# DEVELOPMENT OF A DLC GENE BASED ASSAY FOR DETECTION OF SELECTED *PLASMODIUM* SPECIES.

**KARIUKI MAUREEN WANJA**

**MASTER OF SCIENCE**

**(Molecular Biology and Biotechnology)**

**PAN AFRICAN UNIVERSITY**
**INSTITUTE FOR BASIC SCIENCES, TECHNOLOGY AND INNOVATION**

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# DEVELOPMENT OF A DLC GENE BASED ASSAY FOR DETECTION OF SELECTED *PLASMODIUM* SPECIES.

**KARIUKI MAUREEN WANJA**

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## DECLARATION

This thesis is my original work and has not been submitted to any other university for examination.

_____ _____

Kariuki Maureen Wanja

This thesis has been submitted for examination with our approval as supervisors.

_____ _____

Prof Esther Magiri

Jomo Kenyatta University of Agriculture and Technology

Department of Biochemistry

_____

Dr. Kem Githui

National Museums of Kenya

Department of Molecular Genetic

# DEDICATION

To my father

mother

and

siblings.

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# TABLE OF CONTENTS

# LIST OF TABLES

**Table** **Page**

# LIST OF FIGURES

**Figure** **Page**

# LIST OF APPENDICES

# ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| RDK | Rapid diagnostic kits |
| RDT | Rapid diagnostic tests |
| RBC | Red blood cells |
| PCR | Polymerase chain reaction |
| HRP2 | histidine-rich protein 2 |
| Pldh | parasite specific lactate dehydrogenase |
| ACT | Artemisinin-based combination therapy |
| DLC | dynein light chains |
| WHO | World Health Organization |
| DNA | deoxyribonucleic acid |
| QBC | Quantitative buffy coat technique |
| HC | Heavy chains |
| LIC | Light intermediate chains |
| LC | Light chains |
| IC | Intermediate chains |
| TNF | Tumor necrosis factor |

# ABSTRACT

Malaria is a disease caused by *Plasmodium*a protozoa parasite in the phylum *Apicomplexa*. The disease has a profound impact on public health, economyand social structures of many developing countries. To ensure patients receive appropriate treatment of malaria, accurate differentiation of the *Plasmodium* species is key. There are various techniques applied for the diagnosis of the disease among them polymerase chain reaction method. This technique has proved to be the most sensitive and specific compared to microscopy. PCR can detect low parasitaemia at levels undetectable by microscopy, it can also differentiate among *Plasmodium* species and it is a valuable tool for epidemiological diagnosis. Many laboratories are currently developing more specific PCR using alternative target genes to improve on species identification. Based on an *In silico* study, the gene sequence of TcTex 1 gene was found to be conserved within the *Plasmodium* species. In this study, PCR primers were applied with the aim ofdetecting *P. falciparum,* the most common malaria causing parasite in Kenya and *P. knowlesi*, *P. cynomolgi*species responsible for the emerging zoonotic transmissions in humans. The study entailed the designing of diagnostic genus and species-specific primer sets within the TcTex1 dlc gene. The diagnostic primers weretested for specificity on the different*Plasmodium*species and population samples usingstandardPCR.The findings demonstrate that PCR targeting *Plasmodium* dlcTctex1 sub-unit gene can be used for *P. falciparum* species identification. The results also indicate that there could be probable *P. knowlesi* infections in the coastal region of Kenya, a malaria endemic area. A bioinformatics approach that entailed Artemis software analysis was used to

determine the targetgene chromosomal location, organization and copy number. The data generated showed that the gene has acoding region and occurs as a single copy in *P. knowlesi* chromosome 9 and *P. falciparum* chromosome 11. The data presented in this study shows that TcTex 1 dlc gene can be used as an alternative malaria diagnostic target.

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

Malaria is an ancient disease and upto date it is stil a clinical problem in many countries in the world.The disease is caused by a protozoan parasite of the genus *Plasmodium* and is spread to humans through the bites of infected *Anopheles* mosquitoes. The common malaria causing parasites in humans are, *Plasmodium falciparum*, *Plasmodum vivax*,*Plasmodium malariae* and *Plasmodium ovale.* However, there have been reports of *Plasmodium knowlesi*(Lucci *et al*., 2012)and *Plasmodium cynomolgi*, zoonotic malaria transmissions to humans (Coatney *et al*., 1961, Schmidt *et al*., 1961, Eyles *et al*., 1960).

The disease is seen to occur mostly in tropical and sub-tropical parts of the world. This is due to a number of factors present in those regions. These factors are such as, the weather conditions that favor the transmission of the disease, scarce resources and social- economic instability that hinder efficient malaria control activities ( CDC,2014). The disease affects both the social and economic aspects of a country.It is responsible for employee absenteeism, increased health care spending and decreased productivity, all of which impact negatively.

The disease had at one timebeen reported to be the leading parasitic cause of morbidity and mortality worldwide (Snow *et al*., 2005). In theWHO 2012 report, there were anestimated 207 million malaria cases reported globally, an estimated 627,000 deaths and most of the deaths had occurred insub-Saharan Africa. In order to reduce the morbidity and mortality cases, various measures have been put in place among them, accurate diagnosis.

Accurate diagnosis of malaria is important in ensuring patients receive appropriate treatment of the identified parasite and reduce morbidity cases. It is also key in ensuringthat health facility data can be relied upon as an effective source of information for evaluating malaria intervention programs (Yaw *et al*., 2013). However, the reliability of the hospital-based data for true malaria incidence is often questioned because of diagnosis accuracy issues. For example, the use of health facility data was challenged by Rowe *et al*., (2006) who reported that some of the clinical data that were obtained from some countries in Sub-saharan Africa, were inadequate to be used as a basis for developing estimates and measuring the impact of malaria interventions. This highlights the need for accurate malaria diagnosis in health centers which relies on sensitive and specific diagnostic techniques.

The current gold standard for malaria diagnosis is microscopy, however, it has been found to have relatively low sensitivity. The technique relies on the laboratory technician's eye sight,and as a result accurate species identificationbecomes difficult especially between species that share morphological similarities ( Singh *et al*., 2004). Rapid diagnostic test (RDT) is another commonly used malaria dignostic technique in most health facilities. However, there have been false positive reports from the

RDTs(Rock *et al*., 1987).Another shortcoming of RDTs, is that they have to be used in conjunction with other methods to characterize the infection and monitor treatment.

Polymerase chain reaction (PCR) method is another malaria dignostic technique, however this method has been found to be more sensitive and specific than conventional microscopic examination (Brown *et al*., 1992, Morassin *et al*., 2002). PCR is also a valuable tool for epidemiological studies and can reliably be used for monitoring drug effectiveness or emergence of drug resistance in parasite populations unlike the RDTs. Moreso, it has been found to be more sensitive than some RDTs ( Makler *et al*., 1998, Rakotonirina *et al*., 2008).

PCR is a molecular method that relies on the amplification of a specific sequence. Various genes suchas 18S rRNA, cytochrome b oxidase, circumsporozoite protein, tubulin(Singh *et al*., 2004,Dinko *et al*., 2010)have been used as targets for detection of the *Plasmodium* species. However, short comings in species identification when targeting some of the genes have been reported. For example PCR probing 18S rRNA in *P. knowlesi* identification was found to cross-react with *P. vivax* species ( Lucci *et al*., 2012). Therefore,in an effort to develop alternative target genes to improve on the species identification, some laboratories are using*In silico*analysis of gene databank to identify suitable DNA sequences that are highly specific to a given species of the malaria parasite.

An example is Lucci *et al*., 2012, where a bioinformatics approach was used to search the malaria parasite genome database for suitable DNA sequences relevant for molecular diagnostic test. Multi-copy DNA sequences distributed in the

*P. Knowlesi* genome were identified and several novel primers specific tothe target sequences were designed. The primers were then tested in a non-nested PCR assay and one of the primer sets was found to accurately detect *P. knowlesi*. This study applied the same approach with the aim of detecting *P. falciparum*, *P. knowlesi* and *P. cynomolgi* based on a novel dynein light chain (dlc) TcTex1 sub-unit gene.TcTex1 gene is a subunit gene of the cytoplasmic 1 dynein, and from an *In silico* study done, its sequence was found to be conserved within the *Plasmodium* species. The gene sequence can therefore be used as a target for detection of *Plasmodium* species.

## 1.2 Statement of the problem

The current universal gold standard for diagnosis of malaria is microscopy. However, distinction between the *Plasmodium* species becomes difficult when using microscopy. For example, *P. knowlesi* exhibits stage-dependent morphological similarities to *P. malariae* and *P. falciparum* (Singh *et al*., 2004, Lee *et al*., 2009). These similarities have contributed to misdiagnosis of *P. knowlesi* as *P. malariae* or *P. falciparum*(Singh *et al*., 2004).*P. cynomolgi* is also many of the times misdiagnosed as *P. vivax* due to the morphological similarities of the asexual stages shared by the two species (Coartney *et al*., 1961). In these cases, molecular diagnostic methods are the most accurate way to distinguish betweenthe species (Contacos *et al*., 1963, Chin *et al*., 1965, Coatney *et al*., 1961, Kantele and Jokiranta 2011).

PCR is a molecular method that has been found to be a more sensitive method than microscopy and canaccurately differentiate among species. Different laboratories have applied various PCR methods detecting genes such as 18S rRNA, cytochrome b oxidase, circumsporozoite protein, tubulin (Singh *et al*., 2004, Dinko *et al*., 2010, Lucci *et al*., 2012). However, some studies have identified shortcomings in some of the genes targeted for malaria diagnosis. For example difficulty in *P. knowlesi* diagnosis when probing the 18S ribosomal RNA has been noted (Sulistyaningsih*et al*., 2010, Lucci *et al*., 2012). There is therefore a need for alternative PCR targets to improve on the species identification.

## 1.3 Justification

In an effort to improve on species identification, various laboratories are identifying alternative sequence targets using a bioinformatics approach. This study applied a similar approach and focused on detecting *P. falciparum, P. Knowlesi* and *P. cynomolgi* species based on a novel gene, dlcTctex1 sub-unit gene.

*P. falciparum* has been reported to be the causal of many deaths globally and it is the most common malaria causing parasite in Kenya.The WHO 2010 report, indicated there were 6 million malaria cases and 26,017 deaths caused by *P. falciparum* in Kenya. This species is therefore a great public health concern in the country and an assay that will aid its accurate identification is important.

Recent studies have also reported emerging zoonotic transmissions in humans caused by *P. Knowlesi* and *P. cynomolgi.* These infections should not be ignored especially with the increasing interactions between man and wild animals in the process of urbanization. New diagnostic methods are hence necessary to distinguish between the human and monkey malaria species.

## 1.4 Objectives

### General objective

To detect *P. falciparum,P. knowlesi* and *P. cynomolgi* species using standard PCR based on the amplification of TcTex 1 dlc gene in view of improving malaria diagnosis.

### Specific objectives

i. To validate the effectiveness of the novel diagnostic TcTex1 dlc gene species-specific primers in detection of *P. falciparum, P. knowlesi* and *P. cynomolgi* species in culture and laboratory animal isolates and also in human blood samples using standard PCR.

ii. To confirm the region amplified by the novel diagnostic TcTex1 dlc gene species-specific primers through sequencing.

iii. To determine the location and organization of the TcTex1 dlc gene in the chromosome using Artemis software.

## 1.5 Significance of the study

This study is of great importance since it is geared towards addressing the challenge of misdiagnosis faced in many countries by offering an additional diagnostic target for malaria. The study will aid in ensuring proper diagnosis of *P. knowlesi*, *P. Falciparum* and *P. cynomolgi* species .*P. knowlesi* and *P. cynomolgi* are emerging zoonotic diseases that can lead to fatal cases and hence an assay that will aid in their identification is of huge public health importance.

Due to misdiagnosis cases reported in health facilitiesthere is a possibility of undiagnosed or misdiagnosed *P. cynomolgi/ P. knowlesi* cases in the population, therefore it is necessary to determine if there are any cases of un-reported *P.knowlesi/ P.cynomolgi* infections in the country.

Accurate specification of the *Plasmodium* species will also ensure patients receive appropriate treatment of the identified parasite hence better care to patients.This will also facilitate the relibility of health facility data for monitoring trends in malaria morbidity and for evaluating impacts of malaria interventions. Accurate malaria diagnosis will also reduce the number of annual employee absenteeismreports, it will save on resources spent during health care and increase the country's productivity.

The findings will also contribute to addition of scientific knowledge regarding TcTex 1 sub-unit gene as a malaria diagnostic target and offer a promising alternative to the current malaria targets available.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 History and discovery of malaria

Various studies done have supported the existence of malaria almost back to the beginning of human kind. Studies done on Egyptian mummies that were dated 3000 years old revealed the presence of enlarged spleen presumably due to malaria (Sherman, 1998). Malaria antigen has also been detected from the skin and lung samples of mummies from 3200 and 1304 BC (Miller *et al*., 1994). The disease probably originated from Africa and accompanied the human migration to the mediterranean shores, India and south East Asia(Davis *et al*., 2004) and possibly spread to Southern Europe via Nile valley. Malaria became world wide by 18th century (Sherman, 1998).

