High Frequency of the FY^{ES} Duffy Antigen Receptor for Chemokines allele in the absence of *Plasmodium vivax* in Kilifi, Kenya

By

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A thesis submitted to the Centre for Biotechnology and Bioinformatics in partial fulfillment for the award of Master of Science in Biotechnology, University of Nairobi.

DECLARATION

I declare that this research is my own work and has not been submitted for examination in any other university

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Signature …….. Date…….

SUPERVISOR'S APPROVAL

We confirm that this thesis has been submitted with our approval as university supervisors;

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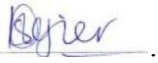
Signature…  …… Date……15th December 2017……

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Signature…….. Date……..

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DEDICATION

For you, Mum and Papa.

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ABBREVIATIONS

bp	–	Base pair
CEBIB	–	Centre for Biotechnology and Bioinformatics
DARC	–	Duffy Antigen Receptor for Chemokines
DBP	–	Duffy Binding Protein
DNA	–	Deoxyribonucleic acid
dNTPs	–	Deoxynucleotide triphosphates
EBLs	–	Erythrocyte Binding-Like proteins
G6PD	–	Glucose-6-Phosphate dehydrogenase deficiency
KHDSS	–	Kilifi Health and Demographic Surveillance System
MgCl2	–	Magnesium chloride
µl	–	Microlitre
ml	–	Millilitre
PCR	–	Polymerase Chain Reaction
P. vivax	–	*Plasmodium vivax*
P. falciparum	–	*Plasmodium falciparum*
P. ovale	–	*Plasmodium ovale*
P. malariae	–	*Plasmodium malariae*
pLDH	–	parasite lactate dehydrogenase
RBCs	–	Red Blood Cells
RDT	–	Rapid Diagnostic Test
RFLP	–	Restriction Fragment Length Polymorphism
rpm	–	Revolutions per minute
SNP	–	Single Nucleotide Polymorphisms
Taq	–	*Thermus aquaticus*
WHO	–	World Health Organization
w/v	–	weight/volume

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ABSTRACT

Malaria is a human disease that is caused by the *Plasmodium* parasite and it is a life-threatening disease. *Plasmodium vivax* being one of the parasites among other *Plasmodium* parasites is a major cause in spreading malaria in humans. The other four parasites that affect the human species include *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*.

Plasmodium vivax is predominant in the Asian and the American continents whereas *Plasmodium falciparum* is predominant in the African continent and is mainly being tackled along the coastal and western regions of Kenya. *Plasmodium vivax* is usually neglected in the African continent since it has been shown that the African population is negative for the Duffy blood-group antigen genotype with the presence of the FY^{ES} allele. Since this gene is silenced, it does not mediate invasion of erythrocytes by the *Plasmodium vivax* parasite.

Due to the high prevalence of the absence of the Duffy blood-group antigen on the surface of the red blood cells, *Plasmodium vivax* is not highly considered as compared to *Plasmodium falciparum* which is highly prevalent in the African continent, especially in Kenya. This study was geared towards finding the frequency of the FY^{ES} allele in malaria patients from Kilifi County Hospital.

Parasite genomic DNA was extracted from 204 blood samples. The DARC gene was amplified by Polymerase Chain Reaction (PCR). Samples that generated good amplicons were analysed by Restriction Fragment Length Polymorphism (RFLP) to validate whether the individuals being tested were Duffy positive or Duffy negative. The samples tested matched the pattern of the Duffy negative control.

The samples were then tested for *Plasmodium vivax* infections using *vivax* primers and a positive control sample. All the samples were negative for *P. vivax* infections. In conclusion, *P. vivax* is not prevalent in the Kilifi region since the FY^{ES} allele is expressed at a high frequency in this region.

CHAPTER 1

LITERATURE REVIEW

1.1 General introduction

Malaria is a devastating disease that is caused by the *Plasmodium* parasite. This parasite transmits malaria to humans through a bite of an infected *Anopheles* mosquito. Since this parasite is carried by the mosquito, it is the major vector of malaria (Cornejo et al., 2006). The 5 major parasite species that cause malaria in the human species are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi.* Out of these 5 species, *P. falciparum* and *P. vivax* are found to be the greatest threats globally due to their mortality rates and also their ability to develop drug resistance (Cornejo et al., 2006).

Approximately 70 to 130 million occurrences of *P. vivax* are reported annually with about 40% of the world's population being at risk (Price et al. 2007). Sixty percent reduction in malaria mortality rates have been observed globally due to increased prevention and control measures (Guerra et al., 2006).

In Kenya, *P. falciparum* is the most dominant parasite that causes malaria. If left untreated, it is very fatal and may cause death. The highest burden is in children below five years of age and pregnant women (Kenya Malaria Fact Sheet, 2009). According to the Kenya Medical Research Institute (KEMRI), 25 million Kenyans are at risk of malaria. This research institute also states that malaria causes 20% of mortality rate in children who are under five years of age in Kenya (Kenya Malaria Fact Sheet, 2009). Since *P. falciparum* is the most common parasite in Kenya, *P. vivax* is found to be rare but since it is a major threat around the world because of its drug resistance, recurring and persisting properties, it is important to be cautious.

1.2 Plasmodium vivax

Plasmodium vivax is a widely distributed human parasite worldwide, which is a major cause of recurring malaria, also known as benign tertian malaria (Ryan et al., 2006; Batchelor et al. 2014). *P. vivax* remains responsible for major mortality rates in *vivax*-endemic areas which include India, Asia and South America (Anstey et al. 2012). This malaria has long been considered absent or extremely rare in most parts of Africa (Culleton & Ferreira 2012). This rarity is because of a mutation in the Duffy blood group gene which confers resistance to *P. vivax* (Batchelor et al. 2014).

1.2.1 Life Cycle

The *Plasmodium vivax* life cycle as shown in Figure 1.1 involves two hosts, the mosquito also known as the definitive host of plasmodia and the human also known as the intermediate host (Westenberger et al., 2010). During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host (Westenberger et al., 2010). The sporozoites that have been inoculated by the *Anopheles* infect liver cells and this leads to the sporozoite either entering a dormant hypnozoite state or maturing into schizonts (Westenberger et al., 2010). The dormant hypnozoites can cause relapses which usually correspond to the seasonal local abundance of the anophelines mosquitoes (Baird 2009) and the mature schizonts lead to the rupturing and the release of merozoites respectively (Westenberger et al., 2010). After this initial replication in the liver (exo-erythrocytic schizogony), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony). The merozoites infect red blood cells and thereafter the ring stage trophozoites mature into schizonts, which rupture releasing merozoites (Westenberger et al., 2010). Some parasites differentiate into sexual erythrocytic stages known as the gametocytes (Westenberger et al., 2010). The gametocytes, male (microgametocytes) and female (macrogametocytes) are ingested by an *Anopheles* mosquito during the blood meal where they multiply in the mosquito and this is known as the sporogonic cycle (Westenberger et al., 2010). While in the mosquito's midgut, the microgametes penetrate the macrogametes generating zygotes. The zygotes in turn become motile and elongated known as the ookinetes which invade the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture and release

sporozoites, which make their way to the mosquito's salivary glands (Westenberger et al., 2010). Inoculation of these sporozoites into new human hosts perpetuates the parasite’s life cycle (Westenberger et al., 2010).

