 20 years ago [41] when with the discovery of the PCR, molecular biological techniques moved into an era of practicable laboratory based diagnostics [40]. The PCR, a quantification test which served as a prototype of molecular diagnostics has over the years heralded the birth of an amazing array of

increasingly complex tools for diagnostics starting with the rt-PCR leading to the RT-PCR [41] today. So, in essence contrary to the standpoint of some scientists who still cite molecular diagnostics to be in its infancy, it can be safely assumed that it has surpassed the initial stages with the rather explosive appearance of novel techniques from research around the world [42]. An interesting feature of this approximately 10-20% development every year in the last five years [40] in the field of molecular diagnostics is the shift from labor-intensive to automated procedures[41]. In essence, whether you call it molecular clinical chemistry, molecular diagnostics or molecular medicine, all these advanced state-of-the art diagnostics can be attributed to five major innovations [43]:

1. The Real-Time Polymerase Reaction
2. Melting Curve Analysis
3. Array Technology
4. Integration of Molecular Diagnostics on a Microchip
5. Circulating Nucleic Acids

Such diagnostics will have far-reaching effects in the practice of clinical medicine as while at one end of the spectrum these tests allow for the identification of the pathogens, there is also the realm of detection of drug resistance [44] at the other. This provides a new avenue in the fight against emerging drug resistance patterns in various pathogens.

IV.3.2 Techniques to Study Resistance [36]:

This section is a broad over-view on the different types of tests available for the analysis of resistant species. The description of individual tests is beyond the limits of this document, and so I have made an attempt to outline the basic principles and procedures therein. Molecular biological tests have just been listed here and are

dealt with in greater detail in the section on methodology in this report. Tests used to study resistance are :

- In Vivo Studies and
- In Vitro Studies

In Vivo Tests

The tests in vivo have the inherent advantage of evaluating the resistance taking into account the host immune response which is strongly related to the intensity of the transmission. Developments in the test protocols were improved and adapted during the past few years to account for the intensity of the transmission and also the age of the patients. However, these tests require clinical and laboratory follow-up with consultations required throughout the course of treatment, which is possible only if the access to health care is amenable [45].

The principle underlying the in vivo tests is the clinical and laboratory follow-up of the patients in whom a diagnosis of malaria has been made and appropriate treatment initiated. They thus don't present an ethical dilemma to the researchers as the patients can be supervised over several days. Also there is an exclusion of patients presenting with severe malaria or an aggravation of symptoms during follow-up. The first standardised tests were for the evaluation of resistance to Chloroquine in Plasmodium falciparum. Developed around the end of the 1960s, they were improved and adapted rapidly to include tests for resistance to other antimalarials.

But it has not been smooth sailing for these test protocols as they need daily blood samples from about 7-28 days. This is a heavy constraint in resource poor settings like Sub-Saharan Africa especially as it implies hospitalization for about a month. Moreover, the results obtained on asymptomatic carriers cannot be extrapolated onto symptomatic patients to draw the same conclusions. A protocol

adapted to tide over these constraints was presented in October 1996 in Manila and then adopted at Phnom Penh in October 2000. The need for a single classification, that could be used in zones with a high as well as a low intensity of transmission were focussed upon. [45]. It also allowed for a comparison of different zones.

The objective of these in vivo tests is to determine the effectiveness of a therapeutic regimen in order to continue with it or to modify it for better effectiveness. Lately, a very important improvement was made thanks to the development of an in vivo test that takes only 14 days, is more convenient to perform and simpler and is performed on symptomatic patients[46]. This test consists of measurements of the body temperature of the subject regularly and examinations of the blood films under the microscope.

In Vitro Tests

There are two types of in vitro tests:

a) *The Phenotypic Tests*, which consists of making cultures of the parasite in the presence of increasing concentration of various drugs.

These tests make it possible to determine the level of effectiveness of a drug on Plasmodium falciparum without taking account the premunition of the patient. It indicates the threshold of sensitivity of the isolates coming from patients and is used to follow-up the evolution of this sensitivity with time. A fall of in vitro sensitivity can be a predictive element for therapeutic failure in the population. Unlike the in vivo tests, the in vitro test are independent of the level of transmission and of the clinical state of the patients and it allows to test several drugs at the same time. The plots are called isobolograms. It isn't good however to formulate a therapeutic regimen as does not take into account the pharmacological and immunological data. The tests to evaluate the sensitivity of Plasmodium falciparum in vitro was designed by the WHO,

in 2000. It is adapted for the evaluation of all principal antimalarials: chloroquine, mefloquine, quinine, amodiaquine, sulfadoxine/pyrimethamine and artemisinin. It can also test halofantrine and pyrimethamine.

Taking into account specificities of this test, the samples must come from patients not having profited from previous antimalarial treatment. Patients are excluded based on the half-lives of the drugs they have been on. One of the constraints for these tests is that urinary measurements of drug proportions aren't a sensitive indicator of drug presence in the body. The parasitemia is then measured and only patients with a Plasmodium falciparum mono-infection are included in the test. As for the in vivo tests, the ethical aspect has priority and a modified treatment is started immediately after the blood sample is taken for the test. The sample is then incubated in the presence of various concentrations of the drug on microplaque with 96 wells at 37°C. The results are measured by making a blood smear after 24-30 hours of incubation and an evaluation of the maturation of the parasites schizonts is made. One can reach a conclusion that resistance exists if is no more inhibition of the growth below a certain threshold concentration.

The results of in vitro test are expressed in terms of the inhibiting drug concentration in 50%s, i.e. the concentration of a drug which after a given time of action causes an inhibition of activity in 50% of the isolates from the test subject. This thus makes is possible to determine the level of sensitivity of the isolate [47].

b) *The Genotypic Test/Molecular Biological Test*, which serves to identify the mutations that confer resistance.

The PCR and until more recently the RT-PCR are the tests that figure in this category. These tests are applicable only when the molecular markers for resistance are known. The advantages of these systems lie in the preservation and transport of

samples which is a constraint in vitro and in vivo tests [48]. Sequencing as a tool to confirm the findings of PCR and RT-PCR is a known entity.

IV.3.3 Molecular Biology of the Plasmodium Species:

The understanding of the genome of the human plasmodia species [49] had to wait until the development of model parasites which caused malaria in other vertebrate hosts. In addition to being able to get over the problem of host-specificity, the animal models were relatively easier to study due to the ease of handling of laboratory rats and mice [50]. The Simian, Avian and Murine malarial species served as prototypes [51] to the plasmodia genomic project. In 2002 scientists cracked the genome of the human malarial parasite Plasmodium falciparum [52] implicated in the causation of severe malaria.

IV.3.4 Genome of Plasmodium falciparum:

Genetic Material: DNA

Chromosome Number (Diploid Number): 14

Genes: 5300

Most of its genes are involved not in encoding for enzymes or transporters but in coding for proteins involved in evading the host immune response [52], unlike in other microbial agents.

IV.3.5 Molecular Markers known to confer Resistance in Plasmodium falciparum

Since the elaboration of the genome of Plasmodium falciparum towards the beginning of this century, the diagnostic modalities described above have been used to

pin-point molecular markers (mutations) that could be implicated in conferring resistance to the the drug being studied.

Reports of resistance to first line antimalarials have poured in from different parts of the world since the late 1950s. Starting from reports from the Thai-Cambodian region going onto include Africa, South America and the rest of South-East Asia. Scientific literature is rich in the information concerning the status of resistance to antimalarials especially in the endemic countries but on the other hand the molecular bases of resistance to antimalarials are sometimes contradictory.

Table 5 shows data from various studies which highlights the molecular markers identified to be responsible for drug resistance tabulated with the drugs against which the resistance has been identified. This is a broad overview of mutations not including details of mutations of the cytochrome b gene which are the corner stone of this study. These have been addressed in the latter sections of this report.

	MUTATIONS CONFERRING RESISTANCE	
Antimalarials	**Genes showing Mutations**	**Other possible Mutations**
Chloroquine [32, 53, 54, 55, 56, 57,58]	pfcrt thr 76 pfmdr1 tyr86	pfmdr1 phe184, cys1034, asp1042, tyr1246
Amodiaquine [62, 65,70]	pfcrt thr 76	pfmdr1 tyr86, tyr1246
Méfloquine, Quinine [32, 59, 60, 61, 62, 63,65]	Polymorphism of pfmdr1	pfmdr1 tyr86, phe184, cys1034, asp1042, tyr1246
Artemisinine [65,66]		pfmdr1 tyr86, phe184, tyr1246
Sulfadoxine-Pyrimethamine [32, 67, 68, 69, 70, 71, 72,73]	Mutations at codons 108, 51, 59, 164 of the pfdhfr gene Mutations at codons 436, 437, 540, 581, 623 of the pfdhps gene	

Table. 5

pfcrt = Plasmodium falciparum chloroquine resistance transporter gene
pfmdr = Plasmodium falciparum multi-drug resistance gene.
pfdhfr = Plasmodium falciparum dihydrofolate reductase gene.
pfdhps = Plasmodium falciparum dihydropteorate synthetase gene.
tyr = Tyrosine; phe = Phenylalanine; cys = Cysteine; asp = Aspartic acid; thr = Threonine;

IV.3.6 Antimalarial chemotherapeutics; Drug Combinations, Malarone® and Cytochrome b [74]

From quinine in the early days to the combination therapies of present times, antimalarials have had a chequered history. From being effective in the initial years of introduction a great majority of them have now entered the realm of therapeutic failures. All classes of antimalarials from the first known quinolines and the anti-folates more recently have been associated with treatment failures. Therapeutic failure has been attributed to the evolution of resistant species, which come into existence due to a variety of factors like the practice of mono-drug therapy, drug pharmacokinetics and agent factors like development of mutations, spontaneous or de novo under drug pressure [75]. Molecular markers that confer resistance to single drugs as well as to multiple drugs have been identified (Table 5). Matters are compounded by the fact that the pattern of resistance attributed to mutations in the parasite genome, has been documented to vary geographically [36]. This is where it becomes important to have a central database of the markers of resistance that have been identified. This would aid countries in making the necessary changes to their country specific malaria treatment protocols.

The development of resistant species to first line antimalarials has prompted thinking in the direction of combination therapy. Combination therapy (CT) is based on the potential that when two blood schizonticidal agents with independent modes of action are used simultaneously, therapeutic efficacy is improved as also is the development of resistance delayed [76]. Combination between atovaquone and proguanil (Malarone), which is the drug being studied does not qualify to be 'combination therapy' as it relies on a synergy between the two constituents [77,78].

The call for combination therapy has become urgent. Since the first reports of resistance over 50 years back, there has been an alarmingly high reportage of therapeutic failures to all first line antimalarials from across the world. As far as Plasmodium falciparum is concerned, which is the focus of this study, resistance has been reported to all antimalarials in use currently, save for the artemesinins [36]. But what parallels the resistance exhibited by the other species of Plasmodia is that the geographic patterns and the spread of resistance to different drugs are varied.

