The detection of *Plasmodium falciparum* in human saliva samples

By
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Dissertation submitted to the department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand in partial fulfilment of the requirements for the Masters (MSc) degree in Biochemistry

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Abstract

Malaria still persists as a major public health scourge, claiming a global toll of up to 500 million clinical cases, mostly on African children. Current effective malaria diagnostic methods necessitate blood withdrawal from patients for accurate *P. falciparum* identification. Risks introduced by blood withdrawal can causes communities to be less cooperative to donate blood during malaria surveillance studies. Previous studies showed that saliva could be used to detect malaria by molecular techniques. This study, therefore, sought to establish the constituents of human saliva that harbours parasite DNA in malaria infected subjects. Furthermore the study optimised the use of Saliva as an alternative malaria DNA source in malaria infected patients. A total of 88 subjects were enrolled in this study, 35 (40, 7 %) were males and 51 (59, 3 %) were females. The age range was from 3 months to 99 years old (mean = 29.6 years; median = 18 years). Blood was drawn from each subject for subsequent use in microscopic examination and PCR tests. Saliva samples were also collected for PCR on Saliva derived parasite DNA. DNA was extracted using commercial kit on different saliva fractions from 46 malaria positive (thick film positive & blood PCR positives) individuals and 45 malaria negative individuals. Nested PCR was used to amplify malaria the *Plasmodium falciparum* dihydrofolate reductase (*pfdhfr*) gene. Generally PCR conducted on DNA purified from both blood and saliva was more sensitive than conventional microscopy, as previously reported. The pellet fraction of saliva was a more reliable and sensitive source of amplifiable parasite DNA compared to the soluble saliva fraction. After PCR optimisation amplification was enhanced 94.1% (sensitivity)

and 97% (specificity) against DNA derived from blood as the gold standard. This study further confirms that saliva samples are a reliable non-invasive alternative to blood for the PCR detection of malaria, as previously reported. The source of DNA was primarily in the pellet fraction though; however, refinement is still needed to identify the exact source of DNA in that fraction.

Dedication

This dissertation is dedicated to my family

Declaration

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Masters degree in Biochemistry at the University of Zululand. It has not been submitted before for any degree or examination in any other university. All sources have been duly acknowledged.

This _______ day of ____________ 2011

Acknowledgements

First and foremost I like to thank the Lord Almighty for carrying me this far, for without him none of this would have been possible. I would like to thank, my supervisors Dr. Addmore Shonhai and Dr. Sungano Mharakurwa for their support throughout this study. We would like to express special gratitude to the communities, the headman and chiefs in the vicinity of Macha, Zambia for their participation in this study. We would also like to thank Mr. Cliff Sing'anga, Ms. Mwiche Siame, Mr. Gift Moono and the rest of the staff at Macha Research Trust for their hospitality and assistance. Gratitude also goes to Mr. Edgar Jembera for the statistical input. I am grateful to the JHMRI funds awarded to Dr S. Mharakurwa. I would also like to thank the National Research Foundation (R.S.A) and the University of Zululand Research Committee for their financial support and lastly my family and friends for their love and support.

Table of Contents

Table of figures

List of tables

List of symbols

Symbol	Interpretation
%	Percent
µl	microlitre
bp	base pair
^{o}C	degree Celsius
ml	milliliter
w/v	weight per volume

List of research outputs

Publication

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Local Conference Proceeding

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MALARIA JOURNAL

POSTER PRESENTATIONS **Open Access**

Optimization of a polymerase chain reaction based assay for the detection of malaria in human saliva samples

Ofentse J Pooe[1*], Addmore Shonhai[1], Sungano Mharakurwa[2,3]

From Parasite to Prevention: Advances in the understanding of malaria
Edinburgh, UK. 20-22 October 2010

Background

Malaria is responsible for more than 1 million deaths per year; most of the victims are young African children [1]. Recent studies have found that *Plasmodium falciparum* DNA is detectable in human saliva as in human blood [2]. However the nature of the source of the saliva fraction that serve as source of this DNA still unclear. Thus this study sought to establish the constituents of human saliva that harbours parasite the DNA in malaria infected subjects.

Method

To determine whether the source of malaria amplifiable DNA in saliva was intracellular or free DNA in saliva, different saliva extractions were used to extract DNA; i. e. the pellet only , supernatant only and pellet and supernatant mixture. DNA was extracted using the Qiagen DNEasy® kit on different saliva fractions from 46 malaria positive (thick film positive & blood PCR positives) individuals and 42 malaria negative individuals were used in this study. Saliva DNA extracts were subjected to nested PCR in separate batches according to their DNA fractions.

Results

PCR conducted on both blood and saliva (pellet fraction) was more sensitive than conventional microscopy conducted on thick blood films. The pellet fraction was a more reliable source of amplifiable parasite DNA compared to the soluble saliva fraction. However it was noted that by reconstituting the pellet fraction with an aliquot of the supernatant, detection sensitivity from the pellet fraction could be improved. Through optimizing the PCR recipe we were able to increase the PCR amplification using saliva DNA extracts to 95, 65% sensitivity, with PCR on blood as the gold standard.

Conclusion

This study further confirms that saliva is a reliable source for detecting malaria based methods and could potential be used as an alternative non-blood based and non invasive malaria detection assay. Furthermore this study established that DNA is more enriched in the pellet fraction compared to the insoluble fraction of saliva. Future work could involve optimization of this assay to make it cheaper and more robust for use in malaria surveillance.

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Optimization of a Polymerase Chain Reaction based assay for the detection of malaria in human saliva samples

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BACKGROUND

Currently malaria diagnostic methods are conducted on blood drawn from patients. Because of the possible risks associated with sampling blood, communities are less cooperative to donate blood during malaria surveillance studies. Recent studies have found that *Plasmodium falciparum* DNA is detectable in human saliva as in human blood [1]. However the nature of the source of the saliva fraction that serve as source of this DNA is still unclear. Thus this study sought to establish the constituents of human saliva that harbour the parasite DNA in malaria infected subjects and to optimize the use of saliva as an alternative, non-invasive detection source.

METHOD

The samples used in this study were collected from 88 volunteers in the vicinity of Macha, Zambia. Saliva from infected and uninfected subjects, was fractionated into the pellet, supernatant and a third fraction made from pellet enriched with supernatant (Figure 1). DNA was extracted from these saliva fractions using the Qiagen DNEasy® kit from 42 malaria positive (PCR on blood samples) individuals. The DNA extracts were subjected to nested PCR using primers (Table 1) targeting the *Plasmodium falciparum* dihydrofolate reductase (*pfdhfr*) gene. The most sensitive of these fractions were then used for PCR optimization on 51 malaria positive and 37 malaria negative saliva samples. All PCR products were resolved on 1.5% agarose gel.