The first official report on *Plasmodium* parasites was made by Alphonse Laveran a french military doctor and this was published a note at a meeting of the Académie de Médecine in Paris, describing "New Parasite Found in the Blood of Several Patients Suffering from Marsh Fever in 1880." Previous theories had argued that malaria was caused by bad air (Italian mala=bad aria=air) however, based on Pasteurs findings who had earlier reported that most infectious diseases are caused by microbes, Alphonse was convinced that malaria was caused by another causative agent and not bad air. He discovered the presence of granules of black pigment in the blood which

seemed to occur at very different frequencies depending on the cases (Bruce, 1981). He then concluded that the pigmented granules were specific to malaria and originated in the blood.

On 6th of November 1880, Alphonse examined the blood of a patient that had been ill for 15 days and during his observations he saw "on the edges of a pigmented spherical body, displacing the neighboring red blood cells (RBC). The motility of these elements immediately convinced him that he had discovered the agent causing malaria and that it was a protozoan parasite. Laveran made drawings of what would be the first picture of the malaria causing parasite *Plasmodium* (figure 2.1). In 1890, it was concluded that a protozoan parasite was the causative agent for malaria.

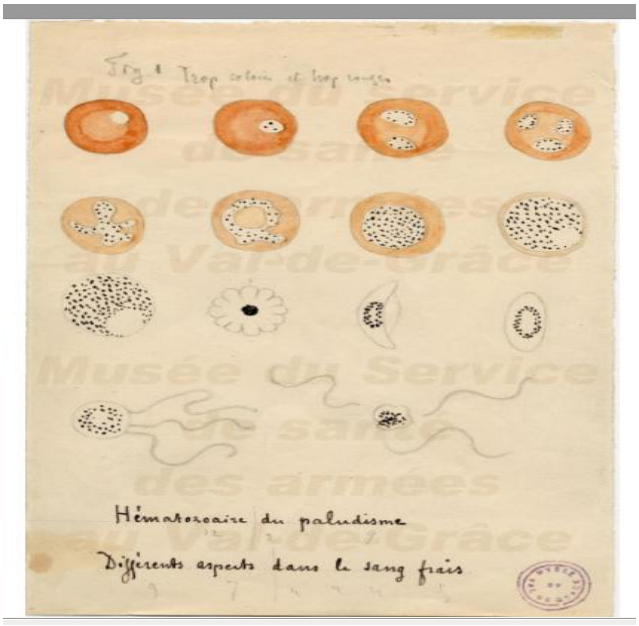


Figure 2.1:Drawings of the different stages of *Plasmodiumfalciparum*as observed by Laveran( http://www.cd.gov).

Three species with specific periodicities and other characteristics were reported to be responsible for benign tertian (Haemamoeba vivax), malignant tertian (Laverania malariae) and quartan (Haemamoeba malariae) malaria now respectively known as *P.vivax,P. falciparum*and *P. malariae*. Grassi in his monograph, *Studi di uno Zoologo SullaMalaria* (Grassi, 1900) summarized the situation as it was in 1900, the work formed a basis for the studies that followed later. In 1918, while working in West Africa, John Stephen discovered a fourth species which resembled *P. vivax* buthe described it as *P. ovale*in 1922(Stephens, 1922). Recent studies done in Southeast Asia have showed zoonotic transmission of a *Plasmodium* species; *P. knowlesi* to humans (Lucci *et al*., 2012).

## 2.2 Global impact of malaria

Malaria disease has been reported to be the leading parasitic cause of morbidity and mortality worldwide (Snow *et al*., 2005). In 2009, it was reported that 3.28 billion people were living in areas that had some risk of malaria transmission and about 1.2 billion people were in areas with a high risk of transmission.In2012, there were an estimated 627,000 malaria deaths worldwide and of the estimated deaths,most (90%) occurred in sub-Saharan Africa (WHO 2013).

Malaria has been shown most to occur mostly in tropical and subtropical areas of the world as shown in figure 2.2.Africa is shown to be the most affected due to a combination of factors. These factors include, the presence of the mosquito *Anopheles gambiae complex* which is responsible for high transmission. It is also the vector for the parasite *P. falciparum*, which is responsible for high mortality cases.

Local weather conditions in Africa also favor the transmission of the disease. Another contributing factor is scarce resources and socio-economic instability that hinder efficient malaria control activities(CDC 2014).

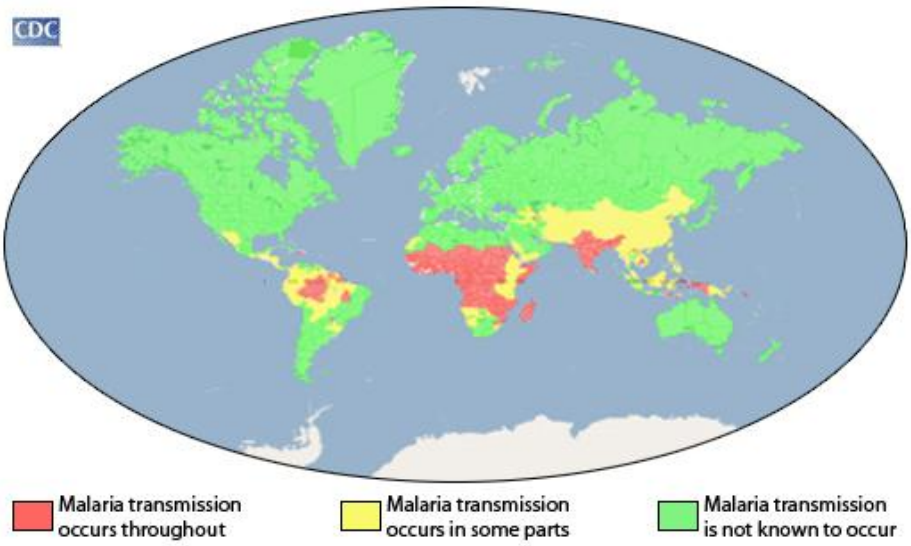


Figure 2.2; A map showing an approximation of the parts of the world where malaria transmission occurs( CDC, 2014)

In Kenya, the epidemiology of the disease varies geographically and four malaria epidemiological zones have been identified as illustrated in figure 2.3(Kenya Malaria Indicator Survey Division,(2007). These zones include, the western highlands of Kenya where malaria transmission is seasonal, with considerable year to-year variation.The increase in the minimum temperatures during the long rains favours and sustains vector breeding, resulting inincreased intensity of malaria transmission.

The other zone consist of the endemic areas in the Coastal region and around Lake victoria. These areashave stable malaria transmission that occur throughoutthe year.

Another epidemiological zone in Kenya, comprises of the arid and semi-arid areas of northern and south-eastern parts of the country. These areas experience short periods of intense malaria transmission during the rainy seasons. The extreme climatic conditions like the El Niño southern oscillation lead to flooding in these areas, resulting in epidemic outbreaks with high morbidity rates because of the low immune status of the population.

The low risk malaria areas in Kenya, covers the central highlands of Kenya including Nairobi. These regions experience temperatures that are usually too low to allow completion of the sporogonic cycle of the malaria parasite in the vector. However, higher temperatures and changes in the hydrological cycle associated with climate change tend to increase the areas suitable for malaria vector breeding that contribute to malaria transmissions in these regions.

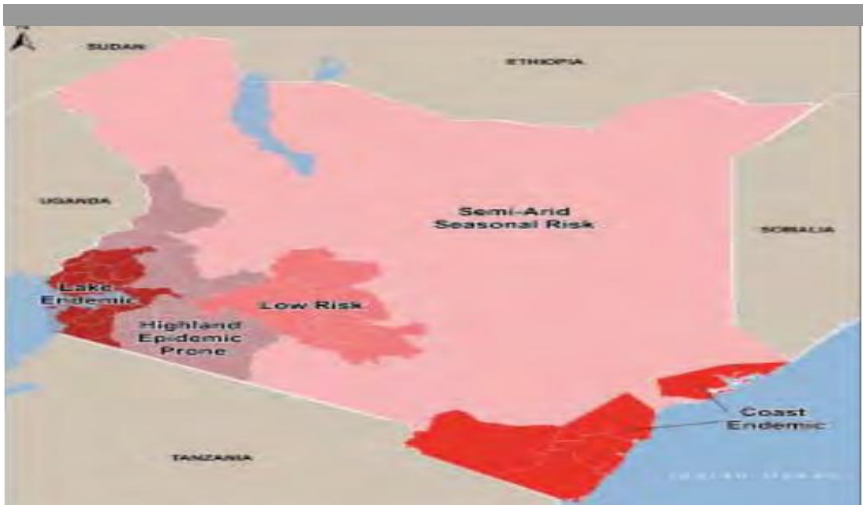


Figure 2.3; A map showing mlaria risk zone areas in Kenya.

(Noor *et al*., 2009).

Malariaaffects both the social and economic aspects of a country. It is estimated that the total cost burden due to malaria in Sub-Saharan Africa increased from US dollars 1.8 billion in 1995 to US dollars 2 billion in 1997 (WHO, 1997). Recent estimates have placed the economic losses in Sub-Saharan Africa to over US dollars 12 billion annually (WHO, 2010). It is responsible for employee absenteeism, increased health care spending, and decreased productivity, all of which impact negatively.

A 2011 Roll Back Malaria report found that in sub-Saharan Africa, 72% of companies reported a negative malaria impact. Overall, households in Africa lose up to 25% of income to the disease( The Abuja Declaration and the Plan ofAction, 2006), this is contributed by the cost of purchase of drugs, expenses for

travel and treatment at clinics and in case of death, expenses for burial. Malaria can strain national economy, impacting some nations' gross domestic product by as much as an estimated 5–6% (World Economic Forum,Global Health Initiative, 2006).The ecomomic impact of the disease in Kenya is as summarized in table 2.1.

Table 2.1: Economic impact of malaria.

(Republic of Kenya, 2001)

| | |
|---|---|
| **Population at risk** | **77%(25 million out of 34 million)** |
| **Per all outpatient visits in public health institutions** | **30-50%** |
| **Per hospital admissions in public health institutions** | **19%** |
| **Estimated working days lost annually** | **170 million** |

## 2.3 Malaria parasite transmission and lifecycle

Malaria is caused by a protozoan parasite from the genus *Plasmodium* and is spread to people through the bites of infected female *Anopheles* mosquitoes, which act as the vectors. Of the approximately 430 *Anopheles* species, only 30-40 transmit malaria. *An.gambiae, An. Arabiensis, An. Funestus* are the main vectors responsible for malaria transmission in Africa whereas *An. Albimanus, An. Culicifacies,*

*An.dirus, An.anthropophagus* are the main vectors responsible for malaria transmission in the rest of the world. The vectors greatly differ in their ability and efficiency to transmit malaria because of their difference in their habitat, feeding habit and lifecycle. They bite mainly between dusk and dawn.

*P. falciparum, P .vivax, P. malariae, P. ovale* are the common parasites that cause malaria in humans, however, it is note worthy that non-falciparum *Plasmodium* species malaria burden is also increasing (Nosten *et al*.,2000, Gething *et al*., 2012). In Kenya, *P. falciparum* and *P. vivax* are the most common malaria causing *Plasmodium spp*(Malaria Fact sheet N°94 2013).

The life cycle of the *Plasmodium* parasite in a human, begins when an infected mosquito transmits malaria sporozoites to a vertebrate host. The sporozoites are transported to the liver, where they invade hepatocytes and multiply thousands of times and mature into schizonts. The cells rupture and release merozoites which infect other red blood cells. The RBC invasion process of the merozoite is as described in figure 2.4. *P. vivax* and *P. ovale* have a dormant stage termed hypnozoites, which can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.

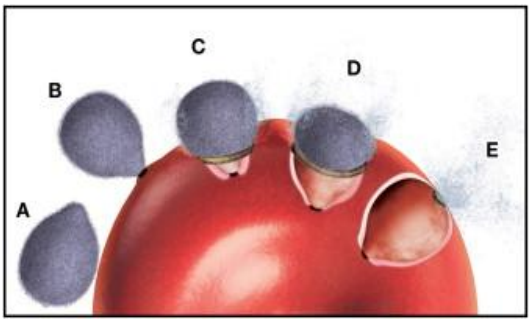


Figure 2.4; Merozoite Invasion of Erythrocytes

(www.cell.com)

(A); An initial “long-distance” recognition of surface receptors followed by a reorientation process whereby the low-affinity contacts are maintained.