As if matters were not already complicated, the family of efficacious antimalarials is very small let alone the fact that there are fewer potential antimalarials in the research and development pipeline[78]. Suffice to say that, we need a massive revamp in the treatment protocols based on evidence of resistant species with geographical specificity [75] to make the best use of the drugs we have today. What needs to be understood is that what may be a highly efficacious regimen in one country when implemented in another could end up being a disaster simply because of the presence of a different strain of the parasite exhibiting a different pattern of drug resistance. This is another reason to call for a reliable database of markers for resistance to aid countries in coming up with a country specific treatment protocol. Change also needs to come in medical practice in making sure that care is taken to refrain from injudicious use of antimalarials which has played an important role in the appearance of resistant species today.

Although combination therapy cannot completed stop the development of resistant species, it definitely does slow the process considerably [79,80]. With this in mind, therapy with combinations have been broadly classified into [81] Artemisinin based (ACT) [82] and non-Artemisinin based (non-ACT) [81, 83] therapies. ACTs are

combinations of antimalarials with artemisinins or its derivatives. Below is a table with a list of drugs under each therapy :

Artemisinin Based Combination Therapy	non- Artemesinin Based combination Therapy
Artemether + Lumefantrine	Chloroquine + Sulfadoxine-Pyremethamine
Artesunate + Amodiaquine	Amodiaquine + Sulfadoxine-Pyremethamine
Artesunate + Mefloquine	Mefloquine + Sulfadoxine-Pyremethamine
Artesunate + Sulfadoxine-Pyremethamine	Quinine + Tetracycline/Doxycycline
	Chlorproguanil + Dapsone
	Atovaquone + Proguanil
	Mefloquine + Sulfadoxine + Pyremethamine

Table 6. List of approved ACTs and non-ACTs.

Malarone®

Malarone has been the drug of choice for drug resistant malaria as well as for [84]. In France, Malarone has the same status in addition to being the drug of choice for the treatment of uncomplicated falciparum malaria unless otherwise contraindicated. This drug combination has two components, hydroxynaphthoquinone named *Atovaquone* ($C_{12}H_{19}ClO_3$) and *Proguanil hydrochloride (*$C_{11}H_{16}ClN_5HCl$) [85] also known as chloroguanide, a biguanide derivative. It is a fixed dose combination of 250mg Atovaquone and 100mg of Proguanil hydrochloride.

Atovaquone acts on the cytochrome bc_1 complex of the susceptible parasite mitochondria. It thus inhibits the ETC (Electron Transport Chain) [86,87] which is responsible for cellular respiration, by collapsing the mitochondrial membrane potential [86]. Eventually this interferes with vital processes concerned with mitochondria like ATP (Adenosine triphosphate) and pyrimidine synthesis. There was very good initial control of parasitemia when atovaquone was used alone but there was also substantial recrudescence and parasites exhibiting recrudescence were found to be resistant to atovaquone in vitro [88].

This was the basis for the formulation of Malarone wherein atovaquone was combined with a dihydrofolate reductase inhibitor [89] proguanil hydrochloride. It is hypothesized that the apparent synergistic action of this drug combination lies in

proguanil's ability to enhance the action of atovaquone on the mitochondrial membrane [90]. This combination fulfills the twin theories that combinations are more effective than single drugs and also that in the event that the parasite develops a mutation for resistance to one drug during the course of the infection, the other would effectively eliminate the parasite. The success story for Malarone with atovaquone as its major component [91] was shortlived with, resistance due mutations at the codon 268 of the parasite P. falciparum cytochrome b gene [92] being observed in vitro and in vivo [93].

With Malarone now entering the list of drugs which have been rendered clinically in-efficacious, researchers around the world have started trying to confirm that reports of clinical failure after the use of Malarone could be well and truly be attributed to mutations of the cytochrome b gene [94,95]. Since reports of mutations of the cytochrome b gene started pouring in, there have been conflicting reports [96,97,98] about the credibility that these mutations were indeed responsible for drug resistance. It is of paramount importance to ascertain these findings as if they are true then Malarone would have to be removed from the current status it occupies in treatment protocols.

The present study is an attempt to do just that. It would merit mentioning at this point that with a limited number of drugs available to combat the parasite and with positive evidence of the parasite having developed resistance against all except the artemesinin derivatives, it is pivotal to monitor drug resistance. And since drug resistance exhibits variations in terms of the mutation specificity and the geography or both, monitoring mutations/molecular markers for resistance is the single best way to prevent further selection of the parasite population with the mutation in question. This can undoubtedly be done through the medium of molecular diagnostics as outlined in

the section titled ‘Molecular Diagnostics’. This study is an attempt to bring to light the usefulness of the RT-PCR as the modality to do just that in the present scenario.

Cytochrome b

As stated before previous studies have demonstrated polymorphisms in the nucleotide sequence of the cytochrome b in *P. falciparum*, some linked to atovaquone resistance. In this context four independent modifications [91] in amino acid sequences have been identified:

a. Y268S
b. Y268C
c. M133I
d. V284K

Of these the mutation designated Y268C is most prevalent amongst the four. Other mutations coexist but serve merely to increase resistance without provoking it. These mutations modify the binding site of atovaquone, structurally similar to ubiquinol [99] on cytochrome b, by either modifying directly the interaction between certain residues of both molecules or by changing slightly the conformation of the binding site [100]. These conclusions have been drawn from studies using inhibitors similar to atovaquone. They were found to exhibit binding at the ubihydroquinone site of the cytochrome b designated as Q_o [101] The mutations cited above are point mutations in close proximity to Q_o [91]. This reduces the affinity of atovaquone to the Q_o site thus making the drug ineffective.

The following two figures are a representation of the structure of the cytochrome b gene in P. falciparum in 3-dimensional view. It depicts the contact residues that are mutated in Atovaquone resistant strains. The four mutations cited above have been represented between the two figures.

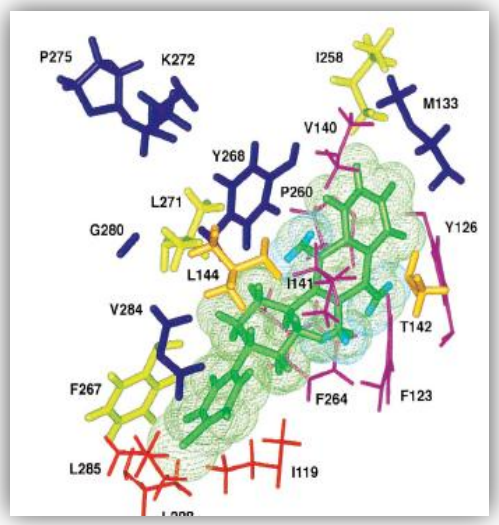


Figure 3

Figures 3[91] and 4[91]: Illustrations of the cytochrome b gene with the putative P. falciparum cytochrome b gene contact residues that were mutated in the atovaquone resistant parasites.

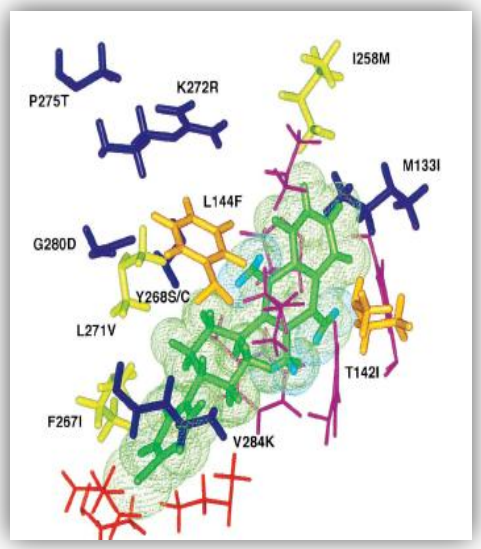


Figure 4

Figure 3 and 4 [91]:
Green Bars: Atovaquone.
Light Blue Bars: Electron pair of atovaquone.
Thin Lines: Elecron densities of atovaquone.
Thin Bars: Contact residues.
Thick bars: Contact residues in the mutated resistant parasites.
Purple bars: Residues mutated in Plasmodium falciparum.

Until now, a technique called the RFLP (Limitation Fragment Length Polymorphism) made it possible to study such polymorphism [79]. This procedure is however long and time-consuming owing to the multiple stages involved therein. This study is an attempt to put forward a new technique based on RT-PCR (Real Time-Polymerase Chain Reaction) and the FRET system (Fluorescence Resonance Energy Transfer). This would allow the detection of the substitution A803C (responsible for Y268S) which is the most common mutation [100] responsible for the P. falciparum resistance to atovaquone.

IV.2.3 Materials and Methods

IV.2.3.1 Background Data

This study is an attempt to underline the importance of surveillance and monitoring as a tool to keep track of the development of strains of P. falciparum resistant first line antimalarials. It bears a special significance to countries like France as even though it is 'non-endemic' for malaria, it has the problem of imported malaria to deal with. In many ways this is more complicated than it appears on the surface due to the fact that we are dealing with a multifactorial situation encompassing imported malaria with a high possibility of resistant species being brought in therein and the non-immune or semi-immune status of travelers to name a couple. Below is an illustration using data mentioned in previous sections in this report to drive home the point in relation to countries like France :

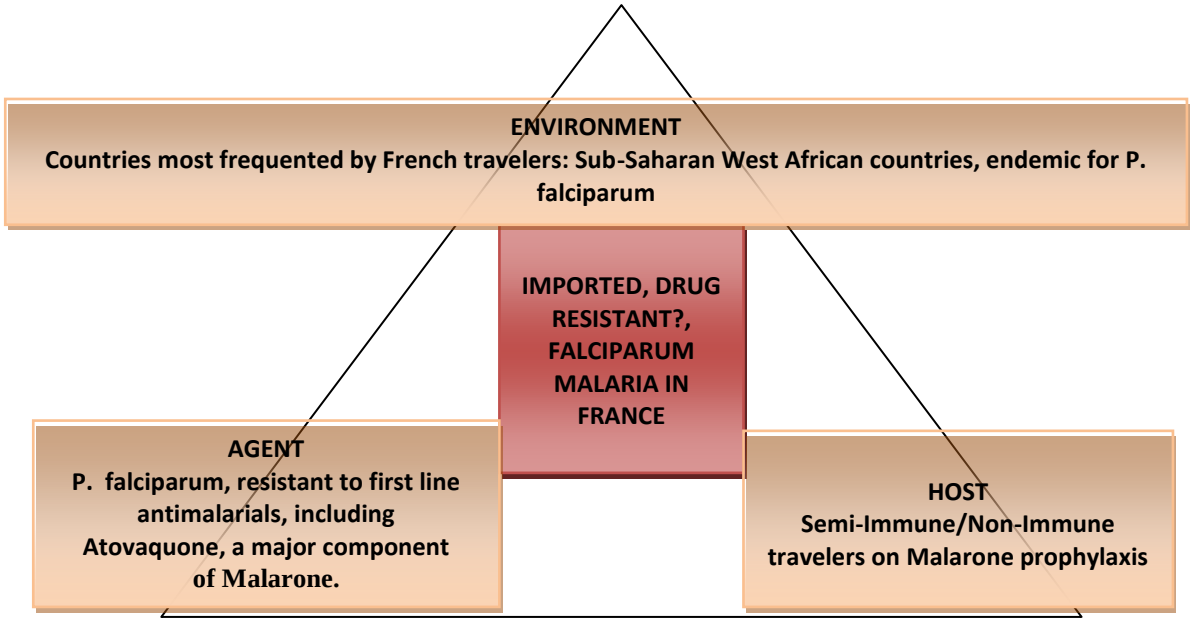


Figure 5. The situation studied under the parameters of the Epidemiological Triad.