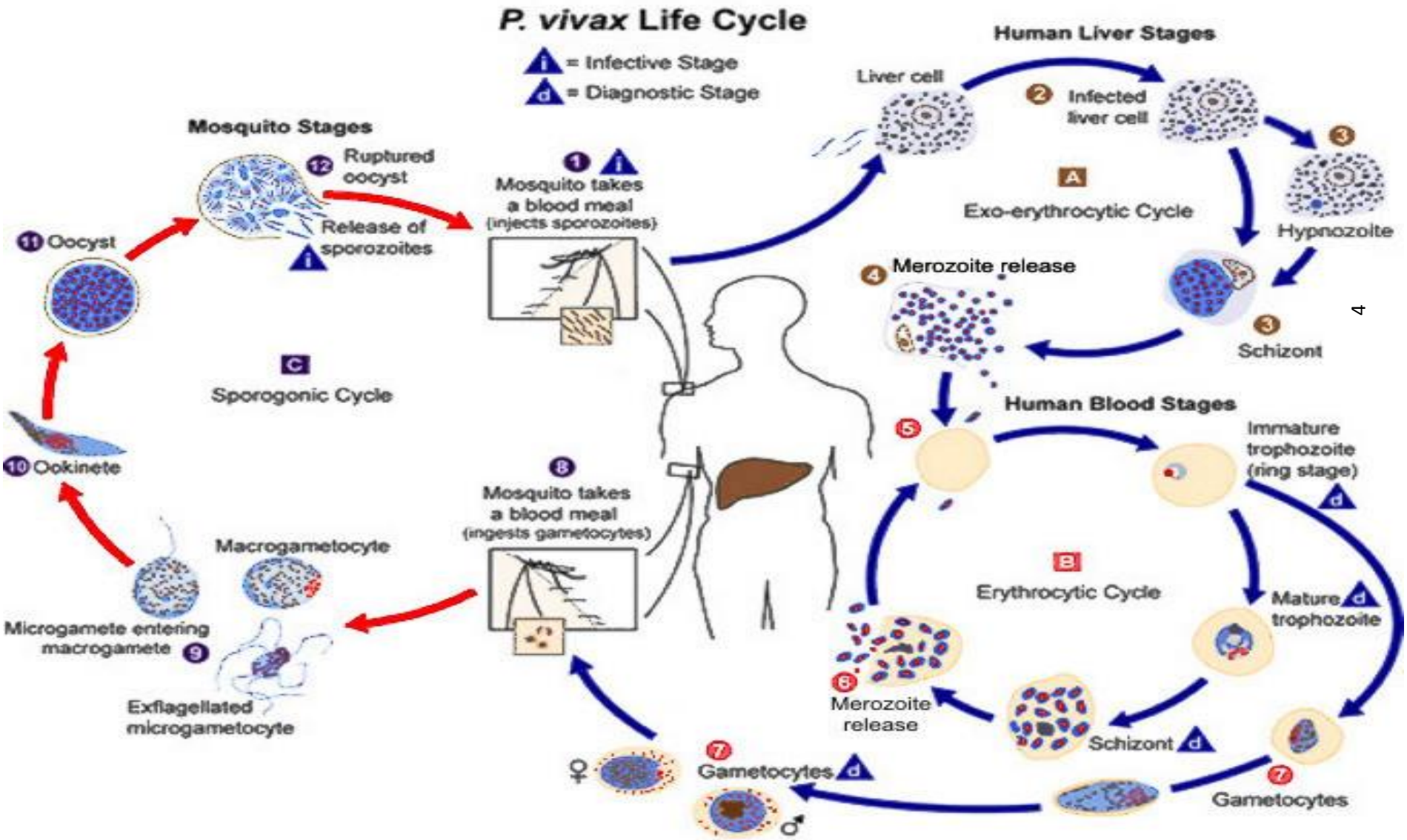

Figure 1.1: Life cycle of *Plasmodium vivax* parasite involving the two hosts, mosquito and the human host. This figure is adapted from the Centre of Disease Control (CDC) website (Baird et al., 2007).

1.2.2 Pathogenesis and symptoms

Symptoms of *Plasmodium vivax* occur through the infection of the red blood cells of humans. Once the red blood cells are infected, they begin rupturing leading to the outbreak of fever. Some infected red blood cells tend to stick to the walls of the capillaries and this process deprives the tissues and the capillaries of oxygen. *P. vivax* is found to populate the bloodstream in the form of asexual-stage parasites (Anstey et al., 2012a). Since this parasite has an unusual biology, it tends to be difficult to interrupt its transmission. During the hypnozoite stage of *P. vivax,* the parasite tends to lie in a dormant state in the infected liver cells with a time span from weeks to months to years after the primary infection. Thus, a single mosquito inoculation may result in repeated cycles of illness (Bousema et al., 2011). This stage is usually responsible for the relapsing of *P. vivax* malaria and hence it tends to be more hazardous as compared to *P. falciparum* since *P. falciparum* infection does not tend to relapse after one mosquito bite. *P. vivax* can cause severe illness and fatal outcomes such as: high fever, violent headaches, chills and profuse sweating (Anstey et al., 2012b). All these symptoms are usually accompanied by vomiting, diarrhoea and the enlargement of the spleen which is known as splenomegaly. In instances where *P. vivax* keeps recurring then it may also cause severe anaemia and malnutrition in children. Other symptoms with hazardous effects include: acute lung and kidney injury and multi organ failure (Anstey et al., 2012b). In pregnant women, the disease may lead to maternal anaemia, miscarriages, low birth weight of the babies and congenital malaria (Anstey et al., 2012b).

1.2.3 Diagnosis and Treatment

Vivax malaria is usually identified through microscopy or rapid diagnostic tests (RDT) (Baird et al., 2007). When there are low numbers of parasites in the blood circulation, it becomes difficult to diagnose or confirm these parasites (Baird et al., 2012). The treatment of this disease requires therapy that can cure the blood infection of this malaria and also prevent subsequent relapses of this malaria (Baird et al., 2012). Radical cure for *P. vivax* requires a blood stage drug and a hypnozoitocide which is chloroquine in default (Howes et al. 2015). Primaquine is the other drug which is administered to prevent hypnozoite-triggered relapse, but due to its assessment on the risks such as haemolysis that affects the G6PD patients therefore, it should be ideally administered to normal patients (Howes et al.

2015). The most common form of drugs mainly used for treating *P. vivax* can either be chloroquine or artemisinin-based treatment combined with primaquine in most cases (Howes et al. 2015). In order to develop safe and effective chemotherapies and drugs for *vivax* malaria these challenges must be overcome which requires a lot of time (Baird et al., 2012). A high-throughput screening specific for *vivax* malaria remains impractical in the absence of continuous *in-vitro* cultivation (Baird et al., 2009). Never the less, serological tests are used to detect antibodies specific to *P. vivax* for screening blood (Rodrigues et al. 2003).

1.3 Geographical Distribution

Plasmodium vivax is one of the parasites in the *Plasmodium* species that causes malaria in humans that is widely distributed geographically (Batchelor et al., 2014).

Geographically, *P. vivax* cases are approximated to be around 70 to 80 million annually (Prajapati et al. 2011). In Africa, cases of *P. vivax* approximate up to 20% as shown in Figure 1.2 (Prajapati et al., 2011). These cases occur mainly in the South and East of the Sahara like Somalia, Ethiopia, Congo and Madagascar (Mendis et al., 2001). Compared to the rest of the globe, the African continent has a very small percentage of *P. vivax* cases and hence it is a rare disease in Africa. The reason for this rarity is due to the silent Duffy blood-group antigen in the African population; therefore it is a neglected disease in Africa.

The rest of the percentage which entails about 80% to 90% of *P. vivax* occurs in the Middle East, Asia, and the Western Pacific and around 10% to 15% in Central and South America as shown in Figure 1.2 (Mendis et al., 2001). Although the effects of recurring attacks of *P. vivax* through human life are rarely lethal, *vivax* malaria can have major disadvantages on personal well-being, growth and development (Mendis et al., 2001). Features that are entailed in the transmission of *P. vivax* give this particular species greater resilience than *P. falciparum* in the face of adverse parasite transmission conditions such as the tendency of the parasites to lie in a hypnozoite stage (Mendis et al., 2001).