(B); Once the apical end is adjacent to the erythrocyte, a tight junction is formed involving high-affinity ligand receptor interactions.

(C and D); The tight junction moves from the apical to posterior pole powered by the parasite's actin-myosin motor and the surface coat is shed at the moving junction by a serine protease, or “sheddase.”

(E); Upon reaching the posterior pole, the adhesive proteins at the junction are proteolytically removed by a resident protease in a process that facilitates resealing of the membrane.

By this process, the parasite does not actually penetrate the membrane but invades in a manner that creates a parasitophorous vacuole. The merozoite also displays a form of phenotypic variation in which different strains express a variant combination of functional ligands that bind to specific receptors on the erythrocyte

(Duraisingh *et al*., 2003). This provides a mechanism to escape host immune detection and to counteract the polymorphic nature of the erythrocyte surface.

During the first 48 hours after infecting a red blood cell, the parasite goes through several phases of development. The first phase is the ring stage, in which the parasite begins to metabolize hemoglobin. This phase is followed by the trophozoite stage, during which the parasite metabolizes most of the hemoglobin, gets larger, and prepares to reproduce more parasites. The parasite then divides asexually to form a multinucleated schizont, which rupture releasing merozoites.

Some of the parasites differentiate into sexual erythrocytic stages (gametocytes) which are taken up by the anopheles. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal. The parasites' multiplication in the mosquito is known as the sporogonic cycle. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes . The zygotes in turn become motile and elongated (ookinetes) which invade the midgut wall of the mosquito where they develop into oocysts . The oocysts grow, rupture, and release sporozoites , which move to the mosquito's salivary glands and are then injected into a new human host and the malaria life cycle begins(http://www.dpd.cdc.gov).

## 2.4 Malaria pathophysiology

The earliest symptoms of malaria are very non-specific and variable, and include headache, fever, weakness, chills, myalgia, dizziness, abdominal pain, diarrhea, nausea, vomiting, anorexia, and pruritus (Looareesuwan, 1999). Cyclic fevers are the hallmark of malaria. The fever occurs at the time of red blood cell schizonts rupture or release merozoites.

It is believed that the glycosyl phosphatidyl inositol anchor that connects parasite proteins to the parasite or red blood cell surface, is exposed at the time of merozoite release and stimulates the production and release of TNF- alpha by macrophages (Kwiatkowski *et al*., 1989, Schofield and Hackett 1993), TNF- alpha then produces the fever associated with synchronous parasite release at the end of the asexual cycle (Kwiatkowski *et al*., 1989). An adult can harbor about $10^8$ malaria parasites in the body (20-20,000 parasites/µl) before the fever begins. However, in endemic areas people with partial immunity usually tolerate up to 10,000µl or $10^7$ paraites per ml of blood without feeling ill (Armstrong *et al*., 1994, Smith *et al*., 1994).

The pathophysiology of the different *Plasmodium* species varies. Cytoadhearance and sequestration are central *P. falciparum* infection and occurs mainly in the venules of vital organs including the brain, heart, kidney and intestine (Miller *et al*., 1994,White, 1998).As the *P. falciparum* parasites mature, knobs appear on the surface of the parasitized red cells and this facilitates the cytoadhearance of *P. falciparum*-parasitized red cells to endothelial cells in capillaries and post capillary venules of the vital organs and thus responsible for vital organs failure.

*P. ovale* and *P. vivax* are clinically and morphologically very similar. They infect only reticulocytes which constitute only 1% of normal RBCs in the blood and the magnitude of the parasitemia they produce is often low (Neva, 1977, Galinski, 1992).But *P. vivax* or*P. ovale* parasitized red blood cells have no knobs and they don't cause microvascular complications (White, 1998). *P. knowlesi* has the shortest life cycle known for *Plasmodium*species and this can lead to high parasitaemia and possible fatality(Cox-Singh *et al*., 2008) while *P. cynomolgi*presents hyponozoites which can initiate relapses just as the case in *P. vivax* (Thuy *et al*., 2014).

## 2.5 Approaches used in malaria diagnosis

Various measures have been put in place in order to reduce malaria morbidity and mortality cases and one of the measures is accurate diagnosis of malaria. Discussed below are some of the techniques used in malaria diagnosis.

### 2.5.1 Clinical diagnosis

This method is based on the patient's signs and symptoms. It is a challenging method of diagnosing malaria because of the non-specific nature of the signs and symptoms that tend to overlap considerably with other common, as well as potentially life-threatening diseases. This impairs diagnostic specificity, which can promote the indiscriminate use of antimalarials and compromise the quality of care for patients in endemic areas(Mwangi *et al*, 2005, Reyburn *et al*., 2004, Mcmorrow *et al*., 2008).

However, the accuracy of malaria diagnosis can be enhanced by combining clinical- and parasite-based findings (Kyabayinze*et al*., 2008).

### 2.5.2 Laboratory diagnosis

In the laboratory, malaria is diagnosed using different techniques, some of these techniques include;

#### 2.5.2.1 Microscopy

Microscopic examination of blood films is the current universal gold standard for malaria diagnosis. The blood sample is obtained by pricking a finger because the density of developed trophozoites or schizonts is greater in blood from this capillary-rich area (Gilles *et al*., 1993).The technique involves preparation of thick and thin blood smears. The thick blood film concentrates the layers of red blood cells on a small surface by a factor of 20 to 30 and is stained as an unfixed preparation using Field's stain or diluted Wright's or Giemsa stain. However, due to lysis of RBCs during staining; it makes identification of the species difficult. The thin blood film is methanol fixed and stained with diluted Giemsa stain using buffered water at pH 7.2 to emphasize the parasite inclusions in the RBC.

The identification of the parasite highly relies on the eye-sight and experience of the technician this results in varying results from different technicians viewing the same sample.Another shortcoming of microscopic examination is its relatively low sensitivity, particularly at low parasite levels and this relies on the the experience of

the technician. An experienced microscopist can detect up to 5 parasites/µl while the average microscopist detects only 50-100 parasites/µl (Payne, 1988).

Species distinction is also difficult, for example *P. knowlesi* exhibits stage-dependent morphological similarities to *P. malariae* and *P. falciparum* (Singh *et al*., 2004, Lee *et al*., 2009). These similarities have contributed to misdiagnosis of *P. knowlesi* as *P.malariae* (Singh *et al*., 2004, Cox *et al*., 2008) or *P. falciparum*. Many parasites may also remain sequestered in the capillary beds and are released into circulation but in insufficient numbers to be detected by peripheral-blood microscopy. In cases where there are mixed infections in a patient this infections tend to be under-reported since the identification of the species via microscopy tend to be aberrant. Hence microscopy alone is not a sufficient method.

#### 2.5.2.2 Quantitative Buffy Coat technique (QBC)

In order to enhance microscopic detection of parasites and to simplify malaria diagnosis, the QBC technique was designed (Clendennen *et al*., 1995). This method involves staining the parasite deoxyribonucleic acid (DNA) in micro-hematocrit tubes with fluorescent dyes, such as acridine orange, and its subsequent detection by epi-fluorescent microscopy. Briefly, finger-prick blood is collected in a hematocrit tubecontaining acridine orange and anticoagulant. The tube is centrifuged at 12,000 g for 5 min and immediately examined using an epi-fluorescent microscope (Chotivanich and Silamut 2006).

The parasite nuclei fluoresces bright green, while cytoplasm appears yellow-orange. In numerous laboratory settings, this technique has been shown to be a rapid and sensitive test for diagnosing malaria (Pornsilapatip *et al*., 1990, Barman *et al*., 2003, Bhandari *et al*., 2008). In the context of epidemiologic studies in asymptomatic populations in endemic areas, the alcidine orange has been shown to be a preferred diagnostic method over light microscopy and immunochromatographic tests probably because of its increased sensitivity at low parasitemia compared to the two (Ochola *et al*., 2006). However, it enhances sensitivity for *P. falciparum* and reduces sensitivity for non-falciparum species (Moody, 2002) and QBC technique is poor at determining species and numbers of parasite.

#### 2.5.2.3 Rapid diagnostic Tests (RDTs)

Commercially available rapid diagnostic immune-capture test strips are now commonly used in health facility settings for malaria diagnosis. The method doesn't require the same level of training and equipment as microscopic examination. These devices detect *Plasmodium* specific antigens in the patient's blood sample. In the early 1990s, paraSight®-F, the first malaria rapid diagnostic device was capable of detecting *P. falciparum* only (Shiff *et al*., 1993). Since then, there are now more rapid diagnostic devices in the market. The major antigens targeted by these devices include: Histidine Rich protein 11(HRP2) (Shiff *et al*., 1993), parasite lactate Dehydrogenase (pLDH) (Piper *et al*., 1999), *Plasmodium* aldolase (Moody, 2002).

Histidine rich protein is a water soluble protein produced by asexual blood stage and young gametocytes of *P. falciparum*(Shiff*et al*.,1993) and is the target used in many

rapid diagnostic devices.But there have been reports of false positives, particularly when using certain HRP2 test strips due to the presence of rheumatoid factor in the blood sample. HRP2 being specific to *P. falciparum* alone has also led to false negative results for the other *Plasmodium*species. Overall, RDT appears to be a highly valuable, rapid malaria-diagnostic tool for healthcare workers, however,most of the test results are qualitative, for any quantification of the parasitemia further laboratory-based tests must be carried out . The intensity of the test band varies with amount of antigen present this may lead to variation in test results. The other disdavantage of RDTs, is that they have a short shelf-life, therefore efficient procurement, transportation, storage and distribution systems are required ( Malaria diagnosis,2007).

#### 2.5.2.4 Molecular methods

The molecular revolution began in 1953 when Watson and Crick proposed the structure of DNA (Watson and Crick 1953). In 1987, a unique DNA amplification method called the polymerase chain reaction method was developed. PCR technique has been reported to be able to detect low parasitaemia at levels undetectable by microscopy (Brown *et al*., 1992). PCR is a valuable tool for epidemiological diagnosis unlike the rapid diagnostic devices. It can also reliably be used for monitoring drug effectiveness or emergence of drug resistance in parasite populations.It has also been found to be more sensitive and specific than QBC and some RDTs (Makler *et al*., 1998, Rakotonirina *et al*., 2008).

PCR has shown higher sensitivity and specificity than conventional microscopic examination of stained peripheral blood smears, and seems the best method for malaria diagnosis (Morassin *et al*., 2002).It can detect as few as 1-5 parasites/µl ofblood (≤ 0.0001% of infected red blood cells) compared with around 50-100 parasites/µl of blood by microscopy or RDT. Moreover, PCR can help detect mixed infections, and the process can be automated to process large numbers of samples (Swan *et al*., 2005).

There are different PCR methods and each relies on the detection of a specific target sequence such as 18S rRNA, cytochrome b oxidase, circumsporozoite protein, tubulin(Singh *et al*., 2004). However, they have been shortcomings that have been reported when using some of the target genes for diagnosis of *Plasmodium* species. For example, Singh *et al*., (2004) reported cross reactivity between 18S ribosomal RNA gene primers and*P. vivax* parasites. In support of these findings, Lucci *et al*., (2012), demonstrated that the 18S ribosomal RNA gene primers cross-reacted with at least four simian malaria parasites (*P. inui*, *P. hylobati*, *P. cynomolgi*, and *P.coatneyi*). At present, there are a few species-specific molecules that can be used as an alternative for the detection of malarial parasites ( Deepak *et al*., 2013).

In an effort to discover alternative target sequencesto improve on the species identification, various laboratories have applied *In silico* approach to search the databases for highly specific sequences unique to a given *Plasmodium* species.Using the approach, Lucci *et al*.,(2012) identified highly-specific, multi-copy sequences from the *P. knowlesi* genome and used the sequence to design novel primers for the detection of *P.knowlesi*. Deepak *et al*., (2013) also used a similar approach and

designed novel primers based on the comparative differences between *P. falciparum* and the human 28S rRNA gene. The novel primers were found to detect *P. falciparum* and *P. vivax* from clinical isolates.

In a recent *In silco* study done on the diversity of dyneins within *P. falciparum*, the dlc gene sequences were examined in public databases and their sequences and motifs compared with those of the human and other members in the *Apicomplexa* phylum. The dlc sequences of TcTex1 and LC7/roadblock proteins were found to be conserved within the *Plasmodium* species (Githui *et al*., 2009). These gene sequences can therefore be targeted for *Plasmodium* species diagnosis in humans and offer an additional method for malaria diagnosis.

## 2.6 Dynein light chain genes; TcTex 1 DLC gene

*Plasmodium* species live as obligate intracellular parasites and they belong to the phylum *Apicomplexa*. This group is characterized by a secretory apical complex as shown in figure 2.5. The infective stages (merozoites) of the malaria parasite actively invade the host cell in which they propagate (Ward *et al*., 1994). During the invasion process, the apical organelle contents are secreted and several protein families associated with the apical complex have been implicated to be crucial for invasion (Chobotar and Scholtyseck 1982, Ngo *et al*., 2003). The biogenesis of the apical organelle is not well understood, but several studies indicate that microtubule based vesicular transport is involved.