Protocol in France stipulates Malarone to be employed as the drug of choice for prophylaxis and treatment of uncomplicated falciparum malaria. Resistance to atovaquone which is a major component of Malarone is a known entity [102,103]. Notwithstanding the issue of species resistant to Malarone, with these travelers being either non-immune or semi-immune[104], the situation is further complicated.

This study done in Bordeaux clearly parallels the trend in the numbers for France [II.2.1] as a whole, in terms of returning travelers and the species of Plasmodium implicated in the etiology of imported malaria therein. The maps that follow, Figures 5[105] , 6[106] and 7[107] reiterate the need for surveillance and monitoring of evolving drug resistance patterns to Malarone amongst returning travelers to be able to deal with the situation appropriately. This would also be in the best interest of the patient as appropriate alternate therapy can be initiated to prevent appearance of fatal complication. This would also serve to minimize the risks of enhancing the selection of resistant strains in the parasite population.

Figure 6. Map showing the Sub-Saharan African countries in green. The West African countries in this zone from where we had an overwhelming number returning travelers have been boxed-in in the rectangle.

Figure 7. Map showing the population at risk in the Sub-Saharan African countries.

Figure 8. Map of Africa showing in dark green the regions of high transmission and in light green the regions of low transmission of Plasmodium falciparum malaria. The black specs represent reports of resistant to different antimalarial drugs.

From figures 6, 7 and 8, there is an overlap and it can be seen to involve the western African countries in Sub-Saharn Africa.

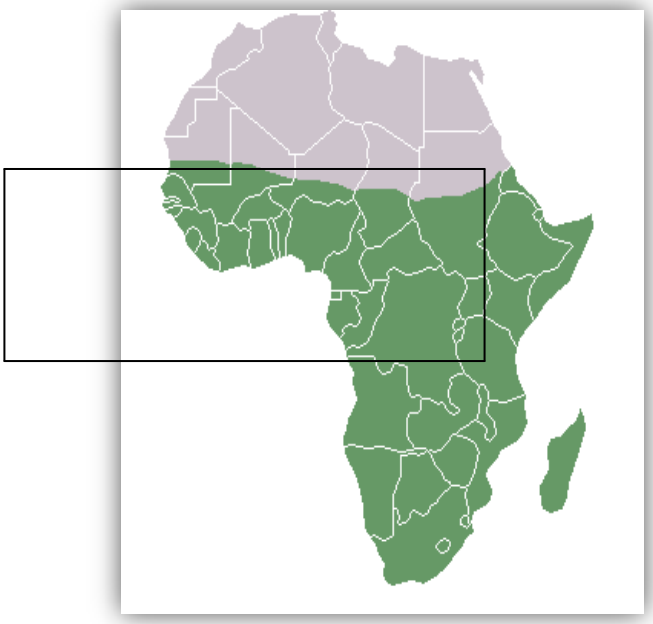


Figure 6

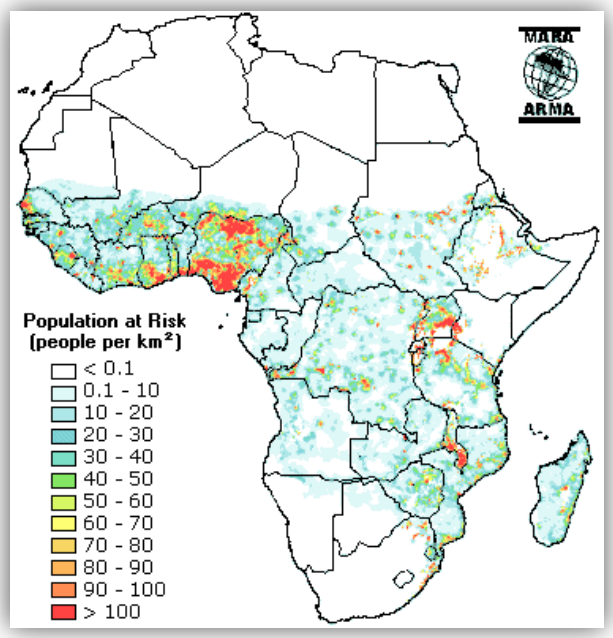


Figure 7

Figure 8

IV.2.3.2 Data from the Saint André University Hospital, Bordeaux

This was the medical facility which the subjects of this study attended and were later diagnosed with malaria on returning from travel to a malaria endemic country. The diagnostic work-up for returning travelers with complaints of fever include the a blood smear to rule out malaria. This is considered the gold standard for the detection of the malarial parasite. In case of a discrepancy between the clinical manifestations and the results of the smear results, the PCR (Polymerase Chain Reaction) is done as a confirmatory test. Tables 7, 8 and 9 represent a break-up of the patients who attended the clinic at the above centre and were diagnosed with malaria:

Table 7. Split-up of the numbers by species diagnosed

Category according to Plasmodium species	Figures
Total number of cases of malaria from June 2005 to July 2007	138
Number of cases of P. falciparum malaria	120
Number of cases of non-P. falciparum malaria	18
Number of cases of P. falciparum with co-infection with other species*	2

* Cases of P. falciparum with a co-infection with other species were included in the same category as exclusive P. falciparum infections ie. The two cases of co-infection in this table are included in the 124 under P. falciparum cases.

Figure 9. Representative of data in Table 6.

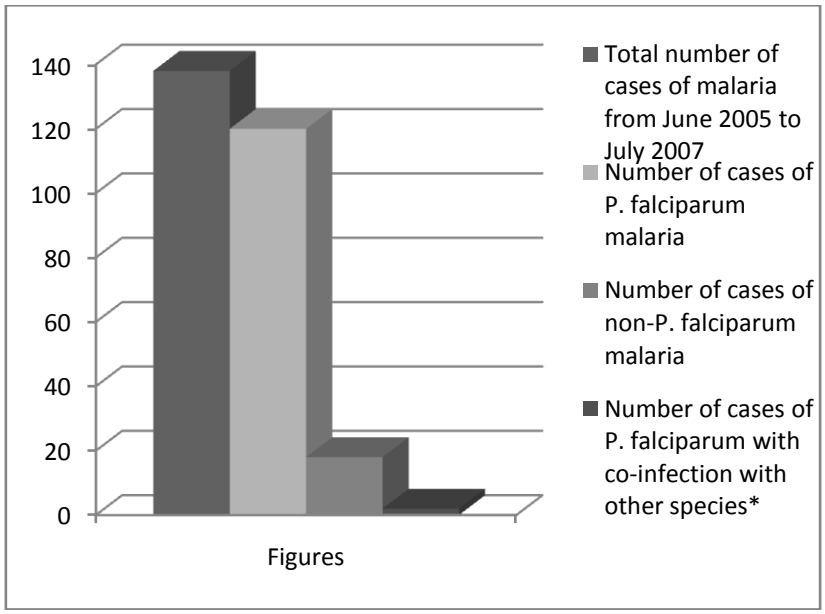


Table 8. Split-up of cases with a P. falciparum disgnosis by geography

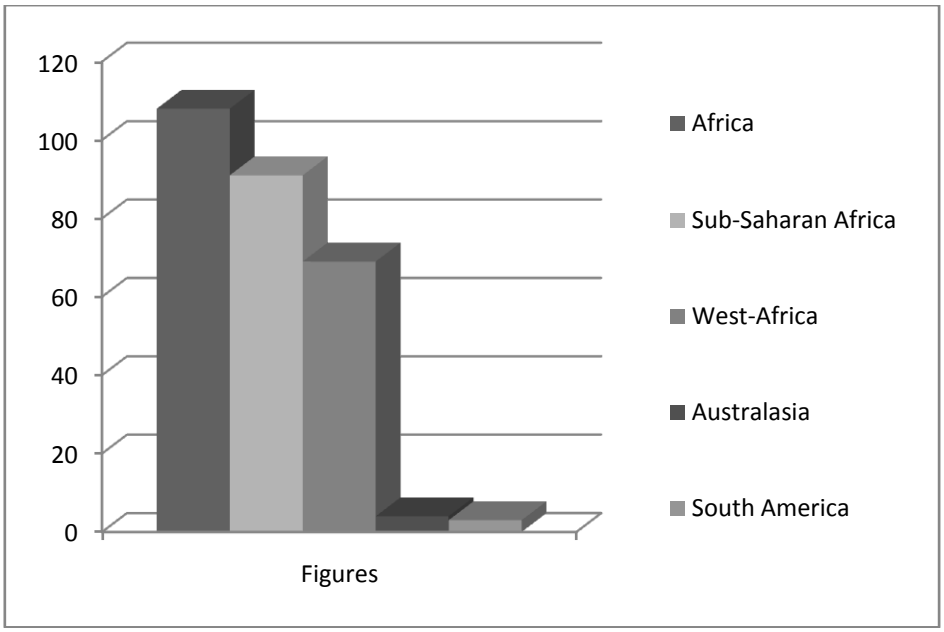
Cases of P. falciparum malaria according to geography	Figures
Africa	**108**
Sub-Saharan Africa	**91**
West-Africa	**69**
Australasia	**4**
South America	**3**

The information regarding country returning from was not available for 5 subjects. They have not been included in the above table.

Table 9. Split-up of the P. falciparum cases according to geographic zones in Africa

	Numbers	Percentage
P. falciparum cases	120	**120/138 = 86.95%**
P. falciparum cases from Africa	108	**108/120 = 90%**
P. falciparum cases from Sub-Saharan Africa	91	**91/108 = 84.25%**
P. falciparum from West Africa	**69**	**69/108 = 63.88%**

Figure 10. Representative of data in Table 7.



IV.2.3.3. Blood Sample Collection/Parasite Samples

Blood samples from 25 patients (who attended the clinic at the St. Andre Hospital, Bordeaux and were later admitted with clinical manifestations of malaria), with a confirmed diagnosis of P. falciparum malaria from slide smear test were further analysed to ascertain if they carried a strain of P. falciparum mutant for the cytochrome b gene that could render resistance to Malarone. The subjects belonged to the bigger cohort who attended the same clinic with complaints of fever on returning from travel to a malaria endemic country, between June 2005 and July 2007. Venous blood samples were collected and stored in EDTA (Ethylenediamine tetra acetic acid) tubes. This was done before and after the initiation of antimalarial therapy. One aliquot of each sample was used for routine diagnostics in the laboratory and the other was stored at –80°C for future molecular biological diagnostics. These samples were subjected to the FRET RT-PCR (Fluorescence resonance energy transfer). The FRET involves a radiation less transfer of energy from a one (donor)

fluorophore/chromophore (molecular beacons) to another (acceptor). The following are the steps involved in the in the FRET RT-PCR analysis of these samples.