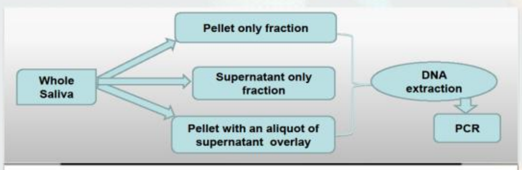

Figure 1: Preparation of DNA for PCR amplification

Reaction	Primer	Nucleotide location	Product size (bp)
1st Nest PCR: U1	5'- GGAAATAAAGGAGTATTACCATG-3'	121...143	273
U2	5'-TAAGGTTCTAGACAATATAACA-3'	393...372	
2nd Nest PCR: U3	5'- GAAATGTAaTTCCCTAGATATGgAATATT-3'	144...172	229
U4	5'- ATTTATCCTATTGCTTAAAGGT-3'	372...351	

Table 1: Primers used adopted from Mharakurwa *et al*, (2006)

RESULTS AND DISCUSSION

PCR conducted on both blood and saliva (pellet fraction) was more sensitive than conventional microscopy, as previously reported [1]. The pellet fraction was a more reliable source of amplifiable parasite DNA compared to the soluble saliva fraction. It was noted that by reconstituting the pellet fraction in a small overlaying volume of supernatant (to make a 10X concentrated suspension), an increase in sensitivity from the pellet could be achieved, presumably due to reduced accidental loss of pellet material during pipetting. Through optimizing the PCR recipe we were able to achieve 94,1% sensitivity and 97% specificity on saliva samples, with PCR on blood as the gold standard (Figure 2).

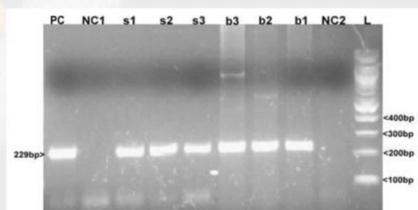

Figure 2: Illustrates amplicons from saliva (s1, s2 and s3) and blood (b1, b2 and b3) extracts from three patients. Lane PC represents positive laboratory control (*pfdhfr* gene product, 229bp), NC1 and NC2 represents Negative controls.

CONCLUSION

This study further confirms that saliva samples are a reliable non-invasive alternative to blood for the PCR detection of malaria, as previously reported [1]. The source of DNA was primarily in the pellet fraction though, further refinement is still needed to identify the exact source of DNA in that fraction. Future work could involve optimization of this assay to make it cheaper and more robust for use in malaria surveillance.

ACKNOWLEDGEMENTS

We will like to thank the communities, the headman and chiefs in the vicinity of Macha for their participation in this study. The staff at Macha Institute at Macha. We are grateful to the JHMRI funds awarded to awarded to S.M. The National Research Foundation (S.A) and the University of Zululand Research Committee.

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February 2011

Chapter 1

Literature review

1. *Plasmodium falciparum* malaria

1.1 Malaria burden on the endemic world

Malaria is an endemic disease caused by *Plasmodium* species. In spite of efforts to control malaria, the disease is still regarded as a public health problem in across the globe. This disease has probably had a greater impact on world history than any other infectious disease, and at present, it is a re-emerging disease; that is re-invading areas where it has been previously eliminated (Suh *et al*., 2004). It has been reported that malaria is responsible for more than 1 million deaths per year; and most of the victims are young African children (Mabunda *et al.,* 2008). Through recent revival of public-private sector initiatives, particularly the Roll Back Malaria programme (WHO, 2008) most endemic countries (Figure 1) are scaling up effective intervention against malaria.

Malaria is a parasitic infection transmitted by mosquitoes that has afflicted humans over the millennia. It remains a major cause of morbidity and mortality in adults and children worldwide (Bryce *et al*., 2005). Malaria in humans is caused by four species of the genus *Plasmodium* which are; *P. vivax, P. falciparum, P. malariae* and *P. knowels*. *P. falciparum* is the most fatal and most common of the four human infecting malaria parasites, and accounts for about 90% of all malaria mortalities across the globe (Mendis *et al.*, 2001).

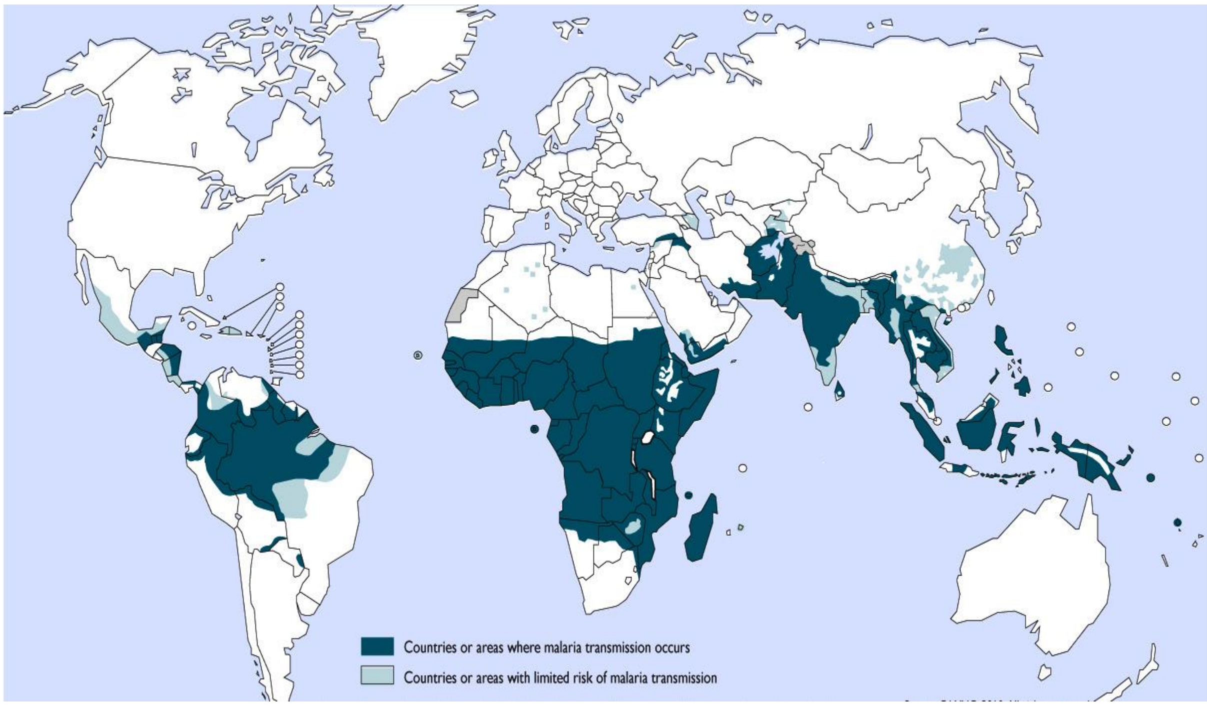

Figure 1 Malaria transmission (WHO, 2010)
The diagram illustrates the global malaria impact across endemic regions. The land was defined as areas with no malaria risk (white), areas where malaria transmission occurs (dark blue) and areas with limited malaria transmission risk.