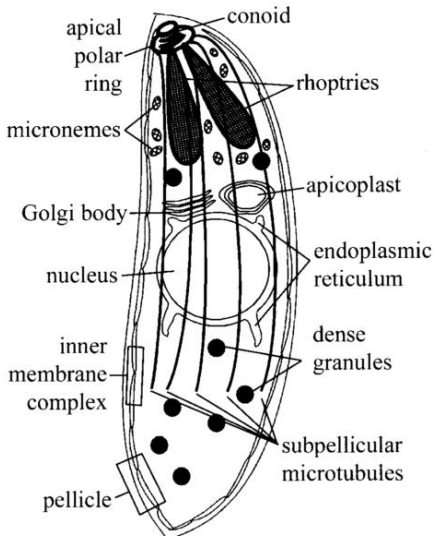


Figure 2.5; Apicoplexan parasite (Naomi *et al.*, 2002).

There are two major superfamilies of microtubule motor proteins kinesins and dyneins.Dyneins have been implicated in apical organelle biogenesis, replicationofthe parasite andintranscellulartransport including the endocytic pathway, organization of golgi, retrograde transport of organelles in axons, and in mitotic spindle assembly (Mocz and Gibbons, 2001, Ma and Chisholm, 2002). In infected erythrocytes, the specific role of these proteins has not been investigated (Adisa *et al*., 2001; Bannister *et al*., 2000).

There are two populations of microtubules in the invasive stage of the *Plasmodium* parasite. The non-dynamic sub-pellicular microtubules that radiate outwards from the apical complex maintain the polarity and the characteristic shape of the parasite, while the dynamic spindle microtubules and the presence of the three subclasses of dlc involved in *P. falciparum* coordinate chromosomal segregation (Fowler *et al*., 2001).

The dynein unit structure includes, two identical heavy chains (HC), each approximately 520 kDa. The C-terminus of each heavy chain forms a globular head containing multiple ATP hydrolysis sites and a small stalk-like structure that binds tomicrotubules,while the N-terminus is a flexible stem that interacts both with intermediate chains,(IC), light intermediate chains (LIC) and light chains (LC) to form the basal cargo-binding domain of the enzyme (Gee *et al*., 1997, Asai and Koonce, 2001,DiBella *et al*., 2001).

Dyneins belong to two main classes(<25 kDa): the axonemal dyneins responsible for sliding microtubules against one another to generate flagellar and ciliar movements that associate directly with individual HCs ( Kristen *et al*., 2008, Vale, 2003, King, 2000). Thesecond class of Dlcs are the cytoplasmic dyneins that are involved in cargo transport along microtubules and consist of three distinct protein families withmolecular weight ranging from 10 kDa to 14 kDa: TcTex1, LC8 andLC7/roadblock (DiBella *et al*., 2001, Wilson *et al*., 2001, Karcher *et al*., 2002, Susalka *et al*., 2002).

TcTex 1 dynein light chain acts as one of several non-catalytic accessory components of the cytoplasmic dynein 1 complex thought to be involved in linking dynein to cargos and to adapter proteins that regulate dynein function. Based on an *In silico* study done by Githui *et al*., (2009) , *Plasmodium* TcTex1 proteins and gene sequences were found to be unique and conserved to the *Plasmodium* species. Therefore the conserved aspect of the TcTex1 dlc gene within the *Plasmodium* species makes it a target for diagnosis.

# CHAPTER 3

# METHODOLOGY

## 3.1 Study area

Blood samples were collected from Sacred medical centre located in Changamwe constituency in Mombasa County. The clinic is a private clinic and caters for patients within the constituency. Mombasa County was selected because it is a malaria endemic area with most malaria transmission occurring throughout the year. Changamwe constituency is located at $4^{o}$ 1' 34'' South, $39^{0}$ 37' 50'' East (Geo Hack). It is a hot and humid area, with a population of population of 147,613 (Kenya national census 2009) and occupies an area of 16.00 sq.Km. It is primarily an industrial area, with a number of modern concrete tower blocks housing residents and refineries.The molecular analysis of the samples was then carried out at the molecular genetics laboratory located in the National Museums of Kenya in Nairobi.

## 3.2 Study population

The study subjects includedpatients who went voluntarily into the clinic for routine clinical checkup and had been referred to the clinic's laboratory for a malaria test by the physician.

## 3.3 Ethical clearance

The study protocol was reviewed by the Kenyatta National Hospital Ethics and Research Committee. The procedure posed minimal risk or discomfort to the study subjects during routine drawing of blood in the clinic.

## 3.4 Sample size determination

The sample size calculation was conducted using the formula by (Banoo S 2006) ;

$$n = (1.96 + 1.28)^2 \frac{P(1-P)}{(P - P_0)^2}$$

Where; n = sample size, (p) is the suspected sensitivity/specificity of the technique and ($p_0$) is the minimal acceptable sensitivity/specificity. In this case; (p) is 0.90 and ($p_0$) is 0.75 and this translates into n = 42. Therefore 42 positive malaria samples confirmed by microscopy were required for the study.

## 3.5 Sample, Sampling technique and sample collection

The samples used in the study included;laboratory clone isolates of *P. falciparum* (3d7 and Dd2) obtained from University of Georgia, 2 animal maintained laboratory culture samples of *P. knowlesi* obtainedfrom CDC Georgia and 1 animal maintained laboratory culture sample of *P. cynomolgi* obtained from IPR/ICIPE, Kenya. These samples acted as the positive controls. 2 samples ( G9 and G10) that had been verified as negative from previous laboratory work by Elijah *et al*.,( 2010) were also

included. The two negative samples had been confirmed through standard PCR and DNA sequencing.

The Population samples included 45 clinical samples obtained from Mombasa and other 10 DNA samples that were already present in the laboratory obtained from different regions in Kenya from a previous study done by Elijah *et al*., (2010). All the population samples were sampled using a similar technique. The DNA samples had been extracted from positive malaria blood samples obtained from Garissa, Kisii, Kisumu, Mumias and Nairobi. The samples were coded depending on the site of collection. Mombasa was assigned (MP and B), Garissa (G), Kisii ( KS), Kisumu (K), Mumias (MU) and Nairobi (H). The total number of population samples used in this study was 55more samples than the required 42. The higher the sample size the lower the percentage error, therefore this improved the specificity test.

The blood sample was obtained from patients who had visited a clinic for their regular check up and were referred to the clinic's laboratory by the physician for a malaria test. An aliquot of theirblood was then used for microscopy test. Thick blood smears were prepared and the reading carried out in accordance to the standard clinical protocol. The samples that tested positive for malaria were included in the study.

From each ofthe positive malaria blood samples500µl of blood was aliquoted and collected in an EDTA vacutainer. The collected blood samples were then transported in a cooler box at -4 $^{o}$c to the Molecular Genetics Laboratory in National Museums of Kenya, Nairobi. On arrival they were stored at -20$^{o}$c in the laboratory's freezer.

## 3.6 PCR analysis of the TcTex1 dlc gene

### 3.6.1 DNA extraction from the blood samples

DNA was extracted from the whole venous blood using a Pure link$^{Tm}$ Genomic DNA extraction kit (Carlsbad, USA) in accordance with the manufacturer's protocol.

The water bath was first set at $55^0$c. 200µl of each fresh blood sample was added into well labelled sterile micro centrifuge tubes. Cross contamination of the samples was prevented by use of sterile pipette tips for each of the samples. 20µl of proteinase k followed by 20µl of RNase were added into each of the tubes containing the blood sample and the tubes closed. The mix was then briefly vortexed and incubated at room temperature for 2 minutes. After the incubation, 200µl of Purelink$^{Tm}$ Genomic lysis buffer/ binding buffer was added into the tubes and the mixture briefly vortexed. To prevent extensive shearing of the DNA, vortexing for more than 10 seconds was avoided. The mix was then incubated at $55^0$c for 10 minutes to promote protein digestion.

After the incubation, 200µl of 97% ethanol was added to the lysate and the reaction mix was mixed well for 5 seconds to yield a homogenous solution. Approximately 640µl of each of the lysate were then transferred into sterile spin columns and the spin columns centrifuged at 10000 revolutions per minute for 1 minute at room temperature. After centrifugation the collection tubes were discarded and the spin columns transferred into clean collection tubes supplied by the kit. 500µl of wash buffer 1 already prepared and containing ethanol was added into the spin columns and centrifuged at 1000revolutions per minute for 1 minute. After centrifugation the

collection tubes were discarded and the spin columns placed into clean collection tubes. 500µl of wash buffer 2 that had earlier been preparedwith 97% ethanol was then added into each of the spin columns.

The spin columns were then centrifuged at maximum speed for 3 minutes at room temperature. The collection tubes were discarded. The spin columns were placed into respectively labelled sterile 1.5 ml micro centrifuge tubes. 100µl of elution buffer was added into each of the spin columns and they were incubated at room temperature for 1 minute then centrifuged at maximum speed for 1 minute. A second elution step was done using the elution buffer. The spin columns were then discarded and the micro centrifuge tubes retained since they contained DNA. The micro centrifuge tubes containing the DNA were then stored at $-20^0$c for use in PCR.

#### 3.6.1.1 Agarose gel electrophoresis

Agarose gel was prepared as described in the laboratory manual ( Sambrook *et al*., 1989).

The mould and the combs were first cleaned and dried. The sides of the mould were then sealed off using a masking tape and the combs put in place. The combs used varied depending on the number of samples to be run. 2.5g of the agarose powder ( Fisher Scientific, United States) was weighed and poured into a clean conical flask containing 250ml of ×1 TAE buffer solution prepared according to lab manual. The conical flask was then placed in a microwave and the solution heated upto boiling point until all the agarose had dissolved. The conical flask was then left to cool upto about $40^0$c. 8µl of ethidium bromide was then added into the flask and the flask

swirled gently. The mixture was then poured into the set mould and the gel was allowed to set for about 30-45 minutes.

The already prepared agarose gel was then placed into the gel electrophoresis machine. 5µl of the DNA sample was mixed with 1µl of the loading dye on a parafilm. The mix was then loaded into the gel using a micropipette. After loading all the samples into each of the wells, the cathode and anode were respectively put into position and the voltage was set at around 100 volts. The gel was then left to run. After completion of the electrophoresis, the gel was viewed under UV and a photo taken by a stand alone camera.

#### 3.6.1.2 DNA quantification using Nano-drop spectophotometry

The quantity and quality of the extracted DNA samples was determined usingNano-drop spectophotometry, firmware USB2000 2.41.3 ND4 at ILRI.

### 3.6.2 Designof the genus and species-specific primers

The sets of primers designed included,2 genus primer set targeting *P. falciparum, P. knowlesi, P. cynomolgi* and 2 species-specific primer set targeting *P. falciparum*.

The primers were designed based on *Plasmodium* dlc gene sequences obtained from NCBI nucleotide data-base (Altschul *et al*., 1997; Schaffer *et al*., 2001). They were assembled and aligned in Clustal-W in the Bioeditor suite program ( Bioedit v7.0.5 copyright (c) 1997–2005) against human and related dlc sequences. The respective conserved sequences weregi: 34190546, 325974481, 332818815, 88758585,

28372534, 124803800, 68073898, 70930600, 221056001, 457870887, 156098455, 82596995, 221054821, 156097557.

The conserved sequence regions identified were evaluated for species-specificity, screened for a satisfying length requirement of approximately 20bp, the GC-content, melting temperature, and primer-dimer forming potential after which they were considered as potential diagnostic targets. The designed primer sequences sent to Eurofilms Inc, UK to be custom made.

### 3.6.3 Analysis of the diagnostic potential of the designed primers using standard PCR.

PCR runs were carried out in the Techne; Tc-4000 thermocycler and the PCR amplification performed in a 25 µl reaction containing 10× Buffer (containing 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$) , 200 µM each dNTP, 0.75 units of Taq DNA Polymerase all from Roche manufacturing company USA, 1µL of 1.25mMof each oligonucleotide primer, and 1 µl(50ng/µl) of the DNA template. Reactions were performed under the following cycling parameters; summarized in table 3.1 and 3.2. After PCR amplification, 5µL of each of the PCR products from the generic and species-specific primers were visualized on a 1.2% agarose gel under UV.