IV.2.3.4 DNA Extraction and template preparation

DNA was extracted from 200 µl of blood using either the QIAamp DNA Mini Kit (QIAGEN) or an automatized DNA extraction machine, the MagNA PURE LC (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. The samples were then analysed with both RT-PCR and conventional PCR assays. Centrifugation for approximately 10 mins at 2000 revolutions/minute provides 80µl of parasite DNA. The DNA would be the designated template for the polymerase chain reaction to follow.

Figure 11. The Roche MagNA Pure LC

IV.2.3.5 Primer and Probe design

The primers and probes were designed using the cytochrome b gene single-stranded sequences of the *Plasmodium* species available from GenBank, accession number AF155925. The *P. falciparum*-specific primers were designed to amplify a 208-bp region from the cytochrome b mitochondrial gene. This was worked out by the

Design Service of SIGMA-GENOSIS and manufactured by SIGMA-PROGLIO. These sequences are summarized in Table 10, a & b:

Table 10.a. Primer Sequences

	Forward	Reverse
Primer	5'TTGGAATTATACCTTTATCACATCCT 3'	5' TATAGTTGTTAAACTTCTTTGTTCTGC 3'

Table 10. b. Probe Sequences

	Probes
Donor	5' ACTCCATCTCAAATTGTACCTGAATGGTACTTTCT-Fluorescein 3'
Acceptor	5' LC RED640-CATTTTATGCAATGTTA**C**AAACTGTTCCAAGT-**P** 3'

The position and alignment results of the cytochrome b gene single-stranded sequences was done using the ClustalW multiple sequence alignment program provided by the European Bioinformatics Institute.

Protein:

1 mnfysinlvk ahlinypcpl ninflwnygf llgiiffiqi itgvflasry tpdvsyayys
61 iqhilrelws gwcfrymhat gaslvfllty lrilrglnys ymylplswis glilfmifiv
121 tafvgfylpw gqmsywgatv itnllssipv aviwicggyt vsdprikrff vlhfilpfig
181 lcivfihiff lhlhgstnpl gydtalkipf ypnllsldvk gfnnviilfl iqslfgiipl
241 shpdnaivvn tyvtpsqivp ewyflpfyam lktvpskpag lvivllsiql lfllaeqrsl
301 ttiiqfkmiy gardysvpii wfmcafyall wigcqlpqdi filygrlfiv lffcsglfvl
361 vhyrrthydy ssqani

Figure 12. Protein Sequence-Cytochrome b gene.

The sequences of the primers and the anchor FRET (Fluorescence Resonance energy Transmission) hybridization probe were designed to be perfect matches (i.e.,

100% homologous) to the *P. falciparum* sequences. The sequence of the acceptor FRET hybridization probe was designed on the basis of one (or two) nucleotide mismatches which could identify the cytochrome b gene of the *P. falciparum* and also distinguish it (wild strain) from the atovaquone resistant (mutant strain). To ensure proper hybridization of the probe to the target sequence, oligonucleotides with a T_m which was at least 5°C higher than the actual annealing/extension temperature were chosen as probes. The 3′ end of the anchor FRET hybridization probe was labelled with fluorescein isothiocyanate (FITC) and the 5′ end of the acceptor FRET hybridization probe with LC-Red640 (LightCycler-RED640). Possible oligonucleotide dimer formation and/or self-complementarity and the theoretical melting temperatures of primers and probes (T_ms) were calculated using Oligo Program, Version 4 (LightCycler Probe Design Software, Roche). Oligonucleotide primers and probes were obtained from Sigma-Genosys and Proligo.

Table 11. Probes with melting temperatures

	Melting Temperatures of Probes
Donor	T_m 63° C
Acceptor	T_m 61° C

Table 12. Primers with melting temperatures

	Melting Temperatures of Primers
Forward	T_m 50° C
Reverse	T_m 52° C

IV.2.3.6 FRET RT- PCR

Amplification and Detection

Purified DNA templates were amplified in a LightCycler Analytical PCR system (version 6.0) (Roche Diagnostics, Indianapolis, Ind.). The Real-Time PCR was performed using the "LightCycler DNA Amplification Kit Hybridization Probes" (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions.

Figure 13. The LightCycler, Roche Diagnostics

Constituents of each 20 µL capillary tube that was placed into the carousel of the LightCycler:
MgCl2 = 4.0 mM
All Primers = 5µM
Acceptor Probe = 5µM
Donor Probe = 5µM
10x LightCycler-FastStart DNA Master Hybridization Probe Mixture = 2µL
Extracted DNA = 10µL

Table 13. Reactants in each tube

The reaction was carried out with a final volume of 20 µl in each capillary tube containing 4.0 mM $MgCl_2$, 5 µM of each primer, 5 µM of the acceptor probe, 5 µM of the anchor probe, 2 µl of 10x LightCycler-FastStart DNA master hybridization probe mixture, and 10 µl of extracted DNA. After a short centrifugation (700*g* for 10 s), the sealed capillaries were placed into the carousel of the LightCycler. The PCR program ran as depicted in Figure 14:

1. 10 minutes at 95°C for enzyme activation and DNA denaturation

2. 45 PCR amplification cycles broken up into: 95°C for 15s, 50°C for 10s and 72°C for 8s.

Temperature transition rate in the denaturation and annealing steps was 20°C/s and 5°C in the polymerisation step.

3. Melting curve in the final cycle was obtained by heating to 95°C for 90s and subsequent cooling to to 45°C for 30s.

4. Final heating step to 95°C with a controlled temperature transition rate of .40C/s

RESULTANT FLUORESCENCE RECORDED STARTED AT 95°C IN STEP 4, USING THE PARAMETER 'CONTINUOUS MODE' AND F3/F1 CHANNEL

READ GRAPH TO SEE CURVES TO IDENTIFY WILD AND MUTANT (?) STRAINS

Figure 14. FRET RT-PCR

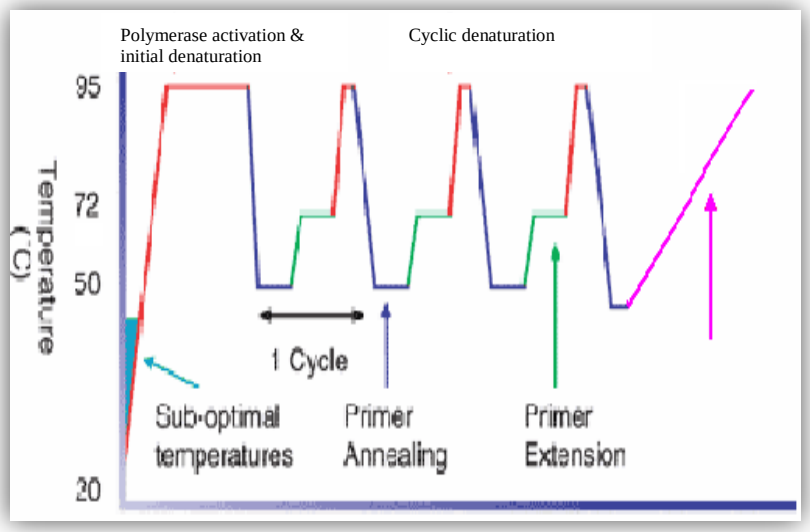


Figure 15. Cycles in the LightCycler

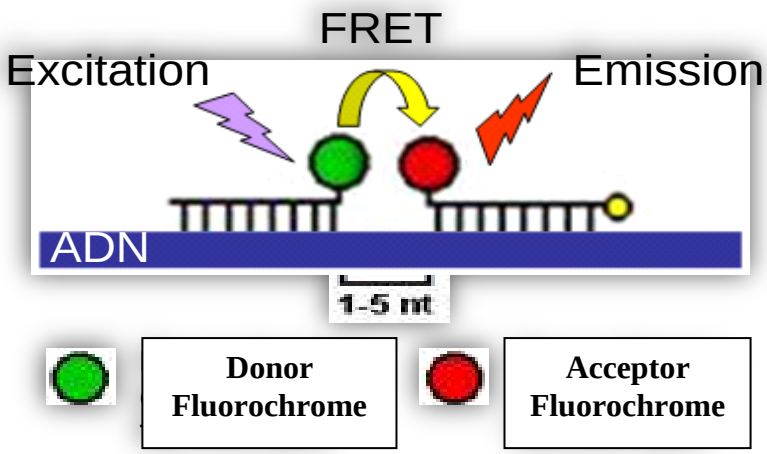


Figure 16. Fluoroscent Resonance Energy Transfer

During amplification, the LightCycler sequence detector monitors real-time PCR amplification by quantitatively analysing fluorescence emissions. The reporter dye signal was measured against the internal reference dye signal to adjust for non-PCR-related fluorescence and well-to-well fluctuations. The threshold cycle represented the refraction cycle number at which a positive amplification reaction was measured and was set at 10 times the standard deviation of the mean baseline emission calculated for PCR cycles 3 to 15.

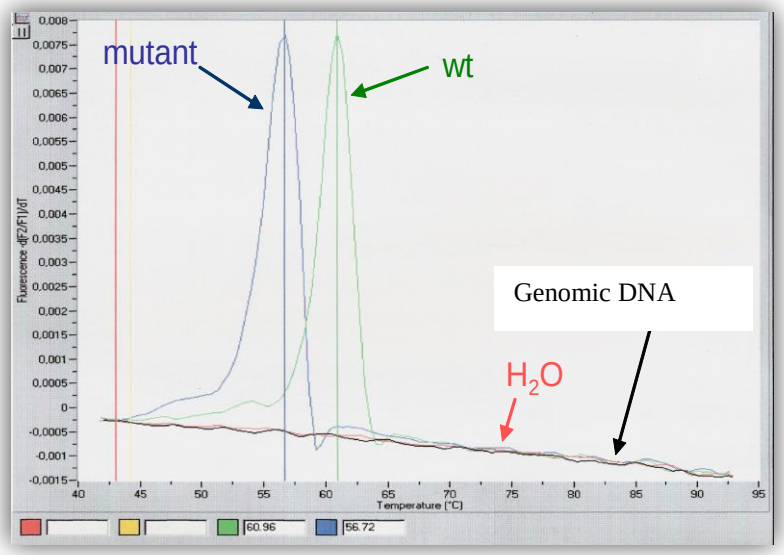


Figure 17. Graph from the FRET RT-PCR, showing 2 curves, for the wild type and the mutant strains of P. Falciparum, confirming the presence of a mutation.

Interpretative Criteria

The presence of an amplification or quantification curve for the LC-Red 640 signal captured in the F3/F1 channel of the LightCycler, in conjunction with a melting curve with a melting temperature (T_m) of about 60.0°C, was considered a reliable positive result for the *P. falciparum* strain. The presence of a quantification curve with a corresponding melting curve with two T_ms of about 60.0°C and 62.0°C was considered a positive for the *P. falciparum* resistant strain. The absence of a quantification curve was considered negative for both the stains.