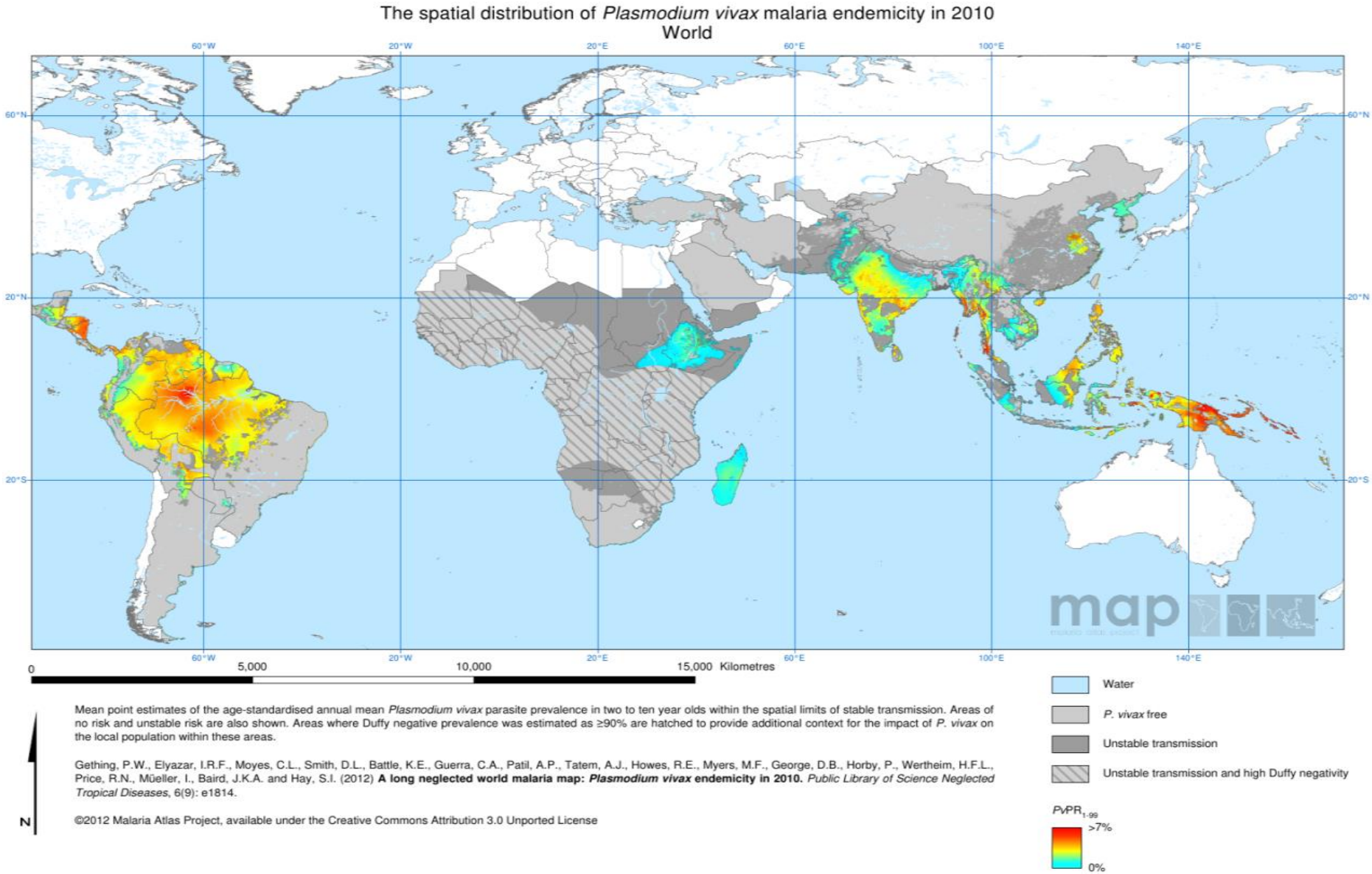

Figure 1.2: The distribution of *Plasmodium vivax* malaria globally. This figure is adapted from (Baird et al., 2012).

1.4 Duffy Antigen Receptor for Chemokines (DARC)

The Duffy blood group antigens mainly function as receptors for the malaria parasites *Plasmodium vivax* and *Plasmodium knowlesi.* FY^A and FY^B are the only two known co-dominant alleles (Tournamille et al. 1995).

The Duffy antigen receptor is a trans-membrane glycoprotein that traverses the membrane. It is found on the red blood cells of the human species that contains an N-terminal domain which consists of a 35-amino acid epitope (Fy6) which then mediates the erythrocyte invasion by *P. vivax* merozoites (Ryan et al. 2006). This particular antigen is mainly used by the *P. vivax* merozoites for invasion and infection of the erythrocytes (VanBuskirk et al., 2004). The major difference in these two alleles is the single nucleotide at the amino acid codon 42. At this point, glycine turns to aspartic acid resulting in the expression of the Fy^a or the Fy^b antigen, respectively (King et al., 2011). The three main phenotypes in the Duffy blood group are Fy (a+b-), Fy (a-b+) and Fy (a+b+). These phenotypes are the products of the co-dominant alleles which comprise the following genotypes: FY*A/FY*A, FY*B/FY*B and FY*A/FY*B respectively (Marsh & Ehrich 2009).

Most of the African populations do not express Fy^a or Fy^b antigens on their Red blood cells (Chown et al. 1965). Therefore, the absence of Fy^a or Fy^b antigens on the surface of the erythrocytes is known as the Duffy negative phenotype which is denoted as Fy (a-b-) and genotype which is denoted as the FY^{ES} allele. The individuals that have this Fy (a-b-) phenotype, ensures that the gene does not get expressed hence it remains silent.

Therefore this is the major reason as to why *P. vivax* is absent in majority of the African continent (Weppelmann et al., 2013). The silenced Duffy antigen expression is a result of the single nucleotide polymorphism (SNP) in the GATA box sequence in the DARC gene promoter region where a Cytosine (C) replaces the Thymine (T) 46 nucleotides before the erythroid cap site (Tournamille et al., 1995; Cavasini et al., 2007).

Chemokines are members of a superfamily of small, secreted proteins that recruit leukocytes to the sites of inflammation (Oppenheim et al. 1991). This superfamily has two branches that promote acute and chronic inflammatory processes (Oppenheim et al. 1991). The African population mostly lacks the chemokine receptor activity on the human red blood cells as shown in Figure 1.3 (Horuk et al. 1993).

In Africa, 95% to 99% of the human population is of the Duffy negative phenotype (Culleton et al., 2012). This condition is known to confer resistance to *P. vivax* infections (Culleton et al., 2012).

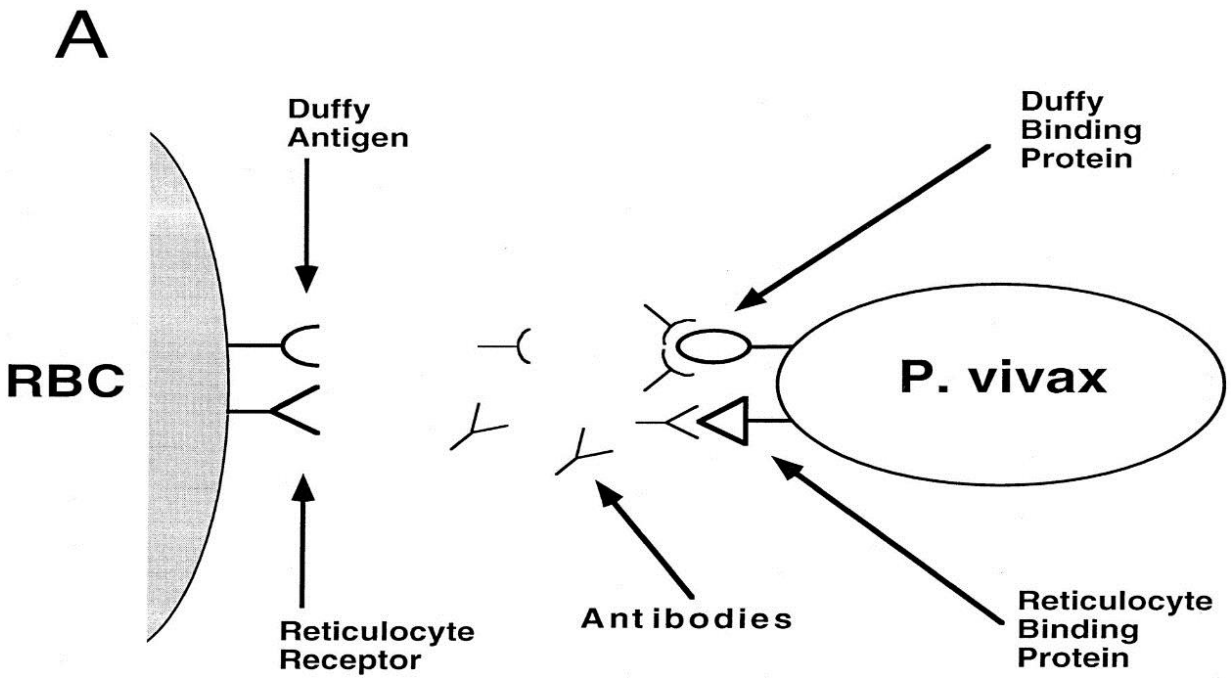

Figure 1.3: Receptors for apical attachment of *P vivax* merozoites to RBCs .The Duffy binding protein and the reticulocyte binding protein. This figure is adapted from the (Galinski et al.,1992).

The *P.vivax* Duffy Binding Protein (DBP) as shown in Figure 1.3 is a critical invasion ligand that recognizes the receptor Duffy antigen for chemokines (DARC) during invasion (Wertheimer & Barnwell 1989). DBP is a member of the erythrocyte binding like (EBL) family of proteins, which localizes to micronemes and use Duffy binding-like domains to bind specific receptors (Tolia et al. 2005). These Duffy binding-like domains are usually located in "region II" of the erythrocyte binding-like (EBL) proteins (Tolia et al. 2005). The DBP is mostly a *P.vivax* therapeutic target mainly because it is the only EBL family member in the *P.vivax* genome (Carlton et al. 2008). Also, DBP is one of the leading vaccine candidates against *P.vivax* malaria (Beeson & Crabb 2007).