Malaria transmission rates vary across different geographical areas, in most countries malaria infection is seasonal (Roca-Feltrer *et al*., 2009). Africa is a continent with spatial and temporal variations; the equatorial belt generally has high rainfall, compared to the northern and southern African countries fairly semi-arid regions. Unfortunately, the high rain-fall and high temperatures promote the survival of the mosquito vectors by providing suitable climates to vector breeding. *Anopheles funestus* and *Anopheles gambiae* are the most effective malaria vectors, responsible for spreading the parasite in most parts of Africa. In spite of efforts to control malaria, the disease is still regarded as a great public health problem in Africa and throughout the endemic world, creating enormous social and economic burdens.

According to the “Roll Back Malaria” (RBM) partnership, it is estimated that 91% of all the malaria mortalities are from Africa. Approximately 10 000 pregnant young women die every year because of malaria during maternity (WHO, 2008). Pregnant women are more likely to be infected than other adults as their immune system is reduced risking anaemia, premature delivery and still-births (Anchang-Kimbi *et al*., 2009). Malaria infected pregnant women are more likely to give birth to low weight babies (Tako *et al*., 2006) and may pass on the disease during birth through congenital malaria. Since most children would not have developed maximum immunological states, they are at greater risk of being infected by the disease. It is estimated that 85% of malaria deaths are children who are less than 5 years of age (WHO, 2008).

Over the past 10 years malaria cases reported in government health institutions have drastically decreased in the disease burden across the endemic world, including countries in the sub-Saharan such as Ethiopia, Zambia and South Africa (Barnes *et al*., 2009). Countries in Africa have managed to reduce more than 50% of malaria cases and deaths, since the year 2000. The reasons are not fully understood, but the disease decline is associated with intense malaria control interventions and implementation of public-private sector initiatives, such as the distribution of insecticide-treated bed nets and increased access to health services may have contributed to the reduced disease transmission the RBM programme (WHO 2010). In 2009, Zambia had an increase in malaria admissions. The reasons for these resurgences are still unclear. Unusual rainfall and temperature conditions could not be linked with these events. Hence even if malaria cases have been drastically reduced a constant surveillance programme is needed. Malaria control programmes should have a surveillance system that is timely, accurate and sensitive. Endemic countries aiming to combat and eradicate malaria need to monitor asymptomatic infection reservoirs as to avoid potential malaria resurges and recurrences.

1.2 Life cycle of *Plasmodium falciparum*

In humans, malaria infection begins when an infected Anopheles mosquito takes a blood meal thereby injecting infected sporozoites into the peripheral circulation; sporozoites may travel directly through the blood to the liver or through lymphatic channels. A low number of sporozoites are injected and the Kupfer cells of the liver tend to clear some of the sporozoites from the pheripheral stream so an even smaller fraction infects the liver; hence this stage rarely causes any symptoms. After invading the liver and the sporozoites undergo asexual multiplication, to form large intracellular sporozoite that contains thousands of merozoites (Vaughan *et al*., 2008). Schizonts mature within a period of 5–15 days after sporozoite inoculation; this phase is known as the pre-erythrocytic phase or exo-erythrocytic cycle (Sacci *et al.*, 2006). Upon maturation the merozoites are released into the blood stream.

The merozoites invade red blood cells (RBC's) and the parasites multiply and develop into trophozoites within the red blood cells (Cowman and Crabb, 2006). The trophozoites later develop into schizonts further complete another round of multiplication within 48-72 hours. The maturation of the schizonts inside the RBC’s results in the destruction of the erythrocyte cells; more merozoites are released into the blood system that invade more uninfected RBC's (Figure 2) thus completing the cycle; the release of merozoites is thought to be responsible for the periodic fevers associated with malaria. The last stage of the cycle is known as the sporogonic cycle, where some merozoites form male and female gametocytes (sexual forms) which are taken up from the blood by a feeding female Anopheles mosquito (Hill, 2006).

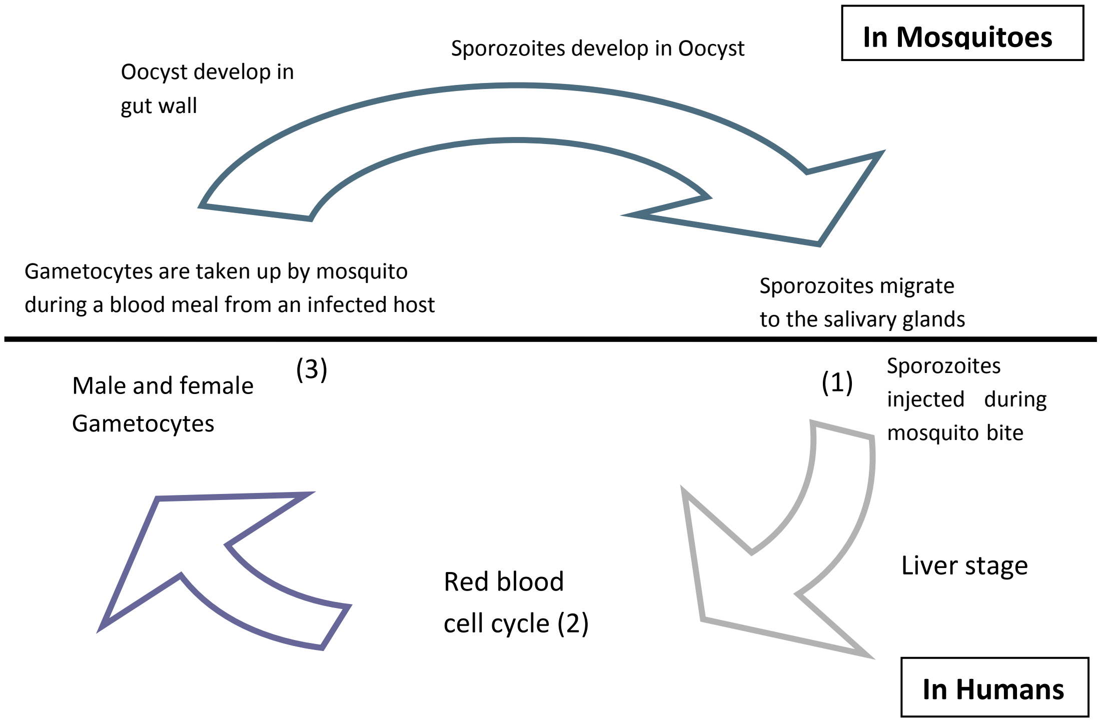

Figure 2: Schematic presentation of the *Plasmodium* malaria transmission life cycle. The cycle shows different stages and forms undertaken by *Plasmodium falciparum* in mosquito and human hosts. (1) When a mosquito bites an uninfected host human, Sporozoites are injected into the bloodstream. The parasite invades the liver where it later develops into schizonts (exo-erythrocytic cycle), which later releases thousands of merozoites into the bloodstream (Vaughan *et al*., 2008). (2) Merozoites circulating in the bloodstream invade RBCs. The parasites multiply through sexual stages within the RBCs, this is known as the erythrocytic cycle (Cowman and Crabb, 2006). These merozoites infect cells and become trophozoites, which later form schizonts that release more merozoites into the blood system. Some merozoites develop into male and female gametocytes. (3) These gametocytes are taken up by mosquitoes during a blood meal. The gametocytes are fertilized within the mosquito where the parasite later forms sporozoites (Sporogonic cycle) that migrate to the salivary glands to await injection into the human host during the next blood meal (Hill, 2006).