Table 3.1; The amplification condtions for Genus specific primer set.

| Activation step | Denaturation | Annealing | Extension | Final extension | Final hold | 45 cycles |
|---|---|---|---|---|---|---|
| $92^0$c for 5min | $94^0$c for 1 min | $50^0$c for 1 min | $70^0$c for 1 min | $70^0$c for 5 min | $10^o$c for ∞ | |

Table 3.2; The amplification condtions for species-specific primer set.

| Activation step | Denaturation | Annealing | Extension | Final extension | Final hold | 45 cycles |
|---|---|---|---|---|---|---|
| $92^0$c for 5min | $94^0$c for 1 min | $52^0$c for 1 min | $70^0$c for 1 min | $70^0$c for 5 min | $10^o$c for ∞ | |

## 3.7 Sequencing of the PCR amplicons

In order to confirm whether the target sequence was amplified at the given optimized conditions, sequencing was carried out on the PCR amplicons.

### 3.7.1 Extraction of the PCR amplicons from the gel

Gel extraction of the PCR amplicons was carried out using the innuPREP gel extraction kit ( Lifescience, Jena, Germany).

The DNA fragments from the agarose gel were excised with a sharp sterile scapel making sure the agarose gel slice was approximately 300mg. The gel slice were then transferred into well labelled 1.5ml reaction tubes and 650µl gel solubilizer added into each tube. This was incubated for 10 minutes at 50µl in a water bath with frequent mixing during the incubation until the agarose gel slice was completely dissolved. After the incubation, 50µl of binding optimizer was added and the suspension mixed by vortexing. The samples were then appplied onto spin filters located in the 2.0 ml receiver tubes. The cap was closed and the sample centrifuged at 12000 rpm for 1 minute. The filtrate was then discarded.

The spin filters were placed back into the respective 2.0ml receiver tubes. 700µl of washing solution was added into the spin filters, the cap closed and centrifuged at 12000 rpm for 1 minute. The filtrate was discarded and the spin-filters placed back into the 2.0ml receiver tubes. The washing step was repeated. A final centrifuge step was carried out to remove all traces of ethanol. This was carried out by centrifuging at maximum speed for 2 minutes and the receiver tubes discarded. The spin filters

were placed into well labelled 1.5ml elution tubes and 30µl elution buffer added. This was incubated at room temperature for 1 minute. Then centrifuged at 8000rpm for 1 minute. The eluted fragments were then stored at $-20^0$c awaiting sequencing.

### 3.7.2 Sequencing of the purified PCR amplicons

A volume of 10 µl of each of the eluted fragments together with the primers were sent to Macrogen Inc., Netherlands for custom sequencing in 454 Sanger's dideoxy sequencing procedures. Where sequences were not clear, re-sequencing was done. A total of 40 samples were sent for sequencing.

## 3.8 Determination of the location and organization of the TcTex1 dlc gene using Artemis software and gene expression studies.

### 3.8.1 Bioinformatic analysis of the PCR amplicon sequences

The blast results based on the nucleotide sample target sequences of *P. knowlesi* and *P. cynomolgi* strains generated the genes IDs of the TcTex 1 dlc as PKH_091120 in chromosome 9 and *P. falciparum* generated the gene ID, PF11_0148 in chromosome 11. Both *P. knowlesi* and *P. cynomolgi* generated similar gene Ids indicating that *P. knowlesi* and *P. cynomolgi* infections are interchangeable.

Artemis software was used to determine the copy number, location, organization and orientation of the TcTex1 dlc gene in chromosomes.Artemis software is a free genome viewer and annotation tool that allows manipulation of the sequence features (Rutherford *et al*., 2000). It is routinely used by the Sanger Institute Pathogen Sequencing Unit for annotation and analysis of both prokaryotic and eukaryotic genomes. The program allows the user to view simple sequences files, EMBL/Genbank entries and the results of sequence analyses in a graphical format. It presents multiple sites/types of information within a single context.

The complete sequences of these chromosomes were accessed using their accession numbers; AM910991.1 and AEO14186.2 for *Plasmodium* chromosome 9 and 11 respectively. The sequences were then uploaded as FASTA files in the Artemis software and analysis done. The open reading frames, splicing sites and CDS were noted. Details of the primer sites were also noted.

# CHAPTER 4

# RESULTS

## 4.1 PCR analysis of the TcTex1 dlc gene.

### 4.1.1 Quality and quantity determination of the extracted DNA samples

Gel electrophoresis of the DNA samples revealed distinctive bands of DNA of high molecular weight above 1000bp. No smears were observed indicating that the extracted DNA samples were not degraded.Below is figure 4.1, a representation of the samples extracted. All the other extracted DNA samples revealed the same trend.

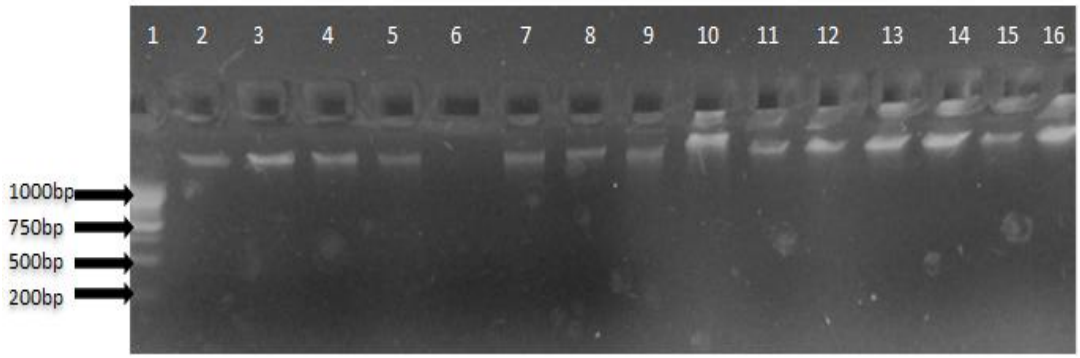


Figure 4.1; Gel electrophoresis of the extracted DNA samples.

Lane 1; molecular marker, lane 2;MB1, lane 3; MB2, lane 4; MB4, lane 5; MB5, lane 6; MB6, lane 7; B1, lane 8; B2, lane 9; B3, lane 10; K13, lane 11; B5, lane 12; MP20, lane 13; B2, lane 14; MP4, lane 15; B3, lane 16; MP4.

The quantity and quality of the extracted DNA samples was determined usingnano-drop spectrophotometry at ILRI. The quantity of the DNA ranged between 155.4-285.8 ng/µl. Using the ratio 260/280 in determining the DNA quality, the samples were found to be in the range of 1.71-1.87**.**

### 4.1.2 PCR analysis of samples using genus and species-specific primer sets

Out of the 4 primer sets designed 2 sets were able to amplify. The two sets include; TctPlasmoHumfwd, TctPlasmoHum rev primer set a genus specific primer designed to detect *P. falciparum, P. knowlesi, P. cynomolgi* and TcHrev and TcHfwd primer a species-specific primer designed to detect *P. falciparum*. The details of the two sets of primers are shown in table 4.1.

Table 4.1; Genus and species-specific primers.

| **Type of primer set** | **Name of the primers** | **Tm conditions** | **Approximated bp size of the amplicon** |
|---|---|---|---|
| Genus set of primer | TctPlasmoHumfwd<br><br>TctPlasmoHumrev | Tm $56^0$c<br><br>Tm $54^0$c | 400bp |
| *P. falciparum* specific | TcHrev<br><br>TcHfwd | Tm $50^0$c<br><br>Tm $52^0$c | 300bp |

#### 4.1.2.1 Genus primer set

The genus specific primer set amplified varying band sizes in the *Plasmodium* species. *P. cynomolgi*a target band of approximately 500bp, *P. knowlesi* laboratory animal maintained sample showed multiple bands, which when sequenced showed confirmed the lower band of approximately 400bp to be the target band. *P. falciparum* laboratory culture controls 3D7 and Dd2 showed distinct bands of approximately 300bps.

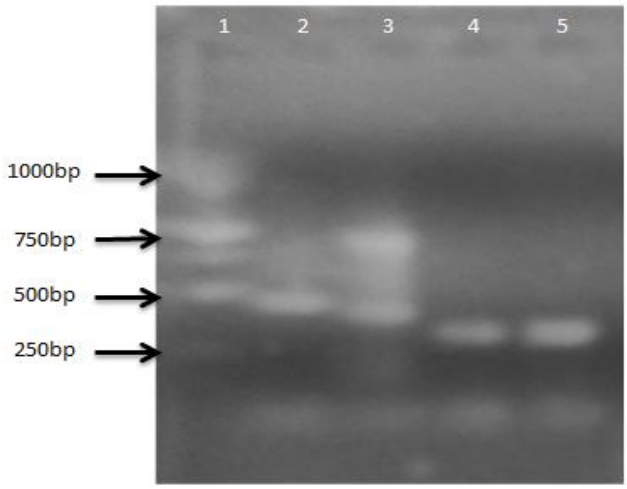


Figure 4.2;Gel electrophoresis of the controls amplified by the genus primer.

lane 1; molecular marker, lane 2; *P. cynomolgi*, lane 3; *P. knowlesi*, lane 4; *P. falciparum* (3D7), lane 5; *P. falciparum* (Dd2).

The genus primer amplified both the target sequence and non-target sequences. Out of 55 population samples 11 samples were amplified by the genus primer. Multiple bands were observed in the amplified samples as shown in figure 4.3. This indicates possible mixed infections in the patients.

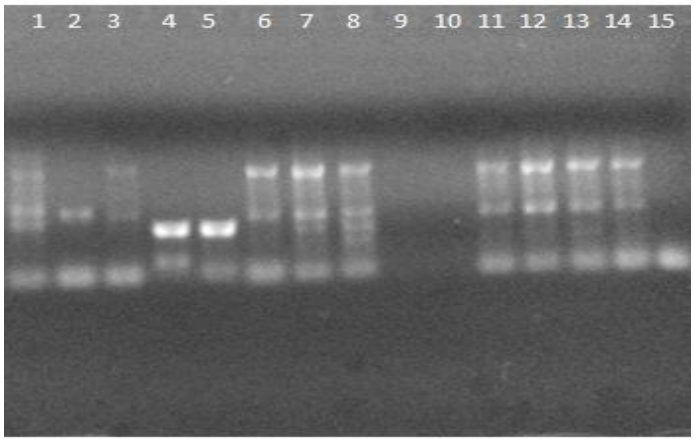


Figure 4.3; Gel electrophoresis of the population samples amplified by the genus primer.
lane 1; *P. knowlesi* , lane 2; *P.cynomolgi*, lane 3; *P.Knowlesi*, lane 4; 3D7, lane 5; Dd2, lane 6; KS4, lane 7; KS3, lane 8; MP20, lane 9; G9, lane 10; G10, lane 11; MP4, lane 12; B3, lane 13; B1, lane 14; PR26D, lane 15; No template control.

### 4.1.2.2 Species-specific primer set

The *P. falciparum* specific primers amplified 3D7; a *P. falciparum* control and produced a distinct band of approximately 300bps as shown in figure 4.4. No cross-reactivity with the other *Plasmodium* species controls was observed.

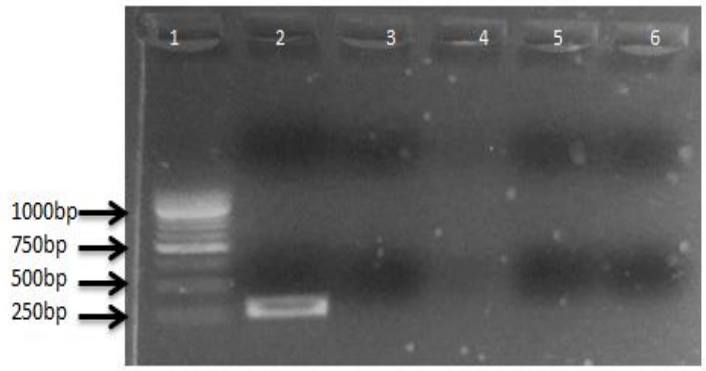


Figure 4.4; Gel electrophoresis of the of controls amplified by the *P. falciparum* specific primer. Lane 1; molecular marker, lane 2; 3D7, lane 3; *P. knowlesi*,lane 4; empty, lane 5; *P. cynomolgi*, lane 6; *P. knowlesi.*

The clinical samples amplified produced clear distinct bands with varying intensity and were all of approximately 300bps as shown in figure 4.5. Out of 55 of the population samples, the *P. falciparum* species-specific primer amplified only 8.

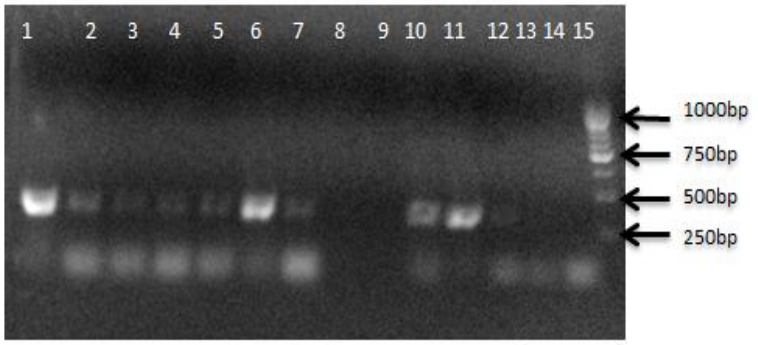


Figure 4.5; Gel photo of the PCR amplicons of the population samples amplified by the *P. falciparum*specific primer. lane 1;3D7, lane 2; KS3, lane 3; KS4, lane 4; H9C, lane 5; K10, lane 6; Dd2, lane 7;PR26D, lane 8; G9, lane 9; G10, lane 10; MU1, lane 11; MU2, lane 12; G24D, lane 13; B3, lane 14; B1, lane 15; molecular marker.