1.5 Justification

P. vivax is a rare disease in Africa because of the genetic background of the African population (Weppelmann et al., 2013). Most of this population could be of the Duffy negative phenotype due to a single point mutation in the GATA box of the promoter region on the DARC gene (Weppelmann et al., 2013). This mutation mainly encodes the FY^{ES} allele, which silences the expression of the Duffy antigen on the RBCs hence abolishing *P. vivax* infections (Weppelmann et al., 2013). Even though *P. vivax* is a rare disease in Africa, it is still the most widely distributed disease globally with the attribute of relapsing malaria in each occurrence. Hence it is an important disease that cannot be overlooked.

The prevalence of the erythroid silent Duffy antigen genotype in Kilifi is not known. Also, *P. vivax* has rarely been observed in Kilifi therefore, this study will determine the frequency of the FY^{ES} allele in the Kilifi population and it will help identify *P. vivax* infections in malaria patients if any. This will provide data on the prevalence of *P. vivax* infections in Kilifi and it is likely to show that the only threat to be dealt within the Kilifi County is *P. falciparum*.

1.6 Research Question

Is the FY^{ES} allele present at a high frequency in the population of Kilifi?

1.7 Hypothesis

There is a low frequency of the FY^{ES} alleles.

1.8 Objectives

1.8.1 Main Objective

This study determined the frequency of the FY^{ES} allele and *P. vivax* infections in malaria patients in Kilifi, Kenya.

1.8.2 Specific Objectives

i. Genotype the DARC gene.
ii. Determine the frequency of the FY^{ES} alleles.
iii. Determine the presence of *P.vivax* infections in Kilifi.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study site

This study was carried out in the county of Kilifi in the coastal region of Kenya (Figure 2.1). The coastal region of Kenya is regarded as a malaria endemic region although over the last 15 years a decline has been noticed (Okiro et al. 2007). The particular study site is the Kilifi County Hospital, which is in a rural area in the Coast Province of Kenya. It was found that malaria in this region occurred seasonally which was mainly in the rainy seasons which were from April to July and from October to November (Awuor 2014).

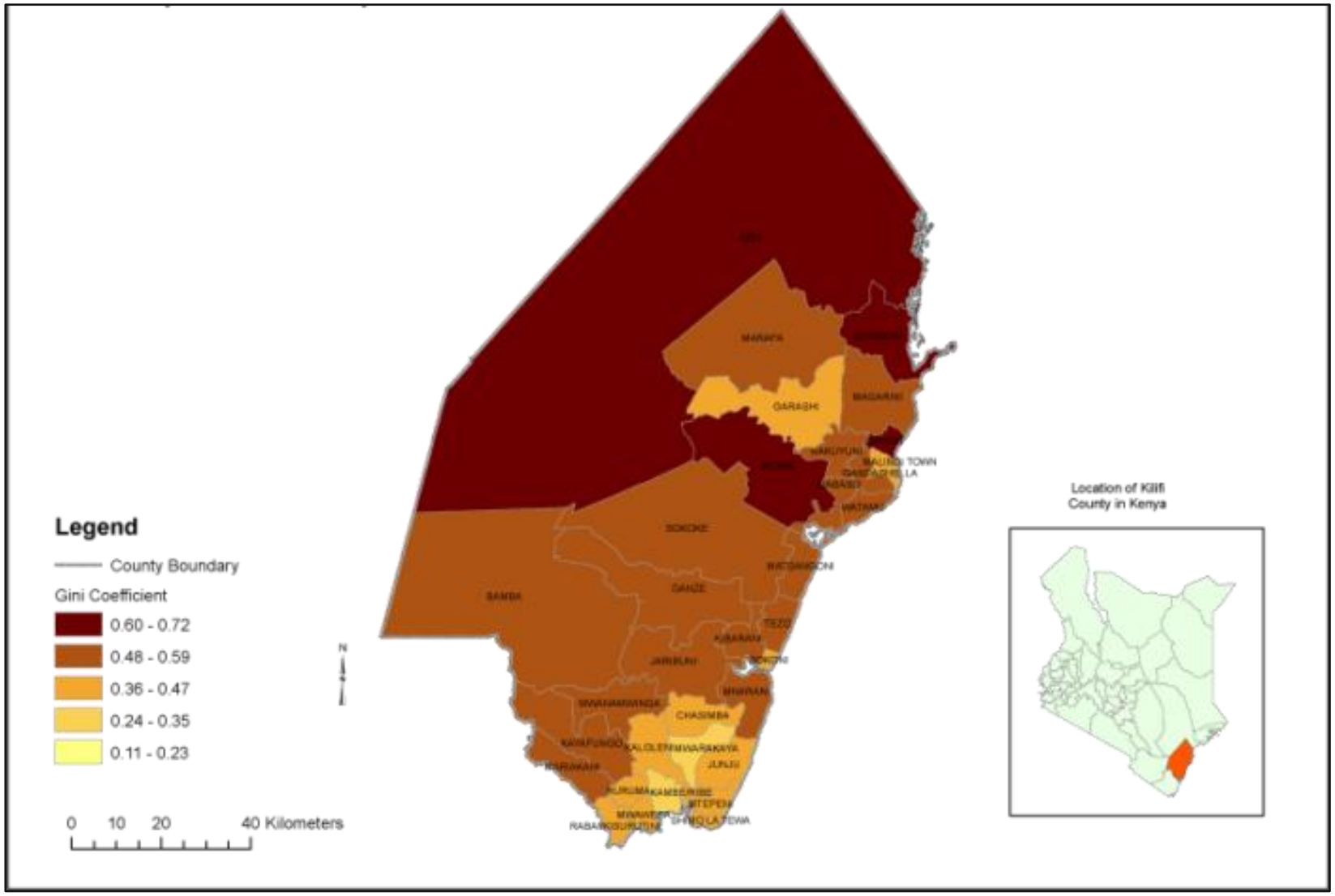

Figure 2.1: The map of Kenya showing the Kilifi County, the study site, which is marked in red bordering the Indian Ocean. This figure is adapted from http://www.adios-ea.com/wp-content/uploads/2015/09/Kilifi-County-map-500x314.jpg

2.2 Study Population

Ninety five human DNA samples were used (Awuor 2014). These were the first set of samples used for the DARC genotyping and were extracted in 2001 from whole blood taken from patients admitted in the High Dependency Unit (HDU) in the Kilifi County Hospital who were suffering from severe malaria (Awuor 2014).

In this case, severe malaria was defined as malaria anaemia, cerebral malaria and/or respiratory distress (Awuor 2014). Fifty two of these patients were males whereas forty one were females and the sex of two patients was undefined. The range of age of the study participants was from 6 months to 8 years with the mean age being 3.5 years (Awuor 2014).

The second set of samples comprised of 109 DNA samples which were obtained in 2013/2014 from Kilifi County Hospital. This set of samples was added as a recent sample set to look for *vivax* infections in Kilifi.

2.3 DNA extraction

The DNA from the blood samples of each individual was previously extracted using the DNA blood minikit from QIAGEN, Inc. The following steps were included in the DNA extraction; the cellular components of blood were lysed using a lysis buffer. Thereafter, DNA was absorbed onto the QIAamp silica-gel membrane in a brief centrifugation step. PCR inhibitors were removed completely in two wash steps due to the salt and pH conditions in the buffers that were used, leaving behind pure nucleic acid to be eluted in the elution buffer that comes with the kit. The eluted DNA was stored at -20°C after making a 1:10 dilution (Awuor 2014).

2.4 Amplification of the DARC gene

The PCR system used for the amplification of the DARC gene was Expand High Fidelity from Roche. The Polymerase Chain Reaction (PCR) mix was made to a final volume of 20µl. Since the reaction mixes were done in a larger number, two sets of reaction mixes were prepared. The preparation of the two sets of reaction mixes usually aids in avoiding the 3” – 5” exonuclease activity of the proof-reading Taq polymerase, which partially

degrades primers and templates during the reaction set-up (Contents & Label 2005). Master mixes were prepared for the multiple reactions.

All the reagents of the PCR system were vortexed and centrifuged at 10000 rpm for 5 seconds to ensure that all the reagents were homogenous. The two master mixes were prepared in two separate sterile eppendorf tubes which were then placed on ice so as to preserve the reagents before placing them into the thermocycler in which the optimum PCR cycling conditions were programmed into the machine.