The gametocytes are fertilised forming a zygote which undergoes meiosis to form first an ookinete and later an oocyst in the gut wall. This phase of multiplication in the mosquito is known as sporogony (Sacci *et al.*, 2006). The oocyst also bursts, liberating large numbers of sporozoites that migrate to the salivary glands to await injection awaits in the salivary gland and where the infection is passed on to a uninfected human host during the feeding (Hill, 2006).

1.3 The socio-economic impact of malaria

The social and economic setback caused by malaria in endemic regions is overwhelming and creates enormous social and economic burdens. Children who survive repeated malaria episodes of fever and illness, reduced social interaction and educational opportunities, are prone to poor social development. It is estimated that 2% of children who recover from cerebral malaria suffer from learning impairments due to brain damage (Murphy and Breman, 2001). According to a WHO report (2010), a single attack of malaria costs a sum equivalent to 10 working days in Africa. The economic loss due to malaria (health care, drug treatment, etc.) is estimated to be 1.3% of GDP growth per year in tropical Africa. It is furthermore estimated that approximately 40% of public health spending in sub-Saharan Africa, making up 20-50% of inpatient admissions, and up to 50% of outpatient visits (WHO, 2010). High fatalities may arise among hospital inpatients due to late hospital admissions and unavailability of effective drugs.

A study survey in Zambia found that poor communities had a higher prevalence of malaria infection than the richer community (Zambia RBM, 2001). Poor people are at increased risk of becoming infected and re-infected with malaria more than the rich community. This could be due to the fact that poor families do not observe basic prevention methods such as use of bed nets to protect themselves against mosquitoes and this could be due to ignorance and failure to afford insecticidal-treated bed-nets. A Tanzanian-based study also showed that child mortality rates due to malaria were 39% in poor households higher than in wealthier households (Mwageni *et al*., 2002). Poor people are less likely to afford effective malaria treatment and have poor access to health facilities that are equipped to effectively treat the disease. A study in Ghana found that while the cost for malaria treatment and care was 1% of the income of the rich household, it represented 34% of the income of poorer households (Akazili *et al*., 2007.). Thus malaria can to some extent be associated with poverty and can be linked to the slow economic developments in most endemic regions.

Fever and septic shock are often misdiagnosed as severe malaria in Africa, leading to failure to treat other life-threatening illnesses. Diagnosis based on symptoms alone without parasitological confirmation; often lead to lack of treatment or inappropriate treatment. The former allows increased disease burden and continued transmission, while the latter leading to misdiagnosis of other treatable diseases (Reyburn *et al*., 2007). Pregnant women are very vulnerable to malaria in endemic areas as the parasite may not be seen in the peripheral blood though present in the placenta (Anchang-Kimbi *et al*., 2009). Misdiagnoses may also play a role for the high proportion of public health expenditure, since many facilities in endemic regions lack laboratory facilities that can distinguish malaria from other infectious diseases. It is estimated that accurate diagnostic tests for malaria have the potential to prevent

400 million unnecessary treatment cases and save 100, 00 lives per year (Rafael *et al*., 2006).

1.4 Methods for malaria diagnosis

Charles Laveran was the first scientist to visualise and identify the *Plasmodium* parasite as the causative agent of malaria the malaria parasite in blood in 1880 (Laveran, 1882). This led to the application of microscopy as a diagnostic tool for malaria. Malaria diagnosis is traditionally achieved by microscopic examination of thick and thin film blood smears. Microscopy is able to detect parasite species and determine parasite densities. The detection limit by thick film microscopy is claimed to be in the 100 to 5 parasites/μl range on a microscopic slide (Wongsrichanalai *et al*., 2007). Quality of the data obtained through microscopy can significantly vary, because microscopic accuracy largely relies on the experience and training microscopists (Babiker *et al*., 2008). Thus in low parasitemia cases, microscopy is ineffective and unreliable in malaria detection. Moreover, studies have shown that microscopy underestimates true parasite counts as parasites are likely to be washed off or lysed during Giemsa staining (Bejon *et al*., 2006).

Rapid Diagnostic Tests (RDTs) are antigen detection tests that are easy to use and provide rapid results in 15 to 20 minutes without the need for electricity, expensive equipment or extensive training. They are very helpful in resource-poor settings, especially where microscopy is unavailable. Most RDTs have a detection limit similar to that of thick film microscopy for *Plasmodium falciparum* but often less sensitive for *Plasmodium vivax* (Wongsrichanalai *et al*., 2007). They are also unable to differentiate between past infection and current infection and may still give positive results even after a previously infected patient has received successful drug treatment.

Current malaria diagnostic methods necessitate the use of blood for diagnosis, using either rapid diagnostic test (RDT) and/or microscopy. Although these methods for malaria diagnosis are sensitive, accurate, relatively cheaper and rapid, (Jelinek *et al*., 1999; Hanscheid, 1999), their greatest disadvantage is their dependency on blood samples for diagnosis. These techniques, therefore, introduce the risk of accidental infection from diseases such as TB, malaria, HIV, and others which abound in malaria-endemic areas, and necessitate rigorous training and biological safety precautions, to ensure proper containerisation and disposal of used needles. In surveillance studies, the use of needles also adds limitations in certain African communities which still regard blood withdrawal as taboo, frequently leading to reduced community participation. This is a problem as surveillance studies require that testing and monitoring to be conducted repeatedly (Wilson *et al*., 2008).

One of the greatest challenges in malaria control is drug resistance. The WHO currently recommends artemisinin combination drugs (ACTs) as the first-line drug for uncomplicated falciparum malaria. Artemisinins kill the asexual stages in blood and affects the amplicon ion of gametocytes in *P. falciparum* infections, which are vital for disease transmission from one person to the other, however Artemisinins do not affect pre-erythrocytic phase of infected individuals hence a system that can detect hidden parasite levels at extremely low levels. Moreover, there are fears that due to artemisinin current dependency, the parasite may sooner or later exhibit resistance to ACTs as has been the case with other classes of anti-malarials. WHO recently reported that parasite drug resistance may be emerging along the Thai-Cambodia border and this is a threat to malaria control (WHO, 2009). Drug efficacy hence has to be continually monitored in endemic countries.