Figure 15 is the graph obtained from the FRET RT-PCR analysis of one of the subjects' blood samples. The presence of two peaks read according to the interpretative criteria given above signifies the presence of a wild type (wt)/control and a mutant (mut)/test strain of P. Falciparum. In order to be sure that the fluorescence is due to the fixation of the probes on the target sequences an agarose gel electrophoresis is done with results as can be seen on the figure below:

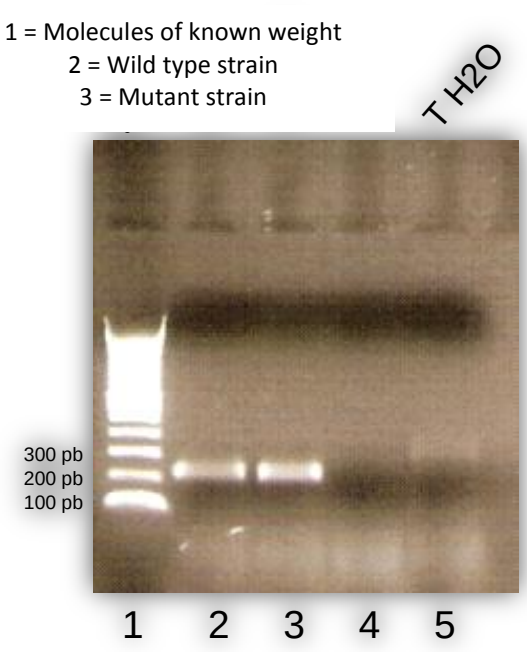


Figure 18. Agarose gel electrophoresis for the wild and mutant strains of P. falciparum

Sequencing

As a final step in the process of characterisation of the mutant strain, a sequencing procedure was done so as to be able to identify the exact mutation present in the mutant parasite. The Purification of amplified DNA was done using column MicroSpinTM S-400 HR from Pharmacia. The reactants for the sequencing were mixed in the following proportions; 10µl of purified DNA, 3.2 pmol of primers in each microvial and the kit CEQ Dye Terminator Cycle Sequencing was used according to recommendations' of the manufacturer (Beckman Coulter.)

The reaction in thermocycler Primus-HT from MWG Biotech was carried out as follows:

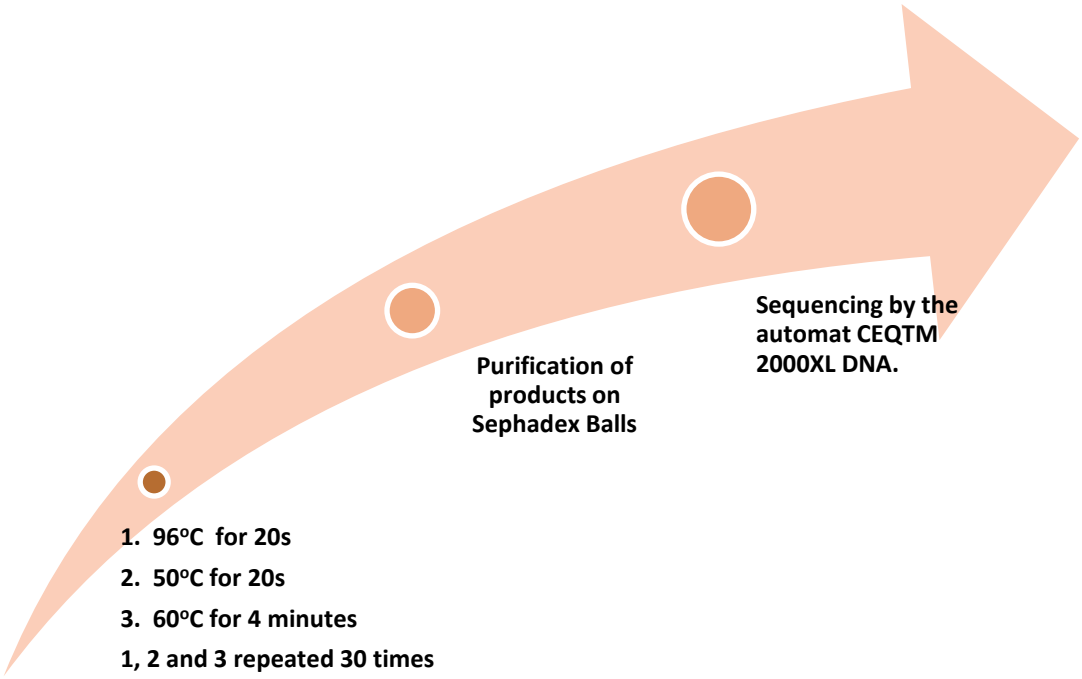


Figure 19. Steps in the sequencing reaction

The sequences of the bases from the above process for both the wild type (WT) as well as for the mutant strain (MUT) of P. falciparum are shown in Table 14.

WT[cytB], 208 bases

TTTGGAATTATACCTTTATCACATCCTGATAATGCTATCGTAGTAAaTACATATGTTACTCC
ATCTCAAATTGTACCTGAATGGTACTTTCTACCATTTTATGCaATGTTAAAAACTGTTCCAA
GTAAACCAGCTGGTTTAGTAATTGTATTATTATCATTACAATTATTATTCTTATTAGCAGA
ACAAAGAAGTtTAACAACTATA

MUT[cytB], 211 bases

TTaTTtGGAaTTATACCTTTATCACATCCTGATAATGCTATCGTAGTaAATACATATGTTACT
CCATCTCAAATTGTACCTGAATGGTACTTTCTACCATTTTGTGCAATGTTAAAAACTGTTC
CAAGTAAACCAGCTGGTTTAGTAATTGTATTATTATCATTACAATTATTATTCTTATTAGC
AGAACAAAGAAGTTTAACAACTATA

Table 14. Results of sequencing for the wild type (WT) and the mutant (MUT) variety of P. falciparum

It can be seen from the sequencing results above that the mutation identified is the substitution A803G responsible for Y268C.

IV.2.4 RESULTS

From the results of the sequencing in figure 18, it is evident that there has been an A803G substitution; G (guanine) substituted for A (adenine) at position 803 leading to the mutation Y268C. Paradoxically the test used probes were designed to detect the A803C substitution responsible for the Y268S which is the most common of the four mutations in the cytochrome b gene in P. falciparum resistant to Malarone or atovaquone. This was due to an error in the design of the probes which could also be proved by the results of the sequencing as seen from the figure 18.

Notwithstanding this error in design, the probes turned out to be a powerful tool in the identification of the mutant strain thus helping in the differentiation between the wild type and mutant strains of P.falciparum. A fresh set of probes with the correction made were ordered and the same procedure was repeated on the samples collected as part of the study. The results with the new probes were in parallel to the results obtained from initial batch of probes.

With the presence of 1 mutant strain in a batch of 25 samples, the prevalence of this mutation amongst the returning travellers who visited the St. Andre Hospital in Bordeaux, with a diagnosis of P. falciparum malaria stands at:

1/25 = 4%

IV.2.5 DISCUSSION/ CASE REPORT

With the FRET RT-PCR serving to identify the mutation in the cytochrome b gene of P. falciparum and subsequent confirmation of the same using the agarose gel electrophoresis and mutation characterisation by sequencing, we decided to go back in retrospect to the clinical records of the subject who carried this strain of P. falciparum deemed to be resistant to Malarone.

Table 15 below is a re-construction of the clinical case history of this subject, with the mutant strain of P. falciparum which carried the A803G mutation known to confer resistance to Malarone. It was clearly a case of treatment failure with Malarone which was brought under control with Lariam. Molecular biological analysis of her blood samples as we have seen in the FRET RT-PCR section, showed that the parasite in this patient was infact a mutant for the cytochrome b gene with a substitution of guanine (G) for adenine (A) at position 803, designated as A803G responsible for the mutation for resistance to Malarone, Y268C.

Patient Profile: 55 year old female.

Presenting complaints: Fever and arthromyalgia. No history of nausea, vomiting or diarrhea.

Travel history: 2 weeks in Burkina-Faso; had returned three weeks before the date of attending the clinic.

Medications taken: Started Savarine® (Proguanil+Chloroquine), a day before departure, but stopped the same due to intolerance 3 weeks after her return from Africa.

Treatment history: She was admitted to the hospital and was diagnosed to be suffering from P. falciparum malaria. Was started on Malarone (Atovaquone+Proguanil) for 3 days. The levels of parasitemia were as follows:

Days in treatment	Parasitemia
Day 1	.03%
Day 2	.07%
Day 4(day Malarone treatment completed)	.05%
Day 5(day discharged from hospital)	.07%

She was discharged with a complete cessation of fever. An analysis of her blood in the laboratory 5 weeks later showed a persistence of parasitemia with rare trophozoites and gametocytes. The patient presented 2 weeks later with complaints of loss of appetite. Her samples indicated a parasitemia of .1% with numerous gametocytes and some trophozoites. She was started on second line treatment with Lariam (Mefloquine). On day 7 there was presence of some gametocytes in the blood and on day 15 there was a total absence of any forms of Plasmodium falciparum in the patient's blood. This total clearance of the parasite from the blood stream along with a resolution of symptoms signified clinical recovery.

Table 15.

This being a pilot study at the laboratories of the CHU, Bordeaux, to ascertain the usefulness of FRET RT-PCR as a tool for the detection of mutations of the cytochrome b marker, the sample size has been small. But having achieved the objective of detecting the above said mutation, the results of this study warrant further research in this area. In addition to becoming the most efficacious molecular biological tool to detect mutations, similar studies with bigger sample sizes would make possible the calculation of the accurate sensitivity and specificity of the FRET RT-PCR as a diagnostic tool for malaria thus elevating it to the status of the gold standard/reference standard along with or even replacing the more traditional Giemsa-stained blood smear examination, as it comes with the inherent advantage of being able to identify known resistance conferring mutations. Having said that, with a price tag of approximately € 10.00, this molecular biological tool is not very amenable for

large scale deployment into the resource-poor malaria endemic countries. Until further research and development of the technique make this procedure more cost-effective, it will remain at the present status of being used only for the detection of mutations and also for the confirmation (CHU, Bordeaux) of diagnosis in cases where the smear examinations are not conclusive. The calculation of sensitivity and specificity would be done based on the information below in Table 16:

Table 16.

***Sensitivity*:** The minimum detection limit of the *Plasmodium* FRET assay was evaluated by use of a 10-fold dilution series of DNA extract from four patients infected with *P. falciparum* (parasitemia: 30% in Giemsa-stained thin blood smear). Each experiment included one reaction mixture without DNA as a negative control, and each specimen was run in duplicate for RT- PCR assay in parallel with the conventional PCR.