The first master mix eppendorf tube (MM1) included PCR water, Buffer 4 (25mM $MgCl_2$ stock solution), dNTP mix (10mM of dGTP, dATP, dTTP and dCTP), Forward and Reverse Primers (Table 2.1) (10µM) in the volumes shown in Table 2.2.

Table 2.1: DARC Primer Sequences.

Gene	**Primer Name**	**Primer Sequence**	**%GC content**	**Melting Temperature (°C)**
DARC	Forward	caggaagacccaaggccag	63.2	61.0
	Reverse	ccatggcaccgtttggttcagg	59.1	64.0

The second master mix eppendorf tube (MM2) included Taq Polymerase (Expand High Fidelity Enzyme solution), Buffer 2 (Expand High Fidelity Enzyme Buffer 10X with 15mM $MgCl_2$) and PCR water in the volumes shown in Table 2.3.

Table 2.2: Reaction volume for 1 reaction of PCR master mix 1 (MM1)

Reagent	**Volume in µl**
PCR Water	6.5
Buffer 4	2.0
DNTPs	0.4
Forward Primer	0.3
Reverse Primer	0.3
Template DNA	0.5
Final Volume	**10.0**

Table 2.3: Reaction volume for 1 reaction of PCR Master Mix 2 (MM2)

Reagent	**Volume in µl**
PCR water	7.7
Buffer 2	2.0
Taq Polymerase	0.3
Final Volume	**10.0**

The PCR reactions were done in 0.2ml PCR tubes for every test sample in the Peltier Thermal Cycler. A PCR optimization process for the amplification of the DARC gene was first done using 3 samples, 2 of which were DNA samples that were taken from the first set of samples and the other a negative control which was double distilled water. Optimization was done by testing the varying temperatures that would be used as the annealing temperature for the PCR reaction. The aim of the optimization process was to identify the correct annealing temperature for the primer set in order to amplify the DARC gene

Once the optimization process was complete, the PCR amplification temperature protocol used was as follows: DNA denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 20 seconds, annealing at 62°C for 15 seconds, and elongation at 72°C for 20 seconds and a final elongation at 72°C for 10 minutes (Weppelmann et al., 2013). Extra reaction mixtures were always made due to the pipetting errors.

This PCR protocol was used to amplify a segment as shown in the Figure 2.2 of the DARC gene in the GATA-1 promoter region which was 200 base pairs and it was set and saved in the thermocycler for the first objective. The PCR reactions for the first 95 samples were run in batches of 14 to 28 samples including a negative control which was double distilled water. The PCR protocol lasted for 1 hour 25 minutes.

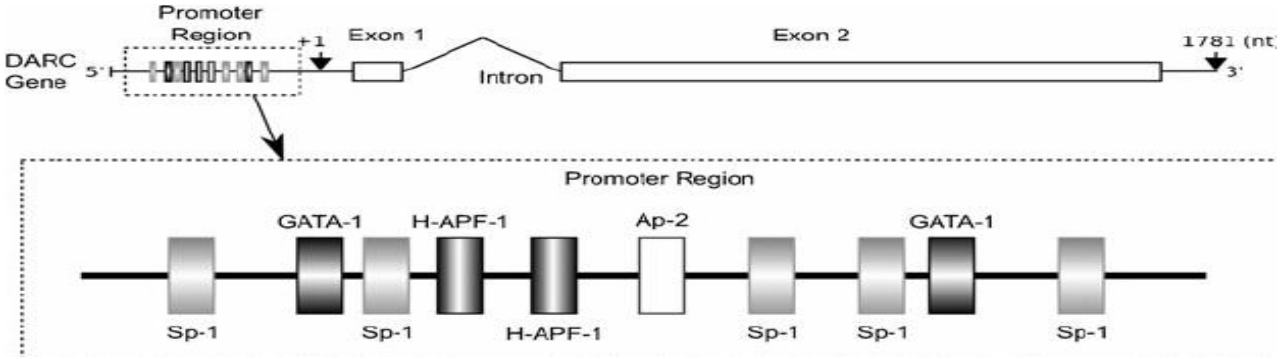

Figure 2.2: The promoter region of the DARC gene showing the GATA-1 region. This image is adapted from https://www.researchgate.net/profile/Thiago_Oliveira9/publication/221682669/figure/fig1/AS:277374915629056@1443142741117

2.5 Agarose Gel Electrophoresis

After PCR, agarose gels were prepared to visualize the PCR bands. The agarose gels were prepared using 1X (working solution) Tris-Borate- EDTA (TBE) buffer of pH 8.0 (Tris base, Boric acid and 0.5M EDTA). 1% w/v agarose gel was made by dissolving 1g of agarose powder (Top Vision Agarose) in 100ml of 1X TBE buffer by boiling the solution. The percentage gel run depended on a few things such as the size of the fragment that was targeted and how well the separation of the fragments was done. Once the solution had been boiled the agarose dissolved in the buffer and it was cooled using running water from the tap. Once the solution was luke warm, 1µl of ethidium bromide was added and the conical flask swirled so as to mix the ethidium bromide well into the gel solution. The gel was then poured into a gel tray which had a comb at one end and was left to set for at least 20 minutes. After the given setting time, the combs formed wells in the gel and the wells were loaded with 1µl of the amplified PCR product which was mixed with 2µl of the loading dye (6X Gel Loading Dye Purple) and was run on the gel in order to check the quality of the gene fragment. These PCR samples were run alongside the DNA ladder (Bioline HyperLadder 100bp) of which 1 µl was loaded. This ladder was used as a standard DNA marker. 1X TBE buffer was used as the running buffer. The gel electrophoresis was run at 80V for 40 minutes. Thereafter, gel visualization was done through digital photography under the UV light using the Molecular Imager Gel Doc (Bio-Rad.,UK). Once the single bands were viewed, the PCR products were then used for Restriction Fragment Length Polymorphism (RFLP).

2.6 Restriction Fragment Length Polymorphism of DARC

After the amplified PCR products were tested for bands, restriction fragment length polymorphism analysis was used to confirm the presence of the – 46 T/C SNP. There were positive and negative controls that were obtained from anonymous donors. One of the donors was a Duffy positive individual whereas the other donor was a Duffy negative individual and these two samples were the positive controls for RFLP whereas the negative control was double distilled water. The PCR products were digested with the restriction endonuclease *sty I* (Weppelmann et al., 2013). The enzyme that was used was according to the manufacturer's specifications which included an *E. coli* strain that carries the cloned StyI gene from *Salmonella typhi* (Weppelmann et al., 2013).

Table 2.4: Reaction volume for 1 reaction for RFLP

Reagents	**Volume in µl**
dH_2O	19.0
Sty I	0.5
10X NEBuffer	2.5
PCR product (DNA)	3.0
Final Volume	**25.0**

Once the reagents were mixed in the above concentration (Table 2.4), the PCR product samples were then incubated at 37°C for 1 hour and thereafter the Sty I enzyme in the mix was heat inactivated, which allowed the restriction nuclease to stop, for 20 minutes at 65°C. , The agarose gel which was stained with ethidium bromide (as described in section 2.5 above) was used and these fragments were then resolved using gel electrophoresis on a 4% w/v agarose gel for 1 hour at 70 volts.

2.7 Detection of *Plasmodium vivax* by Polymerase Chain Reaction

A PCR protocol by Weppelmann et al., was used to detect the presence of *P.vivax* in the DNA samples (Weppelmann et al., 2013). PCR *P.vivax* primers (Table 2.6) targeted the *P.vivax* small subunit ribosomal RNA gene (18S rRNA) (Tajebe et al., 2014) and these primers allowed for the detection of low levels of parasites (Vu et al.,1995). The 18S rRNA gene was used as a target because it contains both highly conserved and variable genes for each of the *Plasmodium* species (Tajebe et al., 2014).

A PCR optimization process was performed to optimize the *vivax* primers which were performed using 3 samples, 2 of which were DNA positive control samples that were donated from Dr. Julian Rayner's laboratory at the Wellcome Trust Sanger Institute, UK and the other sample was the negative control which was double distilled water. Once the optimization process was complete, the PCR temperature protocol used was follows: DNA denaturation at 94°C for 5 minutes, 40 cycles of denaturation at 94°C for 30 seconds,

annealing at 56°C for 30 seconds and elongation at 72°C for 1 minute and thereafter the final elongation at 72°C for 10 minutes (Weppelmann et al. 2013).

A total of 204 DNA samples were run. All the PCR runs being dealt with included a positive control and a negative control which was double distilled water (Weppelmann et al., 2013). The expected size of the amplicon of the positive control was at 130 base pairs (Weppelmann et al. 2013). The total time for the PCR run was 1 hour 56 mins.