1.4.1 Polymerase chain reaction

Polymerase chain reaction (PCR) makes it possible to detect the parasite at very low concentrations (Laveran, 1882). Early and accurate diagnosis of malaria will reduce disease severity in malaria endemic regions. PCR is a DNA-based molecular diagnosis technique that is more sensitive than microscopy and RDT (Coleman *et al.,* 2006), which have been extensively used for diagnosis, verification of diagnosis, epidemiology studies and drug efficaciousness. Amplification of malarial DNA using PCR could be useful in surveillance studies due to its rapidness, accuracy and because it is not confined to the use of blood. PCR is now a widely used technique used in molecular and medical research laboratories for a variety of applications, including DNA cloning genetic fingerprinting and more importantly the detection and diagnosis of infectious diseases (Lima *et al*., 2010; Coleman *et al*., 2006). PCR is a DNA amplifying molecular technique used to amplify small amounts of DNA into millions of copies of a particular DNA sequence; theoretically PCR is capable of amplifying a single parasite in a blood sample or as little a single gene. PCR enables accurate diagnosis of parasite species, enhanced sensitivity in detecting low density parasites and better detection of mixed species/strain infections. It involves a repeated thermo-cycling process. Reagents required for PCR include among others DNA polymerase, dNTP's, template or sample DNA, oligonucleotide primers complementing the DNA sequence adjoining the region of interest (Lavaren, 1982). Confirmation of the amplified PCR amplicon can be carried out by using agarose gel electrophoresis.

1.4.1.1 Principle of polymerase chain reaction

DNA amplification takes place in repeated cycle of three defined stages; denaturation, annealing and extension. The first stage denaturation, utilises high (≥90ºC) temperatures to physically separate the double helix stranded DNA. This is followed by a second stage known as annealing, where the temperatures is reduced lower (35-55ºC) for a short time interval to allow primers to bind to their complementary DNA sequences on the single DNA strands. The specificity of PCR also results from the use of primers that are complementary to the DNA region targeted for amplification. The final stage of the cycle an enzymatic primer extension reaction is carried out to produce complementary copies of the initial single strands from the primer bound strand (Rapley, 1996). The heat-stable DNA polymerase, such as *Taq* polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*, is the key to PCR as the enzyme is able to withstand high temperatures (Laveran, 1982). *Taq* polymerase assembles the new DNA strands from DNA building blocks, the dNTP's. These three stages are usually repeated between 25 and 40 times, the generated DNA is itself also used as the template for following cycles, setting in motion a chain reaction in which the specific target DNA sequence is exponentially amplified. Nevertheless, this advantage also introduces the possibility of exponentially amplifying the wrong sequence. PCR Specificity greatly relies on the specificity of primers (Mharakurwa *et al*., 2006).

1.4.1.2 Strengths and disadvantage of the polymerase chain reaction

PCRs greatest advantage is its sensitivity and specificity, and is relatively rapid. Unfortunately, however, PCRs main strength is also its major disadvantage i.e. since PCR is capable of amplifying a single copy of DNA into thousand of replicates.

Hence any degree of contamination may result in false DNA amplifications. Thus although very specific the PCR method is also prone to giving false positive results and unless extreme aseptic techniques are taken into consideration when setting up PCR reactions to avoid any possibilities of contamination exist, false positive data may be reported (Kwok and Higuchi, 1989). Positive and negative controls are always necessary and should always be included in all PCR runs to monitor contamination. The potential sources of contamination include cross-bench contamination, positive control DNA and previously amplified DNA material (Longo *et al*., 1990). Previously amplified DNA may be found on surfaces, pipettes, and in micro-centrifuges, it is also advised that PCR reaction be set up on areas physically separated from DNA extraction areas. Although more sensitive and specific, nested PCR is also very susceptible to contamination as conventional PCR is, due to sample carry-over, hence strict attention is needed in nested PCR (Neumaier *et al*., 1998)

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A number of factors such parasite resistance, and host immunity can influence parasite clearance rates. PCR can be used predict treatment failure (Kain *et al*., 1994). Hence PCR can be used as a research tool or as a component of a sentinel system to monitor regions with suspected emerging drug resistance. Endemic area with declining malaria prevalence efficient surveillance requires sensitive methods that can detect hidden parasites. Since PCR is more sensitive than microscopy, it can, therefore, be used to detect mixed malaria latent infections as it can diagnose submicroscopic infections (Coleman *et al*., 2006).

1.4.1.3 Nested polymerase chain reaction

Nested PCR is a modification of polymerase chain reaction intended to reduce PCR product contamination, due to the amplification of unexpected primer binding sites (Neumaier *et al*., 1998). Nested PCR involves two sets of primers, used in two successive runs of polymerase chain reaction; the second primer set is intended to amplify a secondary target within the first run amplicon and produce a PCR amplicon that is shorter than the first one (Figure 3). A commonly occurring problem is primers binding to incorrect regions of the DNA, amplifying unintended PCR amplicons (Longo *et al*., 1990). The selection of alternative and similar primer binding sites gives a selection of amplicons, only one containing the intended sequence.

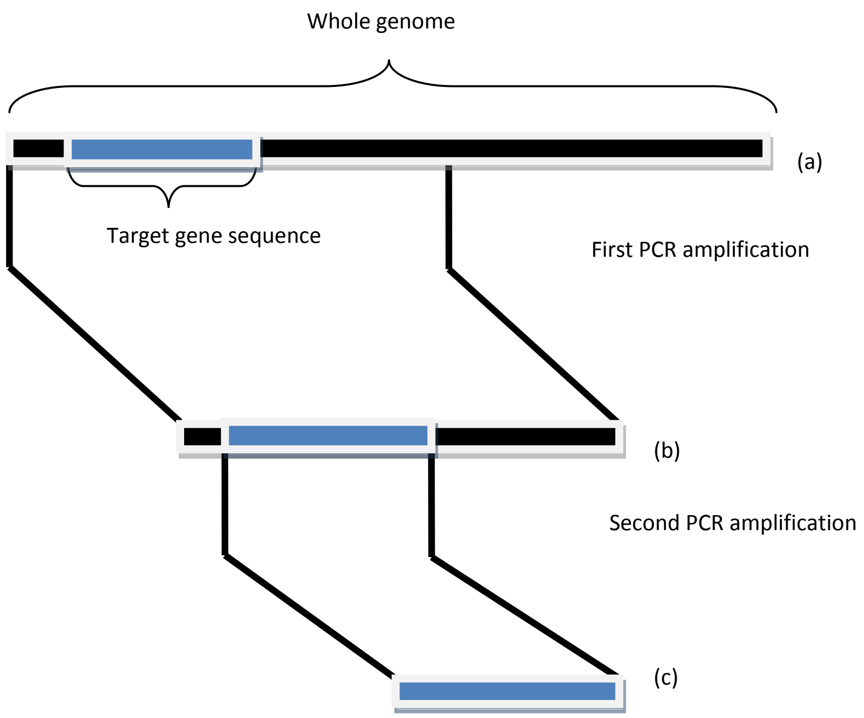

Figure 3 Principle of nested PCR. Diagrammatic representation of a nested-PCR protocol to amplify a Target gene sequence from the genome (a) of a particular organism. In nested-PCR two primers are used to amplify a target gene. The amplicon of the first nested-PCR amplification (b) is used as a template DNA, for the second (nested) PCR, which yields the target gene sequence (c).