***Specificity*:** To estimate the analytical specificity of the *Plasmodium* FRET assay, DNA from European individuals who have never travelled to malaria endemic area or patients diagnosed with other infectious agents (*Toxoplasma gondii*, *Leishmania infantum*, *Pneumocistis carinii*, *Loa loa* and *Trypanosoma brucei gambiense*) was obtained and analysed.

IV.2.6 RECOMMENDATIONS

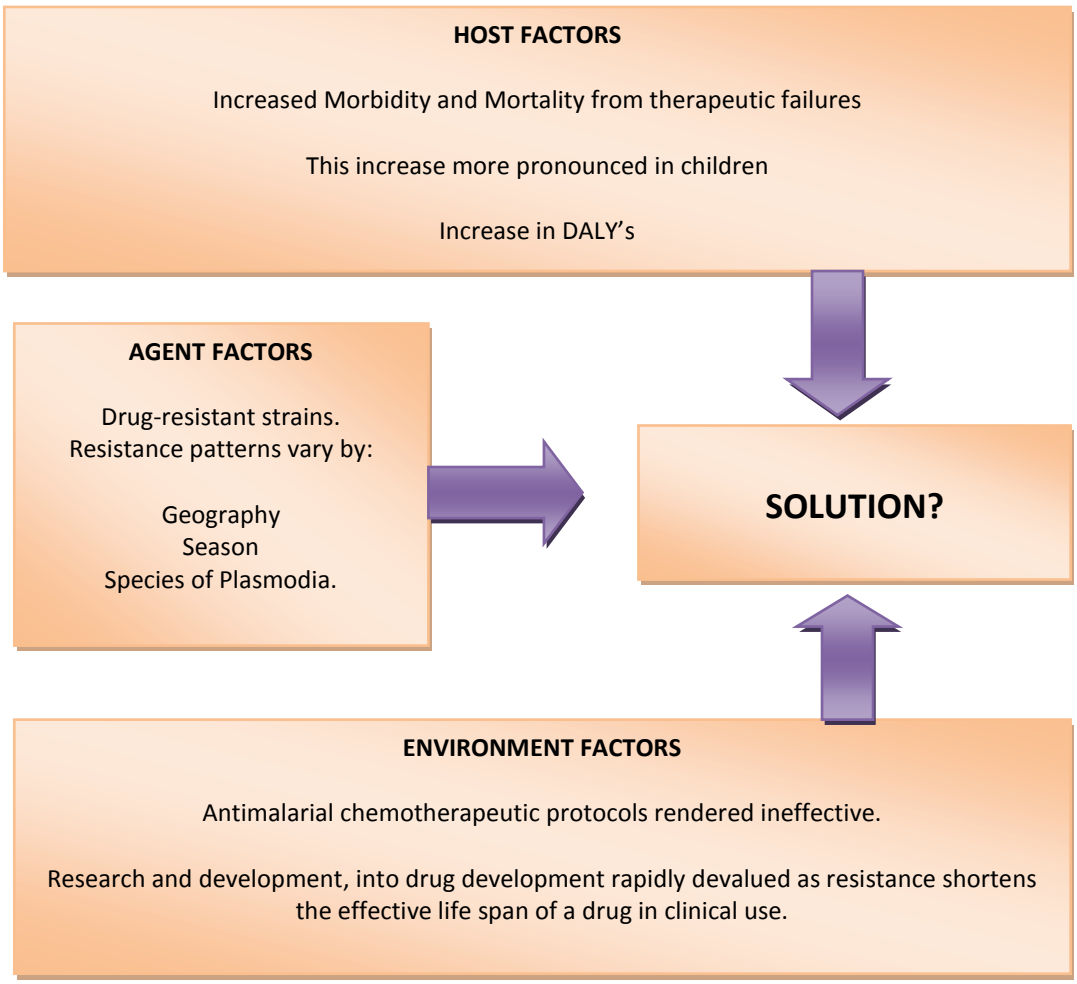
The outcome of the above research project has been:

- **Successful detection of mutation in the cytochrome b gene of P. falciparum conferring resistance to Malarone and thus treatment failure.**
- **Evidence based knowledge from different parts of the world that other molecular markers associated with resistance to antimalarials have been identified.**

From the literature review performed as part of this research project it was obvious that molecular markers bear immense potential in the fight against malaria. But with information in this regard lying scattered in numerous unlinked, independent

databases across the world, it is largely unused. To add to this, most of this information does not go beyond getting published in journals or worse still beyond technical consultations between the researchers in this field. Part of this probably stems from the fact that this data brings to the fore the fact that malaria treatment protocols in many nations would have to be altered to have best effects. This would mean a massive investment into the logistics of removing a drug/s from the therapeutic schedule and replacing it with another if available, or diverting resources into research and development of newer more potent drugs. It goes without saying that this comes with far reaching financial ramifications too.

KEY PUBLIC HEALTH ISSUES IDENTIFIED FROM THIS STUDY



From what has been said above it is clear that the information and resources required are to a great extent available, but what is lacking is the channeling of the

same in a direction that would ensure fixing of the problem. Utilization of information and resources already available and fresh sustained initiative into acquiring more information holds the key to success here. The proposed plan of action would take the form of Table 17.

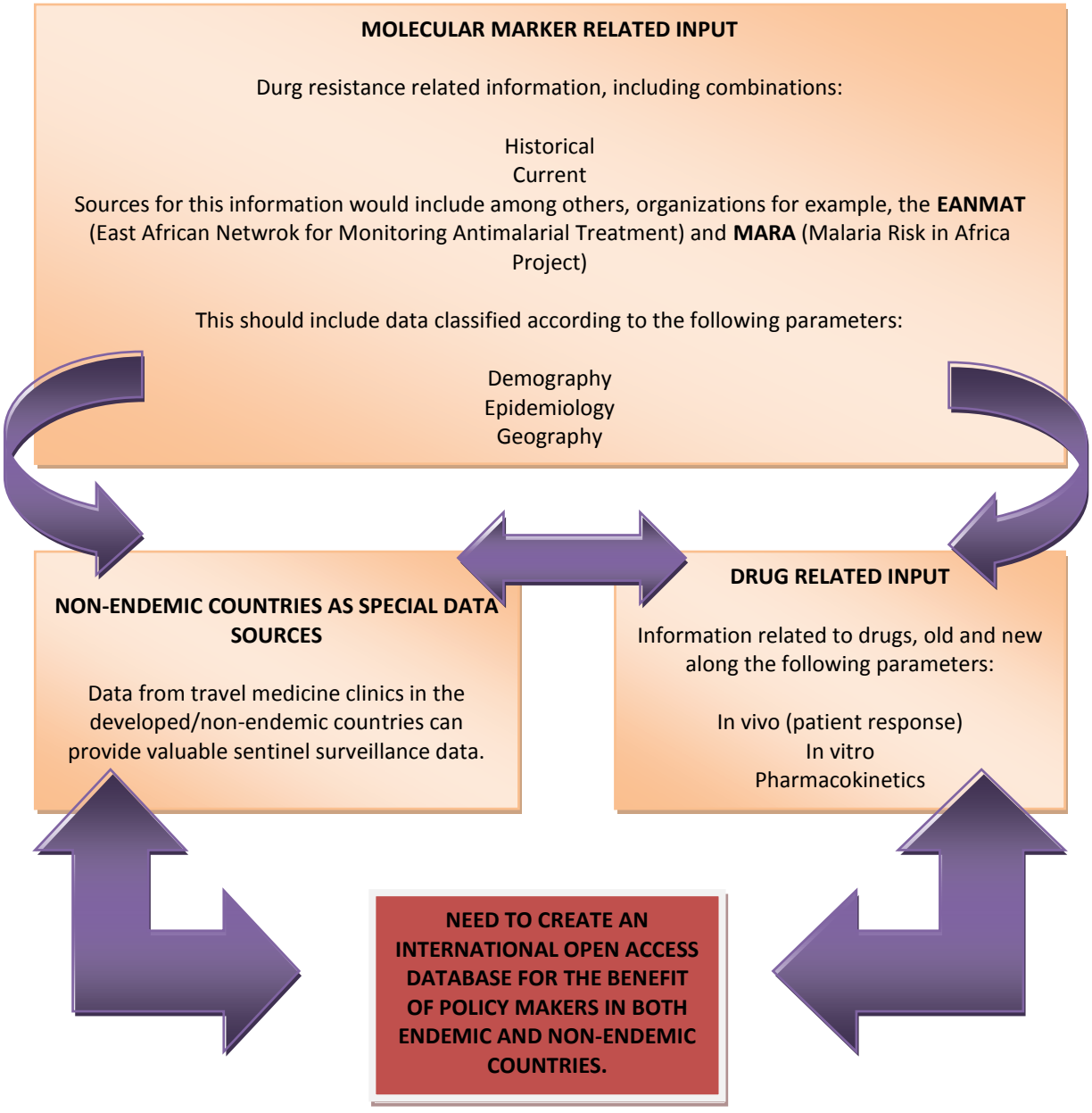


Table 17.

In conclusion, a lot more can be achieved even with the limited resources available especially in the context of the resource-poor countries if they are channeled in the right direction towards optimum utilization.

V.BIBLIOGRAPHY

1. Knell A. J. 1991. Chapter 1: What is Malaria? Malaria. First Edition. Pg. 3. Published by the Oxford University Press, 1991 for the Wellcome Trust.

2. Knell A. J. 1991. Chapter 1: What is Malaria? Malaria. First Edition. Pg. 4. Published by the Oxford University Press, 1991 for the Wellcome Trust.

3. CDC, USA, 2007. The History of Malaria, an Ancient Disease. Available at www.cdc.gov/malaria/history.index.htm. Accessed on the 17/06/2007 at 6: 05 PM

4. Park K. Chapter 1. Man and Medicine: Towards Health for All. Park's textbook of Preventive and Social Medicine. 17th Edition (2002), pg. 2. M/s Banarsidas Bhanot Publishers, Jabalpur, India.

5. Cahill M. K, O' Brien W. Chapter 1. Malaria. Medecine Tropicale-Precis Clinique. 1991. pg. 12. Euro-Edition, Boulevard du Triomphe, 132, B-1160 Bruxelles.

6. Park K. Chapter 1. Man and Medicine: Towards Health for All. Park's textbook of Preventive and Social Medicine. 17th Edition (2002), pg. 5. M/s Banarsidas Bhanot Publishers, Jabalpur, India.

7. Knell A. J. 1991. Chapter 1: What is Malaria? Malaria. First Edition. Pg. 4. Published by the Oxford University Press, 1991 for the Wellcome Trust.

8. Park K. Chapter 2. Concepts of Health and Disease. Park's textbook of Preventive and Social Medicine. 17th Edition (2002), pg. 30. M/s Banarsidas Bhanot Publishers, Jabalpur, India.

9. Gentilini M. Chapter 1. Paludisme. Medicine Tropicale. 5th Edition (1993) pg. 101-103. Medecine-Sciences Flammarion, 4, Rue Casimir-Delavigne, 75006 Paris.

10. Knell A. J. 1991. Chapter 2: Malaria the Problem. Malaria. First Edition. Pg. 11. Published by the Oxford University Press, 1991 for the Wellcome Trust.