Once the PCR runs had been completed, 2% agarose gels were made in order to view the amplified bands at 70 volts for 50 minutes.

Table 2.5: *Vivax* Primer Sequences

Gene	**Primer Name**	**Primer Sequence**	**% GC content**	**Melting Temperature (°C)**
Vivax	Forward Reverse	gcaacgcttctagcttaatc acaaggacttccaagccgaagc	45 54.5	55.3 62.1

2.8 Ethical Clearance

Ethical clearance was obtained from the Kenya Medical Research Institute (KEMRI) ethics review board for systems immunology of malaria (SERU Protocol Number 3149). This study is covered by the approved larger study.

CHAPTER 3

RESULTS

3.1 PCR amplification of the DARC gene

In all the PCR reactions done, positive and negative controls were included. The amplification of the positive control confirms that the reagents are working well, whereas the negative control ensures that there is no contamination.

For DARC, the promoter region was amplified in 95 samples yielding an amplicon of 200 base pairs (Figure 3.1) All 95 samples used in this study were successfully amplified.

During the optimization, DNA bands at 200 base pairs were expected and this PCR was carried out with two DNA samples that were taken from the first set of samples of the 2001 batch as shown in the figure (3.1) below:

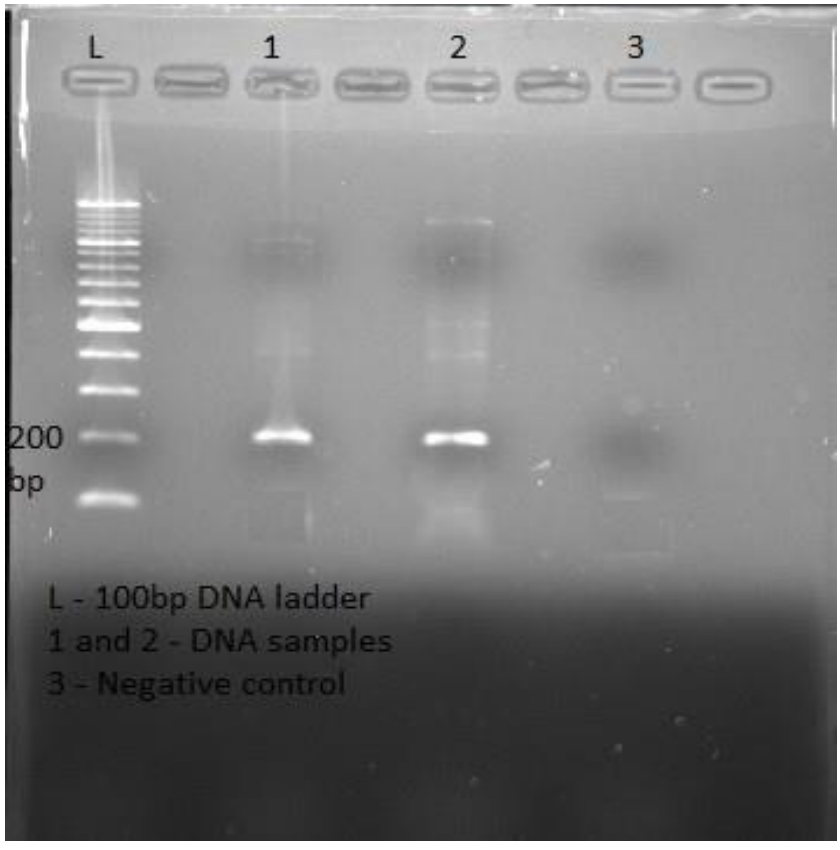

Figure 3.1: The first set of samples after a PCR optimization. The band was shown at 200 base pairs as expected. Samples 1 and 2 were the DNA samples used for optimization whereas sample 3 was a negative control. A clear lane 3 indicated that there was no contamination.

After optimization was successfully carried out, all the 95 samples were successfully amplified with the set PCR conditions (Figure 3.2). Samples in lane 2 and 7 in figure 3.2 did not have bands and hence they were repeated using the same protocol to obtain bands at 200bp. The only change added to the protocol was to increase the amount of DNA from 0.5µl to 0.75µl in order to get the bands required.

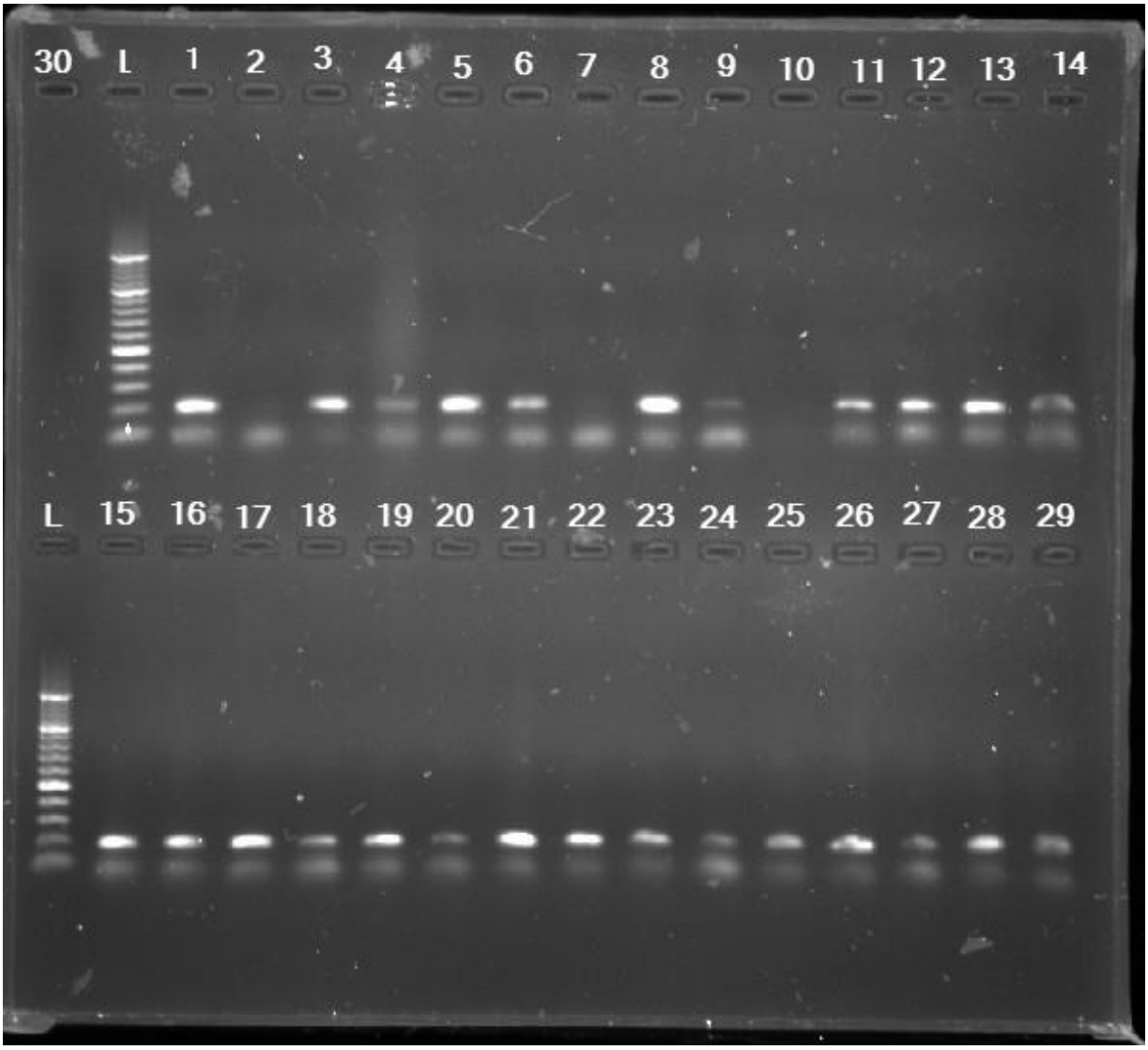

Figure 3.2: Gel image for DARC gene.
Bands in the lanes 1 to 30 represent the amplified products of the DARC gene. The lanes 10 and 30 are the negative controls. The bands in the lane marked L represents the Ladder.

3.2 Restriction fragment length polymorphism of DARC

Once the PCR for the DARC genes was completed and all the bands obtained. Restriction Fragment Length Polymorphism was carried out. The PCR amplicons were subjected to StyI restriction endonuclease digestion. StyI RFLP analysis revealed the polymorphism where the 77bp fragment corresponded with -46T and the 65bp corresponded with the -46C as shown in Figure 3.3.