The amplicon from the first reaction undergoes a second run with the second set of primers. This way, the probability is very low, that unwanted PCR amplicons will contain binding sites for the second set of PCR primers and be amplified by the second pair of primers (Figure 3). This, therefore, ensures that the amplicon produced from the second PCR run has little chances of being contaminated by unwanted PCR amplicons; making nested PCR more sensitive and more specific than conventional PCR (Rapley, 1996). Previous studies have also revealed that nested PCR is more sensitive when compared with microscopy for low density *Plasmodium* parasites (Coleman *et al*., 2006).

1.4.2 Saliva as a diagnosis tool

Great research interest has recently been on human saliva as material for the evaluation of physiological and pathological conditions in humans since it contains hundreds of components that may serve to detect systemic diseases (Schipper *et al*., 2007). Saliva is a very attractive form of sample to collect because it is easier to obtain, has low storage cost and is non-invasive. The advent of modern molecular techniques has seen an increase in the use of saliva for medical investigations (Lima *et al.*, 2010).

The utilisation of saliva as a specimen for diagnosis is due to its exchange with substances within the plasmatic liquid. This occurs due to the presence of a thin layer of epithelial cells separating the salivary ducts from the systemic circulation, making it possible for substances to be transferred to the saliva through active carriage, by diffusion through the membrane via a passive process or against a concentration gradient (Schipper *et al.*, 2007). Previous studies have also shown that whole saliva consists of secretions from the salivary glands, bronchial and nasal secretions, bacteria, viruses, fungi, and epithelial cells. Blood and blood components may enter the saliva through intraoral bleeding (Kaufman and Lamster, 2002). Hepatitis, a disease that causes liver inflammation that has a viral etiology, can be diagnosed through the saliva. Van der Eijk *et al*. (2004) demonstrated the first precise quantitative measurement of hepatitis B virus DNA levels in saliva in comparison with a test conducted on blood samples. The results of this study showed that saliva could be used for diagnosis. Thus saliva has continually been proven to be a valuable non-invasive diagnostic tool (Parry *et al*., 1989).

Data previously published by Mharakurwa *et al*. (2006) shows that *Plasmodium falciparum* malaria infection is detectable by PCR on human urine and saliva samples. Moreover, a study by Nwakanma *et al.* (2009) was able to establish significant correlation in quantitative PCR results for saliva with microscopy counts on blood samples of malaria positive individuals. Previous studies have shown that buccal fluids contain a small amount of macrophages (Kaufman and Lamster, 2002). *Plasmodium* DNA from lysed parasites may, therefore, be carried passively via the serum or within the phagosomes of macrophages of malaria infected human hosts. The exact route of *Plasmodium* DNA into the saliva is not clearly defined at present; hence, in this particular study DNA extracts would be collected from different fractions of the saliva in order to establish the source of *Plasmodium falciparum* DNA in human saliva, with respect to whether its immediate origins is extracellular or intracellular.

1.4.2.1 Purification of DNA from saliva

In general lengthy DNA template sample preparations are not required for PCR to work efficiently, because the sensitivity of the technique. Unfortunately this is always not the case in human saliva for malaria detection, as saliva contains lots of impurities and PCR inhibitors (Ochert *et al*., 1994). For this reason, only commercial kits effectively recover DNA from saliva to detect malaria using PCR, which is a challenge in endemic areas, whose populations tend to be poor. Great caution must be exercised to avert DNA degradation in a purified sample. Firstly, DNA extracts may be contaminated by bacteria during extraction, but storage at -20°C will minimize bacterial metabolism and the release of nucleases, thereby reducing DNA loss. During extraction DNases may be accidentally introduced from the skin. Also recurring thawing cycles may also contribute to DNA degradation (O'Brien, 2002).

1.5 The problem of the study

As of 2008 only 12 countries were still using DDT for the control of malaria. Amongst these were India and some southern African states (WHO, 2009). The use of DDT for indoor residual spraying (Mabaso *et al*., 2004), has been helpful in the eradication of *Anopheles funestus* in countries such as South Africa. This was an important step since *Anopheles funestus* is an all year round malaria vector. Malaria control methods that have been implemented for several years involve targeting both vector and parasite. These efforts have significantly reduced the prevalence of malaria in some parts of countries in the southern tip of Africa, particularly in South Africa (Mabaso *et al*., 2004).

However, the disease still remains a public health burden concern to South Africa as countries that share porous borders with neighbours who still have limited malaria control measures in place (Mabaso *et al*., 2004). Hence, it is important that subject-friendly methods for malaria surveillance and monitoring be put in place to identify emerging and traditional strains of the parasite community (Mabunda *et al*., 2008). Hence this study focused on optimising a PCR based assay properly for the detection of malaria in saliva. Testing malaria on saliva samples would facilitate malaria surveillance and monitoring programmes.

To date the well-known malaria life cycle (Figure 2) does not however account for the presence or the entry route of parasite DNA into the human saliva which was previously reported (Mharakurwa *et al*., 2006) so the outcomes of this study may shed more knowledge on the complexity of the life cycle of the malaria parasite. The mechanism by which DNA for the malaria parasite ends up in saliva is little understood and thus reliability of saliva as an ideal test material needs to be validated. Thus, this study aimed to confirm whether PCR on human saliva samples yields data that is comparable to tests contacted on blood samples derived from malaria patients. It is hypothesised that the utilization of human saliva would enable the same detection of malaria parasites as is currently possible with blood specimens. This study was designed as a qualitative study to assess and explore agreement or association between PCR assays using human saliva compared with blood DNA extracts in malaria infected individuals.

The sensitivity and specificity of *P. falciparum* malaria detection in saliva samples will be validated by conducting nested PCR on blood samples as the gold/reference standard. If these prove satisfactory, a new, simple and non-invasive line of techniques for malaria detection during epidemiological surveys for research and that using saliva can be a valid alternative for obtaining clinical specimens. Data obtained from this study has significance towards establishing a bloodless epidemiological research tool, used for surveillance and possible diagnosis of malaria. The outcomes of this study will aid in optimising DNA extractions and detection sensitivity for potential practical clinical applications of human saliva in malaria research and control. The data may also generate new knowledge on possible mechanisms by which detectable malaria parasite material enters into saliva of infected human hosts.