11. Ipca Laboratories Ltd. Clinical Presentation: Relapse and Recrudescence. Crusade Against Malaria. Document accessed online at www.malaria-ipca.com/cl_as_symptoms.html. Accessed on 21/06/2007 at 6:40 PM.

12. World Wide Malaria Distribution. WHO (2005). Available at http://gamapserver.who.int/mapLibrary/app/searchResults.aspx. Accessed on 21/06/2007.

13. Wiesner J, Ortmann R, Jomaa H, Schlitzer M. 2003. Antimalarial Drugs. Angew Chem Int Ed Engl. 2003 Nov 10;42(43):5274-93.

14. Malaria. TDR (WHO) 2002. Document available at www.who.int/tdr. Accessed on 21/06/2007.

15. Trape J. F, Pison G, Preziosi M .P, Enel C, Desgrees du Lou A, Delaunay V et al. 1998. Impact of Chloroquine Resistance on Malaria Mortality. C. R. Acad. Sci. III 321 : 689-697

16. Paris L, Thellier M, Faussart A, Danis M. World Epidemiology of Parasitic Diseases. Rev Prat. 2007 Jan 31;57(2):131-6.

17. Durant R, Huart V, Jafari S and Jacques Le Bras. Rapid Detection of a Molecular Marker for Chloroquine-Resistant Falciparum Malaria. Antimicrobial Agents and Chemotherapy, Aug. 2002, p. 2684-2686.

18. WHO. Malaria Indicator Definitions. 2005. Available online at www.who.int/wrm2005. Accessed on 21/06/2007.

19. Nardin E. H. Mechanisms of Immunity to Malaria Parasites. Available at http://www.med.nyu.edu/Research/E.Nardin-res.html. Accessed on 13/08/2007 at 9:48 pm.

20. Perlmann P, Troye-Blomberg M. Malaria and the immune system in humans. Chem Immunol. 2002;80:229-42.

21. CDC, USA, 2007. Human Host and Malaria: Immune Responses to Malaria. Avaliable at http://www.cdc.gov/malaria/biology/humanhost/index.htm. Accessed on the 13/08/2007 at 9:59 pm.

22. Hastings IM, Watkins WM. Intensity of malaria transmission and the evolution of drug resistance. Acta Trop. 2005 Jun;94(3):218-29. Epub 2005 Apr 19.

23. WHO. Malaria Indicator Definitions. 2005. Available online at www.who.int/wrm2005. Accessed on 21/06/2007.

24. Parola P, Soula G, Gazin P, Foucault C et al. 2006. Fever in travelers returning from tropical areas: prospective observational study of 613 cases hospitalised in Marseilles, France, 1999-2003. Travel Med Infect Dis. 2006 Mar;4(2):61-70

25. Malvy D, Pistone T, Rezvani A, Lançon F. 2006 Risk of malaria among French adult travellers. Travel Med Infect Dis. 2006 Sep;4(5):259-69. Epub 2005 Sep 23

26. Muenttener P, Schlagenhauf P, Steffen R. Imported Malaria (1985-95): Trends and Perspectives. Bull World Health Organisation 1999; 77: 560-6.

27. Boutin JP, Pradines B, Pagès F, Legros F et al. 2005. Epidemiology of malaria. Rev Prat. 2005 Apr 30;55(8):833-40.

28. Raccurt CP, Le Bras M, Cuisinier-Raynal JC, Ripert C et al. 1990. Imported malaria at hospitals in Bordeaux (France) in 1987 and 1988. Report of 185 cases. Med Trop (Mars). 1990 Jan-Mar;50(1):75-83.

29. Danis M, Legros F, Thellier M, Caumes E. 2002. Current data on malaria in metropolitan France. Med Trop (Mars). 2002;62(3):214-8.

30. Bloland, P. B., 2001. Drug Resistance in Malaria. Ch. 3, p. 12. Available from: www.who.int/drugresistance/publications/WHO_CDS_CSR_DRS_2001_4/en/ (Accessed on November 21, 2006

31. Farooq U, Mahajan RC. 2004. Drug resistance in malaria. J Vector Borne Dis. 2004 Sep-Dec;41(3-4):45-53.

32. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR. 2002. Epidemiology of drug-resistant malaria. Lancet Infect Dis. 2002 Apr;2(4):209-18.

33. Lopes D, Rungsihirunrat K, Nogueira F, Seugorn A. 2002. Molecular characterisation of drug-resistant Plasmodium falciparum from Thailand. Malar J. 2002 Oct 14; 1:12.

34. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR. 2002. Epidemiology of drug resistant malaria. Lancet Infect Dis. 2002 Apr;2(4):209-18.

35. Afonso A, Hunt P, Cheesman S, Alves AC et al. 2006. Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes atp6 (encoding the sarcoplasmic and endoplasmic reticulum Ca2+ ATPase), tctp, mdr1, and cg10. Antimicrob Agents Chemother. 2006 Feb; 50(2):480-9.

36. WHO. Guidelines for the Treatment of Malaria. 2006. Available at http://www.who.int/malaria/docs/TreatmentGuidelines2006.pdf. Accessed on 21/06/2007.

37. M. J. Mackinnon. 2005. Drug Resistance Models for Malaria. Acta Tropica 94 (2005) 207-217. Available online at www.sciencedirect.com. Accessed on 21/06/2007.

38. WHO, 2006. Guidelines for the Treatment of Malaria. Annexure 6. Pg. 156. Available at www.who/htm/mal/2006.1108. Accessed on 15/07/2007

39. Kuhn S, Gill M, J, Kain C. K. 2005. Emergence of Atovaquone-Proguanil Resistance During Treatment of Plasmodium Falciparum Malaria Acquired by a Non-Immune North American Traveller to West Africa. Am. J. Trop. Med. Hyg., 72(4), 2005, pp. 407-409

40. Tsongalis GJ, Silverman LM. 2006. Molecular Diagnostics: A Historical Perspective. Clin Chim Acta. 2006 Jul 23;369(2):188-92. Epub 2006 Mar 28.

41. Csako G. 2006. Present and Future of Rapid and/or High-Throughput Methods for Nucleic Acid Testing. Clin Chim Acta. 2006 Jan;363(1-2):6-31. Epub 2005 Aug 18.

42. Van Belkum A. 2003. Molecular Diagnostics in Medical Microbiology: Yesterday, Today and Tomorrow. Curr Opin Pharmacol. 2003 Oct;3(5):497-501.

43. GingerasTR, Higuchi R, Kricka JL, LO DYM et al. 2005. Fifty Years of Molecular(DNA/RNA) Diagnostics. Clinical Chemistry 51: 3, 661-671(2005)

44. Cockerill FR. 2003. Application of Rapid-Cycle Real-Time Polymerase Chain Reaction for Diagnostic Testing in the Clinical Microbiology Laboratory. Arch Pathol Lab Med. 2003 Sep;127(9):1112-20.

45. World Health Organization. 2001. Assessment of therapeutic efficacy of antimalarial drugs for uncomplicated falciparum malaria. Geneva, Switzerland, 2001.

46. World Health Organization. 1996. Assessment of therapeutic efficacy for uncomplicated falciparum malaria in areas with intense transmission. Geneva ,Switzerland : WHO/MAL/96. 1077. 1996 .

47. Picot S. 2001. Méthodes d'étude de la chimiorésistance de plasmodium falciparum aux quinoleines. Med. Trop. 2001; 61 : 15-20

48. Organisation Mondiale de la Santé. 2001. Surveillance de la résistance aux antipaludiques. Rapport d'une consultation de l'OMS. Genève. 2001

49. Waters, A.P. 2002. Orthology between the genomes of Plasmodium falciparum and rodent malaria parasites: possible practical applications. Philos. Trans. R. Soc. Lond. B. Biol. Sci 357: 55-63

50. Carlton J, Silva J, Hall N. 2004. The Genome of Model Malaria Parasites, and Comparative Genomics. Malaria Parasites: Genomes and Molecular Biology. 2004. Chapter 2. Pg. 34.

51. de Koning-Ward, T.F., Janse, C.J., and Waters, A.P. 2000. The development of genetic tools for dissecting the biology of malaria parasites. Annu. Rev. Microbiol. 54: 157-185

52. Gardner MJ, Hall N, Fung E, White O, Berriman M et al. 2002. Genome Sequence of the Human Malaria Parasite Plasmodium falciparum. Nature. 2002 Oct. 3; 419(8906): 498-511.

53. Guan YY, Tang LH, Hu L, Feng XP et al. [The point mutations in Pfcrt and Pfmdr1 genes in Plasmodium falciparum isolated from Hainan Province] Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi. 2005 Jun 30;23(3):135-9. Chinese.

54. Djimde A, Doumbo OK, Cortese JF, Kayentao K et al. 2001. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med. 2001 Jan 25;344(4):257-63.

55. Millet J, Alibert S, Torrentino-Madamet M, Rogier C et al. 2004. Polymorphism in plasmodium falciparum drug transporter proteins and reversal of in vitro chloroquine resistance by a 9,10 dihydroethanoanthracene derivative. Antimicrob Agents Chemother. 2004 Dec; 48(12):4869 72.

56. Mishra S, Raj DK, Hazra RK, Dash AP et al. 2006. An efficient PCR--SSCP-based method for detection of a chloroquine resistance marker in the PfCRT gene of Plasmodium falciparum. Trans R Soc Trop Med Hyg. 2006 Mar;100(3):243-7.

57. Mayor AG, Gomez-Olive X, Aponte JJ, Casimiro S et al. 2001. Prevalence of the K76T mutation in the putative Plasmodium falciparum chloroquine resistance transporter (pfcrt) gene and its relation to chloroquine resistance in Mozambique. J Infect Dis. 2001 May 1; 183(9):1413-6.

58. Tinto H, Ouedraogo JB, Erhart A, Van Overmeir C et al. 2003. Relationship between the Pfcrt T76 and the Pfmdr-1 Y86 mutations in Plasmodium falciparum and in vitro/in vivo chloroquine resistance in Burkina Faso, West Africa. Infect Genet Evol. 2003 Nov; 3(4):287-92.

59. Nair S, Nash D, Sudimack D, Jaidee A et al. 2007. Recurrent gene amplification and soft selective sweeps during evolution of multidrug resistance in malaria parasites. Mol Biol Evol. 2007 Feb; 24(2):562-73.

60. Price RN, Uhlemann AC, Brockman A, McGready R et al. 2004. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. Lancet. 2004 Jul 31-Aug 6; 364(9432):438-47.

61. Uhlemann AC, Ramharter M, Lell B, Kremsner PG et al. 2005. Amplification of Plasmodium falciparum multidrug resistance gene 1 in isolates from Gabon. J Infect Dis. 2005 Nov 15; 192(10):1830-5.

62. Lopes D, Rungsihirunrat K, Nogueira F, Seugorn A et al. 2002. Molecular characterisation of drug-resistant Plasmodium falciparum from Thailand. Malar J. 2002 Oct 14; 1:12.