## 4.2 Analysis of thePCR amplicons sequences

### 4.2.1 Sequence analysis

Below are figure 4.6 and 4.7 showing the raw sequences of the samples amplified by the genus and the *P. falciparum* species-specific primer stes.The raw sequences show the different base pair sizes of the amplicons as amplified by the respective primer set.

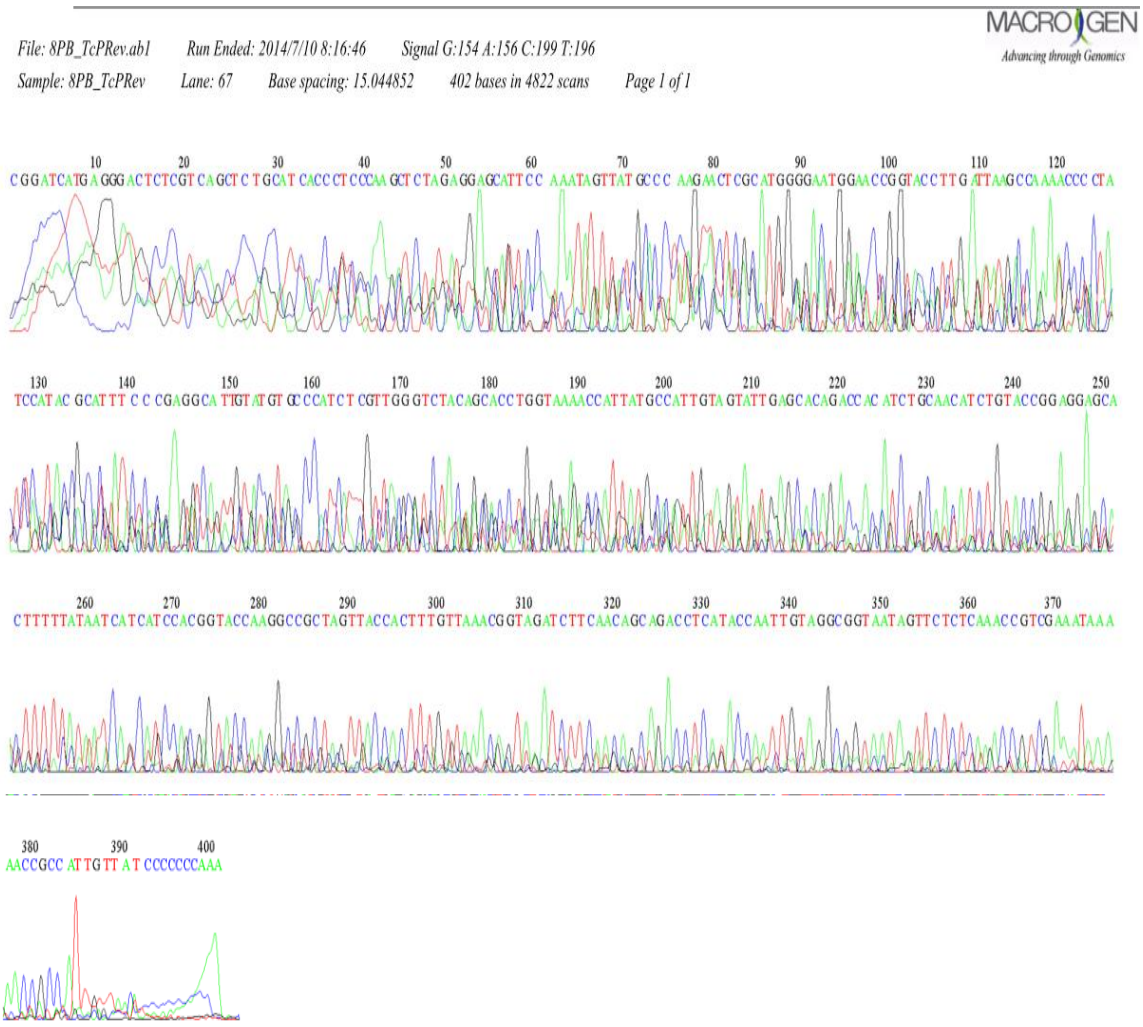

Figure 4.6; Raw sequence of 8Pb a PCR amplicon from the  genus primer amplification.

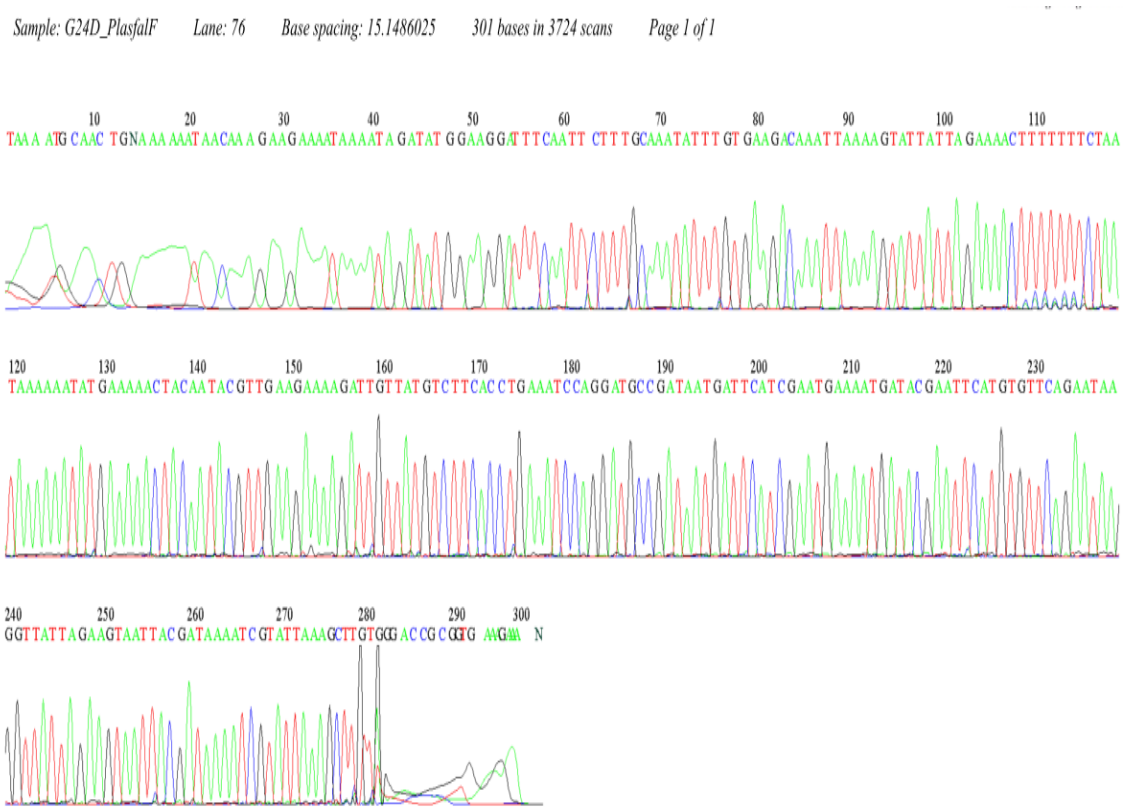


Figure 4.7;Raw sequence of G24D a PCR amplicon from the species-specific primer amplification.

The raw DNA sequences  were Clustal-W aligned in BioEdit Suite program for limited editing of the 5’ and 3’ primer. The respective consensus sequences were then used as queries during BLAST against NCBI non redundant nucleotide data base.E value (expected value), it is a statistical value used to assess whether an alignment constitutes evidence for homology and it helps to know the chances that two sequences share similar ancestry.

Based on the genus primer; the BLASTn results of *P. knowlesi* control (table 4.2) produced a significant score an E values of $9e^{-158}$ for*P. knowlesi* dynein light chain type 2, with the closest neighbour being*P. cynomolgi* with an E value of $4e^{-112}$. Sequences from the other *Plasmodium* species showed alignments with lower E values.

The BLASTn results of the *P. cynomolgi* control (table 4.3) showed a high score of E value = $3e^{-146}$ *P. knowlesi* H dlc type 2 with the next neighbour *P. cynomolgi* having an E value $5e^{-99}$ and non-*Plasmodium* speciesshowed non-significant scores.The *P. falciparum* control produced a score of E value $4e^{-07}$ for *P. falciparum* strain 3D7 (table 4.4).These results showed the genus primer can be used for genus species identification however, further optimization of the primers should be carried out.

Table 4.2; BLAST results for the *P. knowlesi* controlbased on the Genus primer.

P. Knowlesi

| Description | E value | Accession |
|---|---|---|
| *P. knowlesi* strain H DLC type 2 complete cds | $9e^{-158}$ | XM_002259103.1 |
| *P.knowlesi* strain H chromosome 9, complete genome | $9e^{-158}$ | AM910991.1 |
| *P. cynomolgi* strain B DLC type 2 complete cds | $4e^{-112}$ | XM_004222316.1 |
| *P. vivax* SaI-1 DLC type 2 partial mRNA | $1e^{-98}$ | XM_001615210.1 |
| *P. vinckei vinckei* hypothetical protein partial mRNA | 0.016 | XM_008626467.1 |
| *P. chabaudi chabaudi* DLC type 2, partial mRNA | 0.016 | XM_732090.1 |
| *P. yoelii yoelii* str. 17XNL partial mRNA | 0.057 | XM_721401.1 |
| *P.berghei* strain ANKA DLC type 2 mRNA | 0.057 | XM_673772.1 |
| Solanum lycopersicum chromosome, complete genome | 0.69 | HG975521.1 |
| Glycine max putative E3 ubiquitin-protein ligase LIN-like mRNA | 0.69 | XM_006586756.1 |

Table 4.3;BLAST results for the *P.cynomolgi* control based on the Genus primer.

| P. cynomolgi | | |
|---|---|---|
| *P.knowlesi* strain H DLC 2 complete cds | $3e^{-146}$ | XM_002259103.1 |
| *P. knowlesi* strain H chromosome 9, complete genome | $3e^{-146}$ | AM910991.1 |
| *P. cynomolgi* strain B DLC type 2 complete cds | $5e^{-99}$ | XM_004222316.1 |
| *P. vivax* SaI-1 dynein light chain type 2 partial mRNA | $1e^{-86}$ | XM_001615210.1 |
| *P. inui* San Antonio 1 hypothetical protein partial mRNA | $1e^{-67}$ | XM_008819688.1 |
| Trametes versicolor FP-101664 SS1 hypothetical protein partial mRNA | 0.82 | XM_008043674.1 |
| Glycine max putative E3 ubiquitin-protein ligase LIN-like mRNA | 0.82 | XM_006586756.1 |

Table 4.4; BLAST results for the *P. falciparum* control based on the Genus primer.

| *P.falciparum* | | |
|---|---|---|
| *P. falciparum* strain 3D7, chromosome 1 | $4e^{-07}$ | AL844501.1 |
| *P. falciparum* 3D7 erythrocyte membrane protein 1, | $4e^{-07}$ | XM_001350900.1 |

The nucleotide sequences of the population samples based on the genus specific primer produced high scores for *P. knowlesi,* with the closely related sequence being *P. cynomolgi* species and with no significant scores for non-*Plasmodium* species.Among the population samples, the sequences of 2population samples; MP20 and B5b,produced significant scores for *P. knowlesi* strains as shown in table 4.5 and 4.6.

MP20; a Mombasa sample produced a high E-Value score of $3e^{-77}$for *P. knowlesi* strain H dynein light chain type 2, with the nearest neighbor being *P. vivax*having an E-Value of $1e^{-55}$ while the non-*Plasmodium* related sequences showed lower scores of $2e^{-15}$ and $7e^{-09.}$B5b; a Mombasa sample produced a highE-Value score of $1e^{-124}$for *P. knowlesi* strain H dynein light chain type 2, with the nearest neighbor being *P. cynomolgi* having an E-Value of $4e^{-80}$ while the human related sequences showed non-significant scores.This indicates that there are probable *P. knowlesi* infections in the coastal region of Kenya.