1.6 Aims and objectives of the study

The primary objective of this study was to identify the source of amplifiable *Plasmodium falciparum* dihydrofolate reductase (*pf*DHFR) gene (Duraisingh, 1998) in saliva from malaria infected humans, and to measure the sensitivity and specificity of using saliva samples as an alternative diagnosis source for the detection of malaria infection by molecular methods. Furthermore, the study sought to validate and optimise malaria diagnosis using this assay. The results of this study may also give an early detection assay, where drug resistance can be monitored effectively and efficiently in a non-invasive and safe way.

The following specific objectives were pursued in this study:

i. Determining the fraction in saliva specimens of malaria infected human hosts that harbours *P. falciparum* genomic DNA, and development of optimal methods for the collection and extraction of the parasite DNA.

ii. Optimisation of the sensitivity and specificity of *P. falciparum* detection by PCR in saliva, using regular blood-based amplification as gold standard.

iii. Comparing the sensitivity and specificity of detecting malaria in human saliva relative to blood.

Chapter 2

Methodology

The methods used to carry out this project were adopted from the published data by Mharakurwa *et al.,* (2006), which was one of the earliest studies to claim that *Plasmodium falciparum* malaria from human body fluids of malaria infected individuals could be amplified to confirm infection. However, based on the current understanding of the parasite's biology in the human host, it is unknown how the parasite DNA finds its way into saliva. Furthermore, it is also unclear whether the parasite DNA occurs in the soluble or insoluble fraction of saliva constituents. The following is a summary of the study location, materials and methods that were utilised in this study to achieve the intended objective as outlined in Section 1.9

2.1 Study location context

Malaria is a major public health problem in Zambia, where it is responsible for 32-65% of health centre admissions and 40% of all outpatients' attendance. The study samples were collected from the vicinity of Macha (UTMX 818495, UTMY 477000), located in the Zambian southern province. Natural malaria transmission at Macha is hyperendemic, the major vectors being *Anopheles arabiensis* and *An. funestus.* In the study area the collection of blood samples during major malaria surveys or research, frequently lead to tension and reduced community participation, owing to recurrent beliefs usually about Satanism. The development of simple bloodless alternative sampling approaches would lead to greater community participation for research and control of the disease.

2.2 Study sample size

The current study aimed to estimate the sensitivity and specificity of detecting *P. falciparum* in human saliva using PCR. The 95% confidence interval for sensitivity and specificity is given by S ±Z(0.975)*√(S[1-S]/N), where S is the sensitivity (or specificity) and N is the number of positives (for sensitivity) or negatives (for specificity). A sample size of 88 subjects was used. To validate the findings negative and positives controls in the design of the experiments.

2.3 Study design

Samples used in this study were collected during peak malaria transmission seasons from between year 2008 and 2009. Willing parcitipants from areas under headmen areas in the vicinity of Macha were screened for malaria by microscopy and enrolled. Participants of all ages and sexes, with uncomplicated or asymptomatic *P. falciparum* malaria, were eligible to participate, following full explanation of the objectives, procedures, risks and benefits of the study. Data collection was collected using structured questionnaires (for questionnaire design, see Appendix B). Temperature was taken to monitor if the participants were feverish. The history of any symptoms and drug intake in the past 48 hours for all adult participants were recorded. In the case of young children, informed consent was sought from the parents or guardians. The study excluded individuals with severe and complicated malaria, or complicated medical conditions as recommended (WHO, 2003). For active recruitment, only individuals resident in the selected headman area were enrolled. In passive enrolment, only individuals living within walking distance from the hospital, which were able and willing to return for study follow-up, were included. Thick films and filter paper (Whatman ® No 3 MM) blood blots were collected slides were examined at MIAM by an experienced microscopist, followed by feedback to the community, and intervention for confirmed malaria cases. On the day of feedback, whole saliva samples (5 ml) were collected from all willing positive and negative individuals (Mharakurwa *et al*., 2006), in sterile tubes, the saliva specimens were later aliquoted into 1 ml replicate amounts in microcentrifuge tubes at the laboratory and either immediately extracted or stored at -20°C for later extractions (Figure 4).

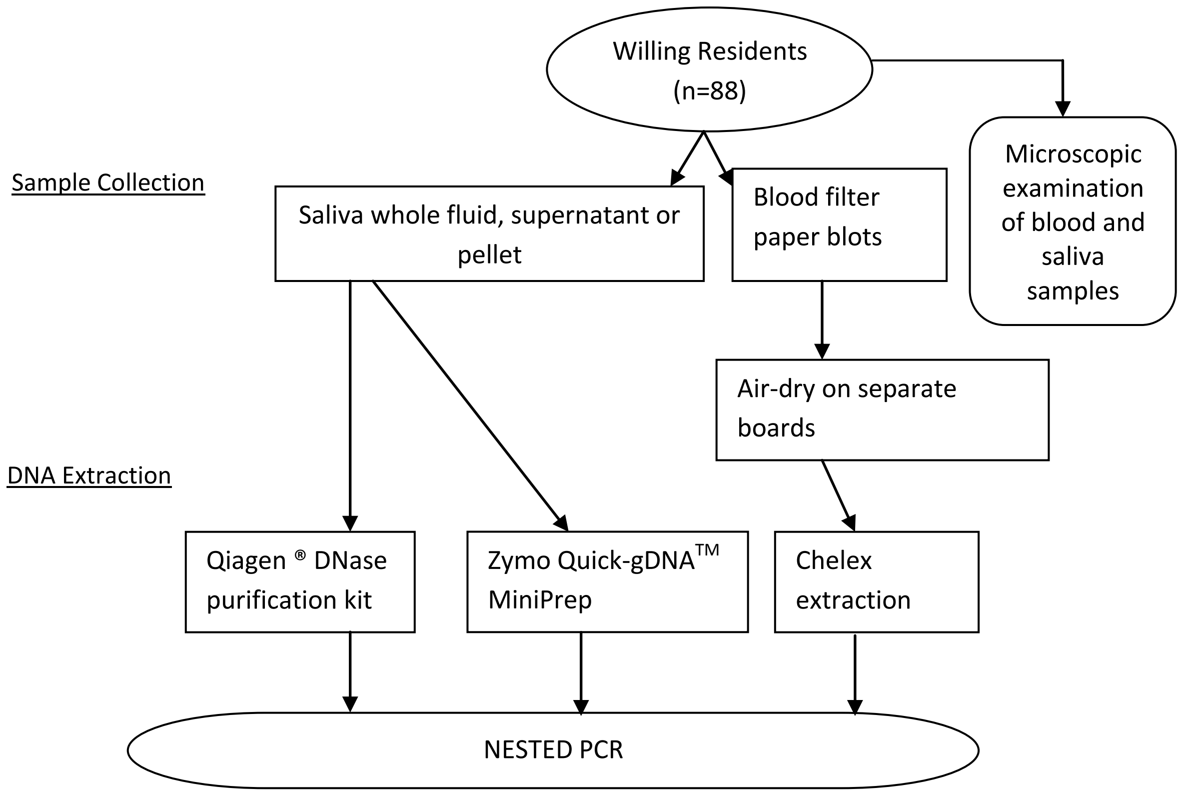

Figure 4 Flowchart of extraction protocol used.