There were two positive controls that were obtained from anonymous donors and a negative control that were used as the markers for the rest of the DNA samples. The positive control in lane A was Duffy antigen positive and the positive control in lane B was Duffy antigen negative. All the 95 DNA samples resembled the bands of the positive control in lane B and hence the DNA samples were all Duffy antigen negative as shown in Figure 3.3.

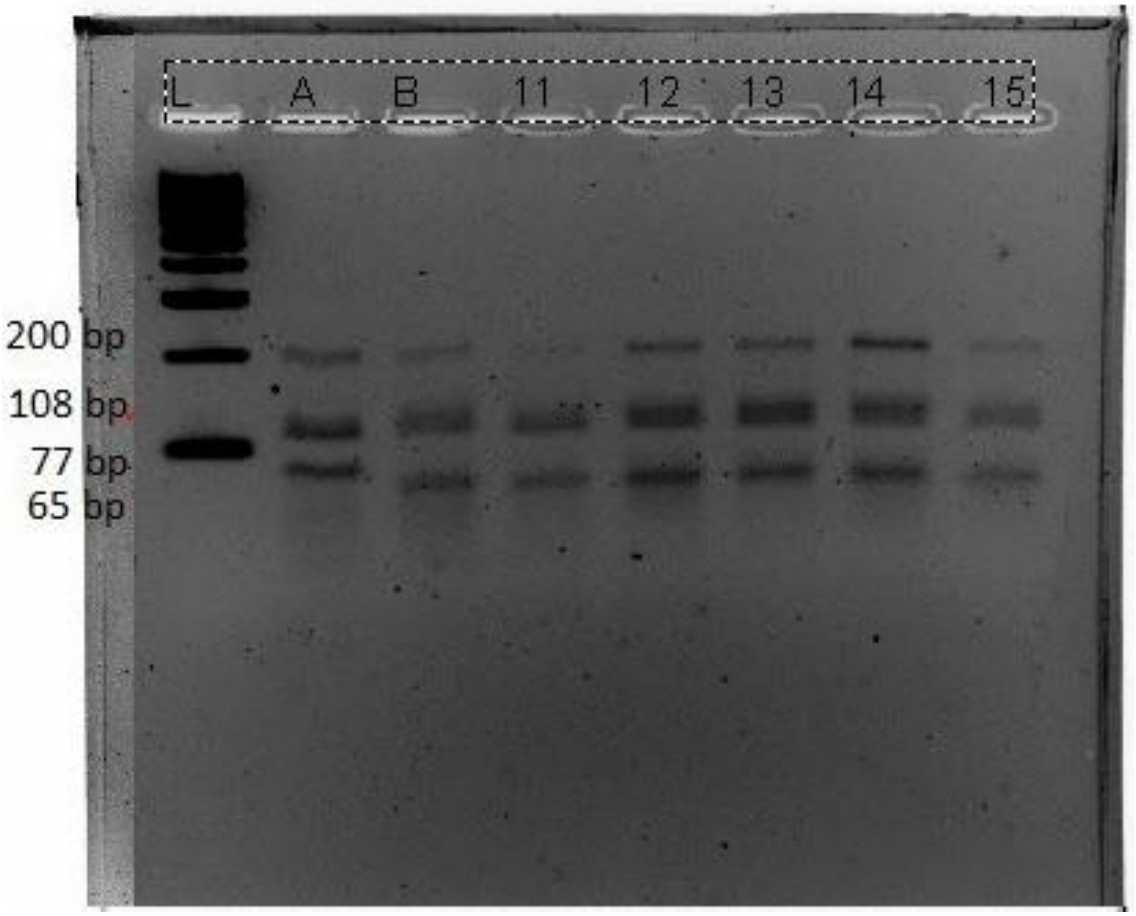

Figure 3.3: This figure shows the results from the StyI digest with the restriction fragments of 200bp, 108bp and 77bp for sample A which was Duffy antigen positive and sample B contained a 65bp band instead of the 77bp which was Duffy negative antigen. StyI RFLP analysis reveals polymorphism where the 77bp fragment corresponds with -46T and the 65bp corresponds with the -46C.

3.3 Detection of P.vivax by PCR

PCR runs with the *vivax* primers were carried out. The positive control showed a DNA band at 131bp which confirmed the *vivax* infection. The negative control used was double distilled water to identify any kind of contamination. This was the first set of DNA samples from the 2001 batch. The well lanes did not show the positive band of 131bp and hence the samples were found to be negative for *vivax* infections though there were smears in the lanes as shown in Figure 3.4. The smears were observed maybe because of the addition of too much DNA template hence the DNA template was reduced to half the volume to improve the appearance of the smears as seen in the Figure 3.4.

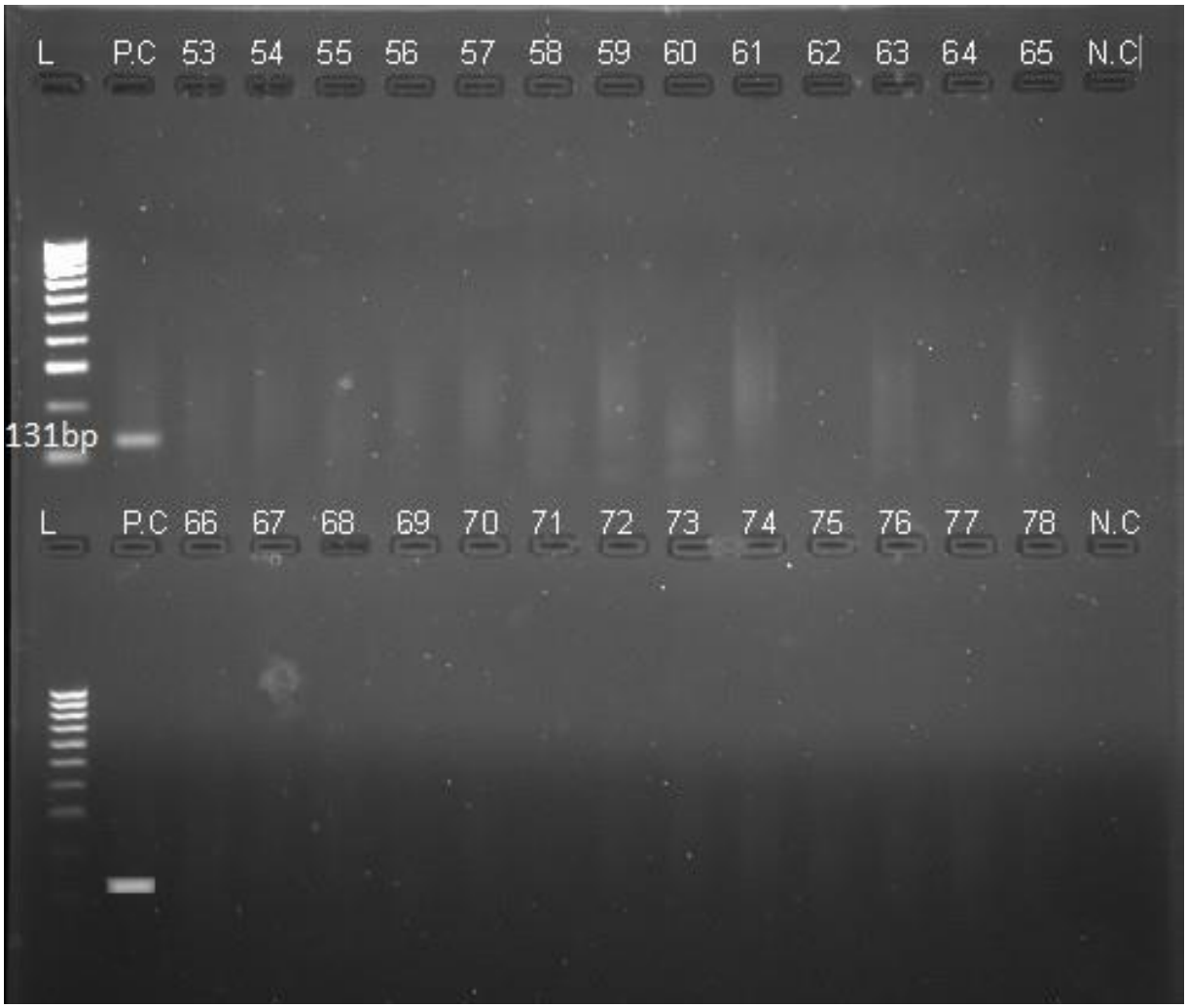

Figure 3.4: Gel image for 2001 sample set. The positive control was a *vivax* positive DNA sample that was set as a marker at 131bp. The negative sample used was double distilled water. The DNA samples did not show any bands at 131bp and hence they were found negative of *vivax* malaria.