Table 4.5; BlAST results of a population sample MP20 based on the genus-specific primer set.

| Description | E value | Accession |
|---|---|---|
| *P. knowlesi* DLC type 2 mRNA, complete cds | $3e^{-77}$ | XM_002259103.1 |
| *P. knowlesi* chromosome 9, complete genome | $3e^{-77}$ | AM910991.1 |
| *P. vivax* SaI-1 DLC type 2 partial mRNA | $1e^{-55}$ | XM_001615210.1 |
| *P. cynomolgi* DLC type 2 mRNA, complete cds | $7e^{-53}$ | XM_004222316.1 |
| *P. inui* hypothetical protein partial mRNA | $4e^{-43}$ | XM_008819688.1 |
| Feline coronavirus UU16, complete genome | $2e^{-15}$ | FJ938058.1 |
| Feline coronavirus RM, complete genome | $7e^{-09}$ | FJ938051.1 |

Table 4.5; BlAST results of a population sample B5b based on the genus-specific primer set.

| Description | E value | Accession |
|---|---|---|
| *P. knowlesi* DLC type 2 mRNA, complete cds | $1e^{-124}$ | XM_002259103.1 |
| *P. knowlesi* strain H chromosome 9, complete genome | $1e^{-124}$ | AM910991.1 |
| *P. cynomolgi* DLC type 2 mRNA, complete cds | $4e^{-80}$ | XM_004222316.1 |
| *P. vivax* SaI-1 DLC type 2 partial mRNA | $4e^{-74}$ | XM_001615210.1 |
| *P. inui* hypothetical protein partial mRNA | $4e^{-61}$ | XM_008819688.1 |
| *P. falciparum* 3D7 chromosome 11, complete sequence | $5e^{-09}$ | AE014186.2 |
| *P. falciparum* 3D7 DLC 2, putative mRNA, complete cds | $5e^{-09}$ | XM_001347783.1 |
| *P. vinckei vinckei* hypothetical protein partial mRNA | $3e^{-06}$ | XM_008626467.1 |
| *P. chabaudi chabaudi* DLC type 2, partial mRNA | $3e^{-06}$ | XM_732090.1 |
| *P. yoelii* genome assembly chromosome 9 | $1e^{-05}$ | LM993663.1 |

Based on the species-specific primer set, the BLASTn results of *P. falciparum* 3D7 control, showed high similarity to *P. falciparum* related sequences and within the target dlc gene regionas shown intable 4.7. The sequences of the population samples amplified by the species-specific primer also produced high scores for *P. falciparum* related sequences with no closely related human sequence observed as shown in table 4.8. This confirmed that the species-specific primer was specific to *P. falciparum.*

Table 4.7;BLAST results for*P. falciparum* control ( 3D7) based on the species-specific primer set.

P. falciparum

| Description | E value | Accession |
|---|---|---|
| *P. falciparum* 3D7 chromosome 11, complete sequence | $3e^{-151}$ | AE014186.2 |
| *P. falciparum* 3D7 DLC type 2, complete cds | $3e^{-151}$ | XM_001347783.1 |
| *P. berghei* strain ANKA DLC type 2,partial mRNA | $4e^{-23}$ | XM_673772.1 |
| *P. inui* San Antonio 1 hypothetical protein partial mRNA | $8e^{-19}$ | XM_008819688.1 |
| *P. knowlesi* strain H DLC type 2 complete cds | $4e^{-16}$ | XM_002259103.1 |
| *P.knowlesi* strain H chromosome 9, complete genome | $4e^{-16}$ | AM910991.1 |
| *P. cynomolgi* strain B DLC type 2 complete cds | $2e^{-13}$ | XM_004222316.1 |
| *P.vivax* SaI-1 DLC type 2 partial mRNA | $2e^{-13}$ | XM_001615210.1 |
| *D. globosa* 5.8S rRNA gene 28S rRNA | 0.001 | KJ136787.1 |
| H. sapiens ankyrin repeat, chromosome 12 | 0.001 | NG_029860.1 |
| H. sapiens 12 BAC RP11-179A1 complete sequence | 0.001 | AC079954.18 |

Table 4.8; BlAST results of a population sample G24D based on the species-specific primer set.

| Description | E value | Accession |
|---|---|---|
| Plasmodium falciparum 3D7 chromosome 11, complete sequence | 4e-136 | AE014186.2 |
| Plasmodium falciparum 3D7 dynein light chain type 2, putative (PF11_0148) mRNA, complete cds | 4e-136 | XM_001347783.1 |
| Plasmodium knowlesi strain H dynein light chain type 2 (PKH_091120) mRNA, complete cds | 6e-14 | XM_002259103.1 |
| Plasmodium knowlesi strain H chromosome 9, complete genome | 6e-14 | AM910991.1 |
| Homo sapiens ankyrin repeat and sterile alpha motif domain | 0.001 | NG_029860.1 |

### 4.2.2 Bank It sequence deposition.

Representative sequences based on the *P. falciparum* species- specific primer set are deposited at NCBI nucleotide data base, genebank id- KM 263606, KM263607, KM263608, KM263609, KM263610, KM263611, KM263612, KM263613.

## 4.3 Determination of the location and organization of the TcTex1 dlc gene using Artemis software analysis.

### 4.3.1 Bioinformatics analysis

The BLAST results of the highly significant sequences based on the genus and species-specific primer sets identified two gene IDs; PF11_0148 in chromosome 11 and PKH_091120 in chromosome 9.

**PKH_091120 in chromosome 9**

The gene occurs at an approximate position 48 megabases within the chromosome of total size 210megabases (figure 4.8).

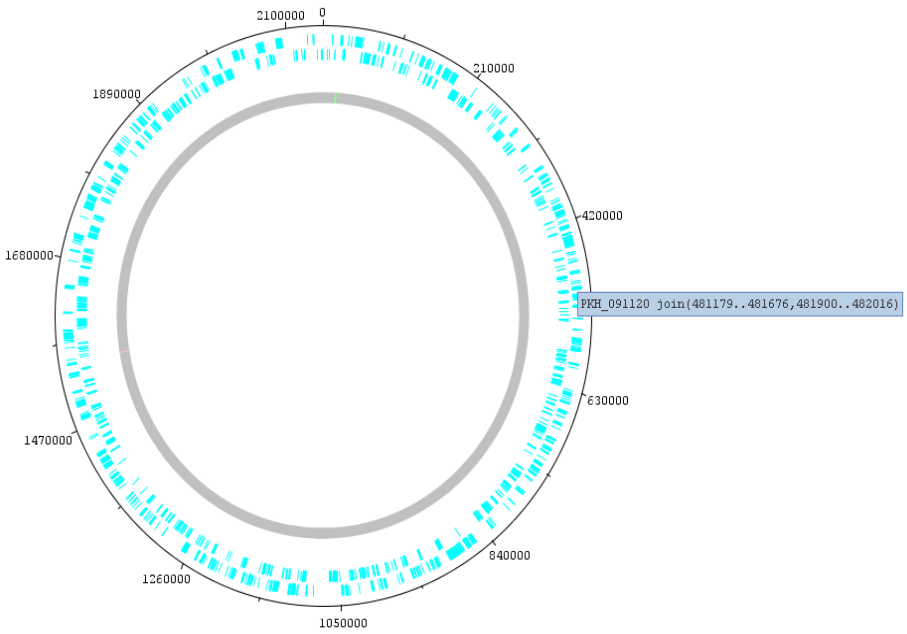


Figure 4.8; A DNA plotter view of the position of the gene PKH_091120 in the *Plasmodium* chromosome 9.

It occurs as a single copy in the first open reading frame (ORF) and the third ORF within the functional proteinand within the coding region. The splicing sites were also noted(figure 4.9) and the primers sites seen to be within the coding region.

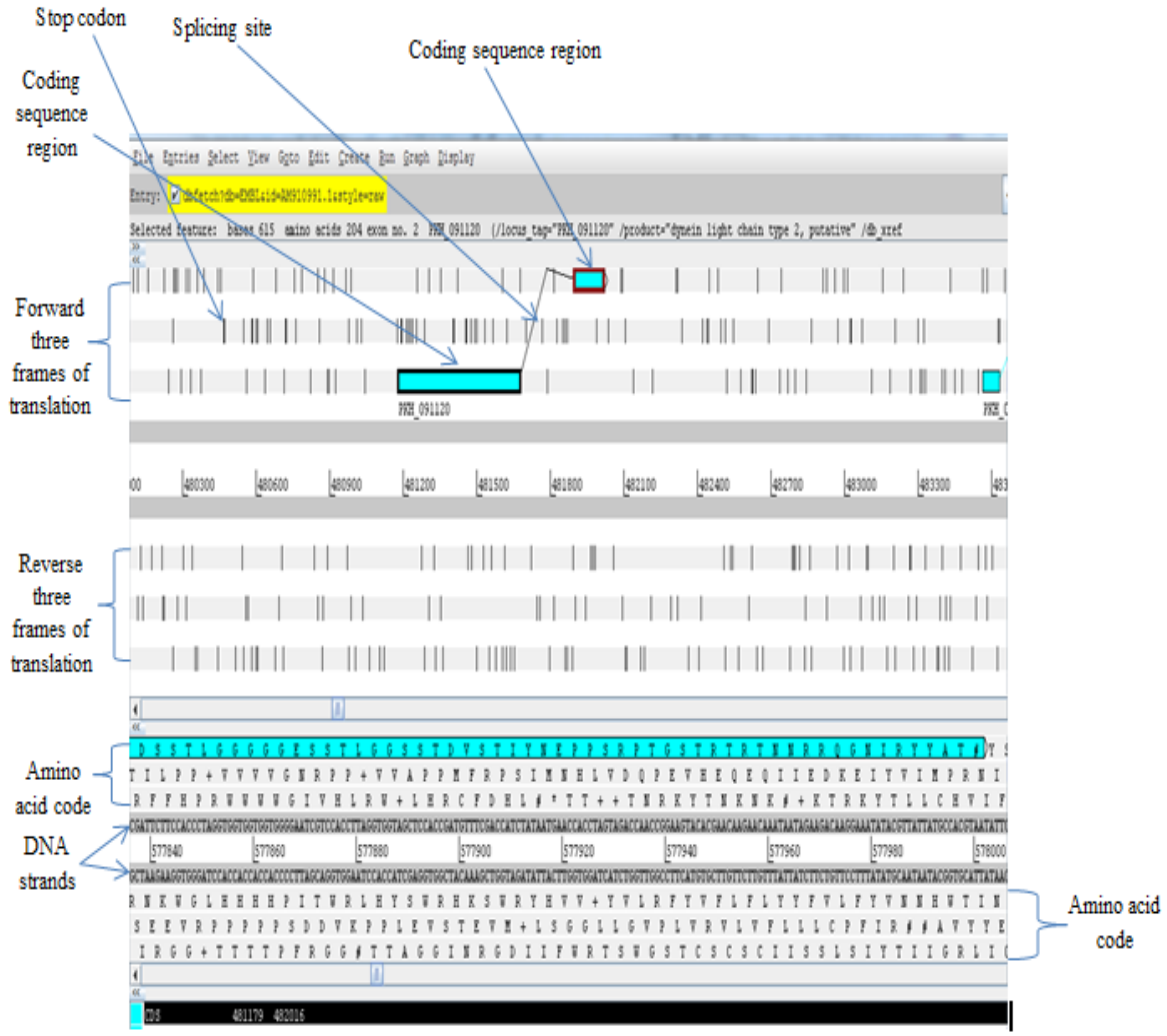


Figure 4.9 ; An Artemis window view showing the organization of the gene PKH_091120 in the *Plasmodium* chromosome 9.

**PF11_0148 in chromosome 11**

The gene occurs at an approximate position 53 megabases within the chromosome of total size 200 megabases (figure 4.10).

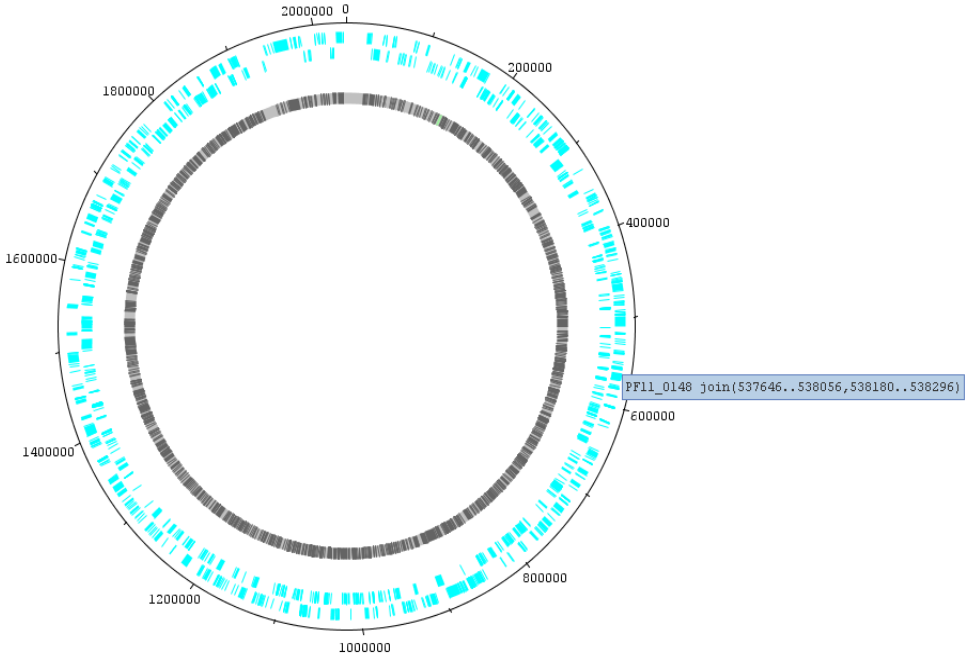


Figure 4.10; DNA plotter view for the gene PF11_0148 in chromosome 11.