63. Congpuong K, Na Bangchang K, Mungthin M, Bualombai P et al. 2005. Molecular epidemiology of drug resistance markers of Plasmodium falciparum malaria in Thailand. Trop Med Int Health. 2005 Aug; 10(8):717-22.

64. Huaman MC, Roncal N, Nakazawa S, Long TT et al. 2004. Polymorphism of the Plasmodium falciparum multidrug resistance and chloroquine resistance transporter genes and in vitro susceptibility to aminoquinolines in isolates from the Peruvian Amazon. Am J Trop Med Hyg. 2004 May; 70(5):461-6.

65. Humphreys GS, Merinopoulos I, Ahmed J, Whitty CJ et al. 2007. Amodiaquine and artemether-lumefantrine select distinct alleles of the Plasmodium falciparum mdr1 gene in Tanzanian children treated for uncomplicated malaria. Antimicrob Agents Chemother. 2007 Mar; 51(3):991-7.

66. Afonso A, Hunt P, Cheesman S, Alves AC et al. 2006. Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes atp6 (encoding the sarcoplasmic and endoplasmic reticulum Ca2+ ATPase), tctp, mdr1, and cg10. Antimicrob Agents Chemother. 2006 Feb; 50(2):480-9.

67. McCollum AM, Mueller K, Villegas L, Udhayakumar V et al. 2007. Common Origin and Fixation of Plasmodium falciparum dhfr and dhps Mutations Associated with Sulfadoxine Pyrimethamine Resistance in a Low-Transmission Area in South America. Antimicrob Agents Chemother. 2007 Jun; 51(6):2085-91.

68. Osman ME, Mockenhaupt FP, Bienzle U, Elbashir MI et al. 2007.Field-based evidence for linkage of mutations associated with chloroquine (pfcrt/pfmdr1) and sulfadoxine pyrimethamine (pfdhfr/pfdhps) resistance and for the fitness cost of multiple mutations in P. falciparum. Infect Genet Evol. 2007 Jan; 7(1):52-9.

69. Heidari A, Dittrich S, Jelinek T, Kheirandish A et al. 2007. Genotypes and in vivo resistance of Plasmodium falciparum isolates in an endemic region of Iran. Parasitol Res. 2007 Feb; 100(3):589-92.

70. Dokomajilar C, Lankoande ZM, Dorsey G, Zongo I et al. 2006. Roles of specific Plasmodium falciparum mutations in resistance to amodiaquine and sulfadoxine pyrimethamine in Burkina Faso. Am J Trop Med Hyg. 2006 Jul; 75(1):162-5.

71. Marks F, von Kalckreuth V, Kobbe R, Adjei S et al. 2005. Parasitological Rebound Effect and Emergence of Pyrimethamine Resistance in Plasmodium falciparum after Single-Dose Sulfadoxine-Pyrimethamine. JID. 2005; 192.

72. Nsimba B, Jafari-Guemouri S, Malonga DA, Mouata AM et al. 2005. Epidemiology of drug-resistant malaria in Republic of Congo: using molecular evidence for monitoring antimalarial drug resistance combined with assessment of antimalarial drug use. Trop Med Int Health. 2005 Oct; 10(10):1030-7.

73. Happi CT, Gbotosho GO, Folarin OA, Akinboye DO et al. 2005. Polymorphisms in Plasmodium falciparum dhfr and dhps genes and age related in vivo sulfadoxine-

pyrimethamine resistance in malaria infected patients from Nigeria. Acta Trop. 2005 Sep; 95(3):183-93.

74. Malarone™; GlaxoSmithKline, Research Triangle Park, NC.

75. Bloland, P. B., 2001. Drug Resistance iin Malaria. Ch. 3, p. 12. Available from: www.who.int/drugresistance/publications/WHO_CDS_CSR_DRS_2001_4/en/ (Accessed on November 21, 2006

76. WHO, 2006. Guidelines for the Treatment of Malaria. Chapter 7(2.1). Pg. 16. Available at www.who/htm/mal/2006.1108. Accessed on 12/05/2007

77. WHO, 2006. Guidelines for the Treatment of Malaria. Chapter 7(2.2). Pg. 16. Available at www.who/htm/mal/2006.1108. Accessed on 12/05/2007

78. Malaria: Combination Therapy for Uncomplicated Malaria Programme in Africa? WHO/AFRO. January 2001. Volume 4, No. 1. Pg. 1

79. Gil JP, Nogueira F, Stromberg-Norklit J, Lindberg J et al. 2003. Detection of atovaquone and malarone resistance conferring mutations in Plasmodium falciparum cytochrome b gene (cytb). Molecular and Cellular Probes. Volume 17, Issues 2-3, April-June 2003, p 85-89.

80. WHO, 2006. Guidelines for the Treatment of Malaria. Chapter 7(2.3). Pg. 17. Available at www.who/htm/mal/2006.1108. Accessed on 12/05/2007

81. Malaria: Combination Therapy for Uncomplicated Malaria Programme in Africa? WHO/AFRO. January 2001. Volume 4, No. 1. Pg. 3

82. WHO, 2006. Guidelines for the Treatment of Malaria. Chapter 7(.3). Pg. 21. Available at www.who/htm/mal/2006.1108. Accessed on 12/05/2007

83. WHO, 2001. Antimalarial Drug Combination Therapy. Report of a WHO Technical Consultation. Pgs. 9-12. Available at www.rbm.who.int. Accessed on 13/05/2007

84. Farnet A, Lindberg J, Gil P, Swedberg G et al. 2003: Evidence of Plasmodium falciparum malaria resistant to atovaquone and proguanil hydrochloride: Case Reports. BMJ 2003; 326:628-9

85. Fivelman QL, Butcher GA, Adagu IS, Warhurst DC et al. 2002: Malarone treatment failure and in vitro confirmation of resistance of Plasmodium falciparum isolate from Lagos, Nigeria. Malaria Journal 202, 1:1

86. Fry M, Pudney M. 1992: Site of action of antimalarial hydroxynapthoquinone, 2-(trans-4- (4'chlorophenyl) cyclohexyl)-3-hydroxy-1, 4-naphthoquinone (566C80). Biochem Pharmacol 1992, 43:1545-1553

87. Srivastava IK, Rottenburg H, Vaidya AB. 1997: Atovaquone, a broad-spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. J Biol Chem 1997, 272: 3961-3966

88. Looareesuwan S, Chulay JD, Canfield CJ, Hutchinson DBA. 1999: Malarone (atovaquone and proguanil hydrochloride): a review of its clinical development for treatment of malaria. Malarone Clinical Trials Study Group. Am J Trop Med Hyg 1999, 60:533-541

89. Basco KL. 2003: Molecular epidemiology of malaria in Cameroon. XVII. Baseline monitoring of atovaquone-resistant Plasmodium falciparum by in vitro drug assays and cytochrome B gene sequence analysis.

90. Srivastava I, Vaidya A. 1999: A mechanism for the synergistic antimalarial action of atovaquone and proguanil. Antimicrob Agents Chemother 1999, 43:1334-1339

91. Korsinczky M, Chen N, Kotecka B, Saul A et al. 2000: Mutations in Plasmodium falciparum cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. Antimicrobial agents and Chemotherapy, Aug. 2000, p. 2100-2108

92. Wichmann O, Muehlen M, Gruss H, Mockenhaupt FP et al. 2004: Malarone treatment failure not associated with previously described mutations in the cytochrome b gene. Malaria Journal 2004, 3:14.

93. Schwobel B, Alifrangis M, Salanti A, Jelinek T. 2003: Different mutation patterns of atovaquone resistance to Plasmodium falciparum in vitro and in vivo: rapid detection of codon 268 polymorphisms in the cytochrome b as potential in vivo resistance marker. Malaria Journal 2003, 2:5.

94. Fivelman QL, Butcher GA, Adagu IS, Warhurst DC et al. 2002. Malarone treatment failure and in vitro confirmation of resistance of Plasmodium falciparum isolate from Lagos, Nigeria. Malar J. 2002 Feb 8;1:1.

95. Gebru T, Hailu A, Kremsner PG, Kun JF et al. 2006. Molecular surveillance of mutations in the cytochrome b gene of Plasmodium falciparum in Gabon and Ethiopia. Malar J. 2006 Nov 21;5:112

96. Pimentel S, Nogueira F, Benchimol C, Quinhentos V et al. 2006. Detection of atovaquone-proguanil resistance conferring mutations in Plasmodium falciparum cytochrome b gene in Luanda, Angola. Malar J. 2006 Apr 5;5:30

97. Musset L, Pradines B, Parzy D, Durand R et al. 2006. Apparent absence of atovaquone/proguanil resistance in 477 Plasmodium falciparum isolates from untreated French travellers. J Antimicrob Chemother. 2006 Jan;57(1):110-5. Epub 2005 Nov 30

98. Färnert A, Lindberg J, Gil P, Swedberg G et al. 2003. Evidence of Plasmodium falciparum malaria resistant to atovaquone and proguanil hydrochloride: case reports. BMJ. 2003 Mar 22;326(7390):628-9

99. Vaidya A. B, Lashgari M.S, Pologe L.G, Morrisey J. 1993. Structural features of Plasmodium falciparum cytochrome b that may underlie susceptibility to 8-aminoquinolines and hydroxynaphthoquinones. Mol. Biochem. Parasitol. 58 :33-42.

100. Kessl JJ, Ha KH, Merritt AK, Lange BB et al. 2005. Cytochrome b mutations that modify the ubiquinol-binding pocket of the cytochrome bc1 complex and confer anti-malarial drug resistance in Saccharomyces cerevisiae. J Biol Chem. 2005 Apr 29;280(17):17142-8. Epub 2005 Feb 17

101. Di Rago J.P, Coppee J.Y, Colson A.M.1989. Molecular basis for resistance to myxothiazol, mucidin(strobilurin A), and stigmatellin. J. Biol. Chem. 264 :14543-14548.

102. Looareesuwan S. Et al. Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, from treatment of acute uncomplicated malaria in Thailand. American Journal of Tropical Medicine and Hygiene, 1996, 54(1) : 62-66

103. WHO, 2006. Guidelines for the Treatment of Malaria. Resistance to antimalarials. Annex 6. A6.3.2. Pg. 161. Available at www.who/htm/mal/2006.1108. Accessed on 25/05/2007

104. Field JW, Niven JC. A note on prognosis in relation to parasite count in acute subtertian malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene, 1937, 30 :569-574

105. Sub-Saharan Africa. A political map showing national divisions in relation to the ecological break. (Sub-Saharan Africa in green). Avaliable at http://en.wikipedia.org/wiki/Sub-Saharan_Africa, accessed on 16/05/2007.

106. Control Malaria. Malaria. ATM-WHO/AFRO. Available at http://www.afro.who.int/malaria/, accessed on 17/05/2007.

107. WHO, 2006. Guidelines for the Treatment of Malaria. Resistance to antimalarials. Annex 6. A6.4 Pg. 167. Available at www.who/htm/mal/2006.1108. Accessed on 25/05/2007