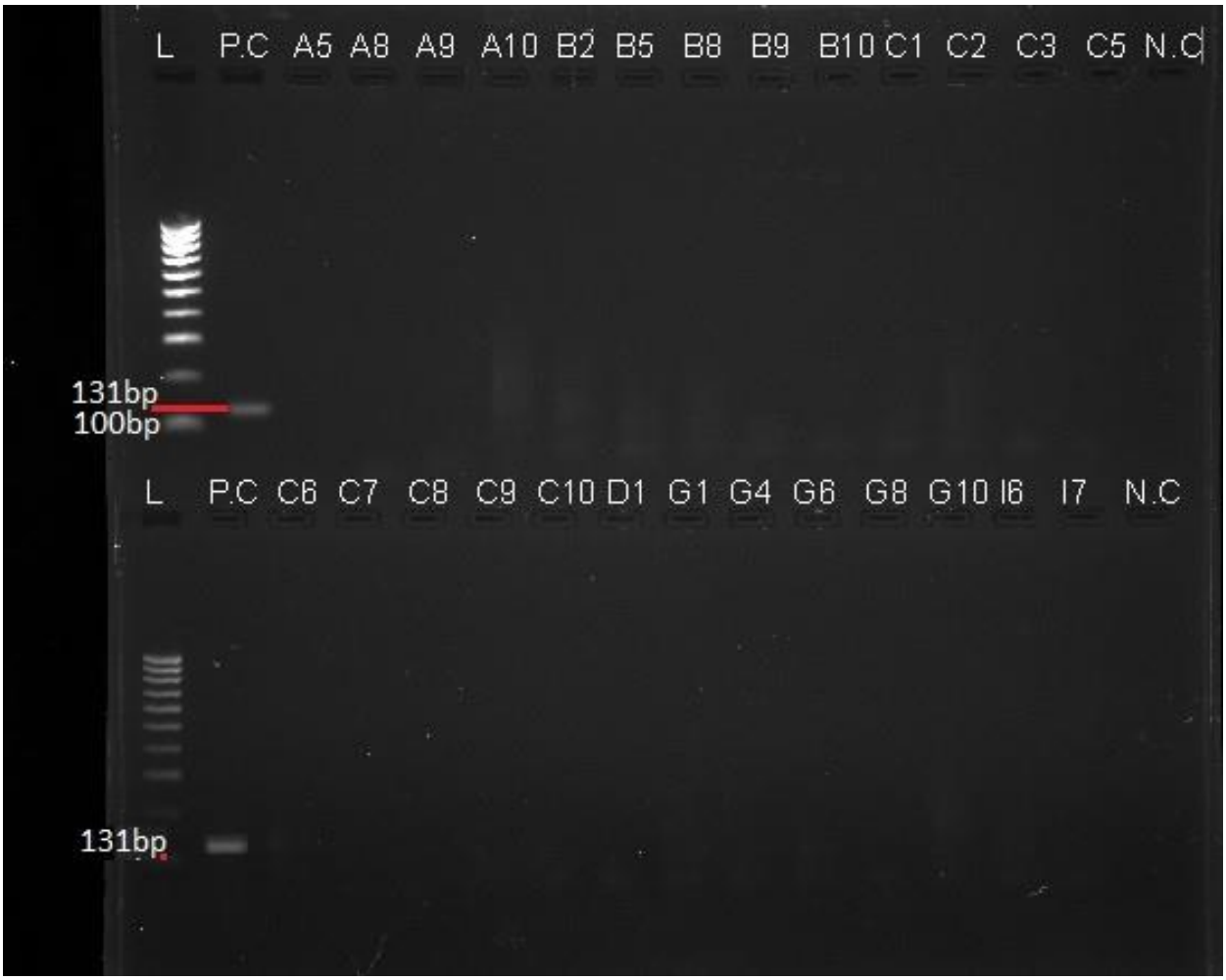

Figure 3.5: Gel image for 2013/2014 sample set. The positive control was a *vivax* positive DNA sample that was set as a marker at 131bp. The negative sample used was double distilled water and no bands in the lane indicated that there was no contamination. The DNA samples did not show any bands at 131bp and hence they were found negative for *vivax* malaria.

For Figure 3.5 the image of the results shown was pertaining to the second set of samples for the batch of 2013/2014 DNA samples. The positive control showed bands at 131bp which confirmed the *vivax* infection whereas the rest of the wells being empty revealed that there was no *vivax* infection.

CHAPTER 4

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

The main aim of this study was to genotype the DARC gene and to confirm the frequency of the FY^{ES} allele in the county of Kilifi which is a malaria endemic region. The Duffy antigen receptor for chemokines is the major receptor that is required to interact with *P.vivax* in order for the parasite to invade the human erythrocytes, thus causing infection (Chitnis & Sharma 2008).

The malaria population from the 2001 batch that was sampled using PCR-RFLP was Duffy negative and their DARC promoter sequence contained the C mutation at position -46. This mutation disrupts the expression of the Duffy antigen. DARC is silenced as a result of a SNP in the GATA box sequence found in the DARC promoter region where a cytosine (C) replaced a thymine (T) at the 46 nucleotides before the erythroid capsite (Weppelmann et al. 2013).

The PCR-RFLP differentiated the 200bp band obtained for the DARC PCR into the following mutations: The T mutation at the 46^{th} position on the promoter region resulted in the 200bp, 108bp and 77bp which signified that the individual expressed the Duffy antigen and the C mutation at the 46^{th} position on the promoter region resulted in the 200bp, 108bp and 65bp which signified that the individual did not express the Duffy antigen and hence the expression of the Duffy antigen was silenced. The positive and negative controls allowed for the differentiation of the 77bp and 65bp bands to determine whether the DNA samples being tested followed the same banding patterns. Sequencing was not a necessary procedure in this study since all the samples from 2001 had already been sequenced in a previous study therefore it was not repeated in this study. However, PCR-RFLP correctly confirmed the sequencing results and either method can be used to detect the Duffy positive or Duffy negative genotype. Since PCR-RFLP is a cheaper method than sequencing, this can be rapidly employed in a field setting in sub-Saharan Africa.

It was also important to ensure there were no *P.vivax* infections in this sample set from 2001. From a study done by Ryan et al.,(2006) there was evidence of *P.vivax* among a Duffy antigen negative population in Western Kenya but since *P.ovale* and *P.vivax*

closely resemble each other microscopically, expert microscopists find it very difficult to differentiate between them (Ryan et al. 2006). The paradox in this study was that *P. vivax* seemed to be transmitted among people who were Duffy negative and had a possible explanation that the parasite was not *P. vivax* because it required Duffy antigen phenotype for invasion, or that the parasite was *P. vivax* that had evolved to use receptors other than the Duffy antigen, or that the study population had a unique mechanism that served in *P. vivax* invasion (Ryan et al. 2006).

In addition to these samples, a contemporary sample set from 2013/2014 was obtained from Kilifi County Hospital that was added to confirm whether the *P. vivax* prevalence may have changed over time.

Only the positive control used showed a band at 131bp and all the samples from 2013/2014 were negative for *P.vivax*, which indicates that even in a more recent sample set there are no *vivax* infections in the Kilifi population that was tested. From the above discussion, the findings in this study indicate that the samples tested are all Duffy negative and hence all the individuals tested do not express the Duffy antigen as expected therefore there are no *vivax* infections in the region of study.

4.2 Conclusion

In conclusion, the results of the DARC-RFLP analysis clearly show the dominance of the Fy (a-b-) allele which is expressed at a high frequency in the Kilifi population sampled, due to the -46C mutation which switches off DARC expression.

Since *P. vivax* malaria is also not prevalent in this region, the risk of infection to travelers or people who are Duffy positive is low.

4.3 Recommendation

Since there is no *P. vivax* malaria in the Kilifi population sampled therefore it can be assumed that there is no risk of this infection in the region. *P. vivax* infections are absent due to the large majority of the population expressing the Duffy antigen negative phenotype. Since DARC is the major receptor for the *P. vivax* invasion ligand (Duffy Binding Protein), the Duffy negative phenotype prevents infection. Therefore, the main

concern for malaria in Kilifi it appears would be *P. falciparum*, *P. ovale* and *P. malariae* species, since as expected no evidence of *P. vivax* has been found.

The technique of PCR-RFLP has proven to be efficient and useful rather than sequencing because it reduces the cost of screening for *P. vivax* in malaria endemic areas in sub-Saharan Africa.

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