The gene occurs as a single copy with a single open reading frame (ORF) within the functional protein. The primary transcript has an extra ORF that occurs within the 3' post-translational site. The splicing sites were also noted (figure 4.11). The gene is present in the coding region and the primers sites were seen to be within the coding region.

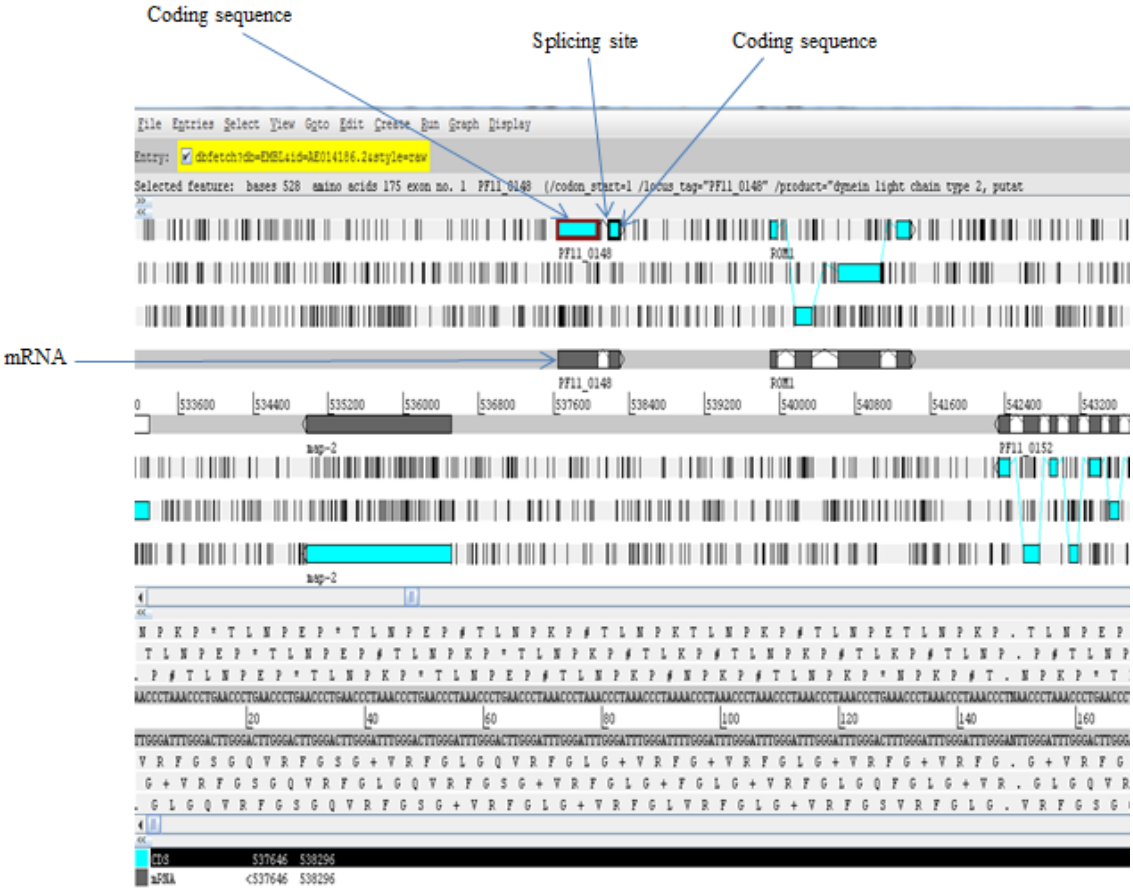


Figure 4.11; An Artemis window view showing the organization of the gene PF11_0148 in the *Plasmodium* chromosome 11.

# CHAPTER 5

# DISCUSSION, CONCLUSION AND RECOMMENDATION

## 5.1 Discussion

This study reports a new PCR assay based on *Plasmodium* dynein light chain Tctex1 sub-unit gene. It entailed designing primer sets based on conserved and unique gene sequences present in the *Plasmodium* species. A bioinformatics approach was used in identifying the conserved sequences as similarly done by Lucci *et al*., (2012). The study done by Lucci *et al*., 2012, had used a bioinformatics approach to search the malaria parasite genome database for suitable DNA sequences relevant for molecular diagnostic test. Multi-copy DNA sequences distributed in the *P. knowlesi* genome were identified and several novel primers specific to the target sequences were designed. The primers were then tested in a non-nested PCR assay.

In this study, the primers were designed based on *Plasmodium* dlc gene sequences obtained from NCBI nucleotide data-base (Altschul *et al*., 1997, Schaffer *et al*., 2001). The unique and conserved dlc gene sequences had been identifiedfrom an *In silico* study done by Githui *et al*., (2009). The study had entailed screening of dlc genes deposited in public databases, examining and comparing theirsequencesandmotifs with those of the human and *Apicomplexa* phylum .

Based on the conserved dlc sequences, novel primers were designed. The primers designed included 2 genus specific primer set designed to detect *P. falciparum*,

*P. knowlesi* and *P. cynomolgi* species and 2 species-specific primer sets for *P. falciparum* detection. The novel primers were then tested for specificity in a standard PCR assay. Out of 4 sets, 2 sets were found to amplify consistently.

The current gold standard for malaria diagnosis is microscopy however, microscopic observation does not reliably distinguish between species (Singh *et al*., 2004, Lee *et al*., 2009). However, PCR done on control and clinical samples using the genus specific primer detected both *P. falciparum* and non-*P. falciparum* species.Notably, the different *Plasmodium* species could be defined by their molecular sizes. These PCR results were further confirmed by DNA sequencing.

The genus primer when tested on the population samples, amplified 16 samples out of the 55 presumptive positive malaria population samples that had tested positive for microscopy. This finding confirms that microscopy alone as tool for malaria diagnosis can lead to inaccurate diagnosis(Payne, 1998). Inaccurate diagnosis of the disease further leads to misdiagnosis cases as reported by Yaw *et al*., 2013, in a study done in Western Kenya that reported 60% misdiagnosis cases in the health centers that were sampled.There is therefore need for an additional diagnostic technique in the hospitals to further confrim the microscopy findings.

Among the population samples amplified by the genus primer, two samples (B5b and MP20) both obtained from Mombasa when sequenced and analysed, both showed high sequence similarities to *P. knowlesi* related sequences. This indicates the possibility of *P. knowlesi* infections in the coastal region of Kenya. However, there have been no reports yet of *P. knowlesi* infections , probably due to misdiagnosis of

*P. knowlesi* infections as *P. falciparum* infections as reported by Cox-Singh *et al*., (2008). The study done by (Cox-Singh *et al*., 2008) in Sarawak found that microscopy-diagnosed *P. falciparum* cases were actually *P. knowlesi*by PCR.This emphasizes the need to screen the population in Kenya for possible *P. knowlesi* infections and the findings will influence the current treatment approaches. For example, in cases where a patient may have severe *P. knowlesi* malaria but was misdiagnosed as *P. falciparum*, he/she may fail to receive immediate parenteral treatment which may result to a fatal outcome ( Doughlas *et al*., 2010).

The importance of zoonotic transmissions by non-human primates is increasing especially in the forested areas of Southeast Asia and therefore should not be ignored. ( Khim *et al*., 2011, Ta *et al*., 2014, Lucci *et al*., 2012). Such occurrence is also possible in areas in sub-Saharan Africa, especially due to the close proximities between human and wild animals as people are encroaching forested areas for farming and urbanization.

*P. falciparum* is another known malaria causing parasite responsible for many fatal cases. Most of the deaths reported globally are often caused by *P. falciparum* (Nosten *et al*., 2000, Gething *et al*., 2012).The World Malaria report of 2010 stated that there were an estimated 6 million malaria cases, 26,017 deathsreported in Kenya and most of them caused by *P. falciparum*. This highlights the importance of developing a tool for specific identificationof *P. falciparum*.

The *P. falciparum* species-specific diagnostic primer set designed in this study showed a 100% specificity in detection of *P. falciparum.* This was evident bythe clear, intense single bands of approximately 300bp size produced by the amplified 3D7(*P. falciparum* clone) control and no cross-reactivity was observed with the *P. knowlesi* and *P. cynomolgi* species.The results were further confirmed by BLAST analysis. The BLASTn results of 3D7 control showed high similarity to *P. falciparum* strains.

The primer also amplified population samples that produced single distinctive bands of approximately 300bp. When sequenced they showed high sequence similarity with *P. falciparum*related sequences and no significant score was observed between the sequences with the human related nucleotide sequences.This indicates that the conserved TcTex 1 dlc sequence region can be used as a target for species differentiation among the *Plasmodium* species. Though present in humans and in the parasite, the TcTex1 dlc gene sequence is unique and conserved and this element aids in differentiating the parasite infection from the the human genome.

Based on the BLAST results of the highly significant sequences, two gene ids, PF11_0148 in chromosome 11 (*P. falciparum*) and PKH_091120 in chromosome 9 (*P. knowlesi)* were identified and analyzed using Artemis software. ThePKH_091120 gene occurs at an approximate position 48 megabases within the chromosome 9 of total size 210 mega bases and as a single copy and is positioned in the first open reading frame (ORF) and the third ORF within the functional protein. This indicate that the gene ancestry is different among the species. The gene is present in the coding region. The gene PF11_0148 occurs at an approximate position 53 megabases

within the chromosome 11 of total size 200 megabases. The gene occurs as a single copy with a single open reading frame (ORF) within the functional protein.The primary transcript has an extra ORF that occurs within the 3' post-translational site. The gene is also present in the coding region. This indicates that the gene can possibly be expressed and this can further be explored into development of a RDT. However, further gene studies and wet laboratory analysis should be carried out.

## 5.2 Conclusion

Molecular diagnostic methods are the most accurate way to distinguish between species that are morphologicallyidentical such as *P. knowlesi/ P. falciparum/ P. malariae* or *P. vivax/P. cynomolgi* (Lee *et al*., 2009, Singh *et al*., 2004, Kantele and Jokiranta 2011,Chin *et al*., 1965, Contacos *et al*., 1963, Coatney *et al*., 1971). PCR has been found to be a more sensitive method and it relies on the amplification of a target sequence. There are various target sequences applied in malaria diagnosis. The TcTex1 dlc assay described in this study offers a suitable alternative for accurate diagnosis of *P. falciparum*. The assay can also be applied in genus species identification, however, further optimization of the novel genus primer set will be required.

The findings from the study also indicate that other variant *Plasmodium* species are considered presumptive *P. falciparum* cases in routine microscopy procedures in the hospitals and this contributes to under reports of non-falciparum infections in the population. They also indicate possible *P. knowlesi/P. cynomolg, P. falciparum/ P. knowlesi* mixed infections in individuals in the coastal population a malaria

endemic area. However, further refinement of this data will give clear evidence of the zoonotic transmission of primate malaria.

## 5.3 Recommendations

A limitation of the study was that the novel primers were not tested on other species in the *Apicomplexa* phylum. Therefore, further validation of these primers on other species will be required.

Determination of the assay sensitivity and further optimization of the genus primer should also be done.

Inorder to further validate the utility of this assay in clinical diagnosis, additional laboratory and field-based testing of this assay will be necessary.

The findings from this study have also highlighted a need to screen the Kenyan population for possible *P. knowlesi*/ *P.cynomolgi* infections.

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# APPENDIX

## APPENDIX A: CLINIC'S CONSENT.

Sacred Medical Center,

P.o Box 3112-80100

Mombasa.

14/04/2014.

REF: **ACCEPTANCE TO CO-OPERATE.**

I hereby accept to assist Maureen Wanja Kariuki a student at JKUAT-PAN AFRICAN university pursuing a masters degree in Molecular Biology and Biotechnology. Having understood the nature of her project and the need to have human blood samples in actualization of her project, the clinic administration has allowed her to obtain the samples from the clinic.

Yours sincerely,

Mr.Kariuki,

Clinic's owner,

Sacred Medical center.