2.4 Materials

Zymo Quick-gDNA™ MiniPrep, Qiagen DNeasy® Blood & Tissue Kit (cat. no. 69504), Whatman 3MM, Chelex 100 resin (Biorad® catalog no. 1422832), TBE Buffer, Saponin, distilled water, 15ml collections sterile tube, Giemsa stain, Microscopic slides. Marker, methanol, weighing boats, 100 bp DNA ladder, DNA loading dye, Pipettes, Techne Flexigene Thermal cycler TC-5000 (Bibby Scientific Ltd, UK), Electrophoresis, UV transilluminator, a computer-linked Kodak EDAS 290 gel imaging system (Eastman Kodak Company, USA). Pipets and pipet tips, vortexer, microcentrifuge tubes (1.5 ml or 2 ml), microcentrifuge with rotor for 1.5 ml and 2 ml tubes, ethanol (96–100%), sample-specific lysis buffer.

2.5 Experimental design and methodology

2.5.1. Specific aim 1: To examine thick blood films and saliva spit films for the presence of *Plasmodium falciparum* parasites under light microscopy.

i. Rationale

This experimental procedure was designed to examine blood samples for any cellular evidence of *P. falciparum,* so as to diagnose malaria infection microscopically. Additional human saliva samples were microscopically investigated for the presence of malaria parasite cells or whether as trace amounts of infected red blood debris or phagocytised parasite debris.

ii. Procedure

Blood samples were collected for microscopic evaluation for all participants of this study. Thick blood films were prepared and air dried (see Appendix A.1.) for detailed preparation thick blood films). The blood films were stained in 5% Giemsa solution for 30 minutes then rinsed carefully and gently in water. The slides were then blindly examined for *Plasmodium falciparum* infection under light microscopy by an experienced microscopist. The same experiments were repeated using saliva instead of blood as starting material to examine the presence of any material of parasite origin in human saliva.

After microscopic examination on the thick blood films, 30 saliva samples (15 malaria infected and 15 malaria uninfected samples) were collected from patients before malaria treatment was administered. The saliva spits were Giemsa stained and microscopically examined, none of the observed organisms could clearly be identified and characterised as *P. falciparum* parasites as saliva contains artifacts which impair visualisation.

2.5.2. Specific aim 2: Assessing the sensitivity of using short polymerase chain reaction amplicon primers to amplify *pf*DHFR gene from blood spots collected on filter papers

i. Rationale

Data previously published by Mharakurwa *et al*., (2006) showed that *Plasmodium falciparum* amplicon yield was influenced by the PCR primer set used for amplification. This study aimed to further validate if short amplicon primer set, enhanced amplicon yield.

ii. Procedure

DNA impregnated collected on filter papers was extracted (see Appendix A.3.) using the chelex 100 resin (Biorad®) DNA extraction protocol (Kain and Lanar, 1991). Standard *pf*DHFR (Duraisingh, 1998) primers or longer amplicon primers (M1/M5, F/M4) were compared to shorter amplicon primer set (U1/U2, U3/U4). The shorter (U1-4) amplicon primers were designed to amplify a primary (370 bp) and secondary (229 bp) amplicon, whereas the longer amplicon primer amplifies a primary (643 bp) and secondary (326 bp) product (Table 1). DNA amplifications were conducted on physically separate batches. The sensitivity and specificity of *P. falciparum* detection by PCR saliva extracts were compared to data derived from assays conducted using PCR amplification from blood and microscopy as corresponding gold standards.

Table 1 Primer used in nested PCR *for P. falciparum* DHFR amplification. Lower case denotes nucleotide mismatches

Reaction	**Primer**	**Nucleotide location on the *P. falciparum* genome**	**Amplicon size (bp)**
1st Nest PCR: U1	5'- GGAAATAAAGGAGTATTACCATG-3'	121...143	273
U2	5'-TAAGGTTCTAGACAATATAACA-3'	393...372	
2nd Nest PCR: U3	5'- GAAATGTAaTTCCCTAGATATGgAATATT-3'	144…172	229
U4	5'- ATTTATCCTATTGCTTAAAGGT-3'	3…18	
1st Nest PCR: M1	5'-TTTATGATGGAACAAGTCTGC-3'	121...143	643
M5	5'-AGTATATACATCGCTAACAGA-3'	645…625	
2nd Nest PCR: F	5'-GAAATGTAATTCCCTAGATATGgAATATT-3'	144…172	326
M4	5'-TTAATTTCCCAAGTAAAACTATTAGAgCTTC-3'	469…439	

Nucleotide mismatches were incorporated into the DHFR primer designs as a system for recognising and repairing erroneous insertion and mis-incorporation of bases that may arise during DNA replication and recombination. The PCR amplicons obtained were resolved by electrophoresis on 1.5% ethidium bromide agarose gels and visualised by UV transillumination on a 1D Kodak (EDAS 290) imaging system (see methodology in Appendix A.2.).

2.5.3. Specific aim 3: To determine the fraction containing *P. falciparum* genomic DNA in human saliva specimens

i. Rationale

So far detection of malaria in saliva relies on amplifying parasite DNA in this fluid. In order to confirm the source of parasite DNA observed, the saliva samples were divided into pellet, and soluble fractions. It was assumed that the occurrence of parasite DNA in pellet and soluble fractions would infer as to the immediate source of the parasite DNA

ii. Procedures

A 1ml of whole saliva sample was equivalently aliquoted into two sterile tube (0.5 ml) i.e tube **(a^1)** and **(b^1)**, both tubes were centrifuged for 3 minute at 14,000 rpm. 250 µl of the supernatant was aspirated from tube **(a^1)** and **(b^1)** into a new sterile tube **(c)** then stored for DNA extraction at -20°C. 200 µl of the remaining supernatant was discarded from tube (**a)** and **(b)**. Tube **(b)** was then stored for DNA extraction at -20°C while Tube **(a)** was resuspended in 950 µl of Phosphate buffer saline (PBS), the solution was then centrifuged at 14,000 rpm for 3 minutes, 950 µl was discarded. The remaining supernatant was resuspended in 950 µl of PBS and centrifugated at 14,000 rpm for 3 minutes after which the supernatant was discarded without disturbing the pellet fraction. Immediately, DNA was extracted from the saliva pellet fraction. DNA was extracted from all saliva samples using the crude cell lysates protocol (see Appendix A.4.) following the manufacturer's (Qiagen DNeasy® purification kit) instructions. Kit Buffer ATL® (200 µl) was used for the initial lysis step on the pellet and final DNA elution was carried out in 25 µl volumes. Blood spots collected on filter paper were extracted using the Chelex method (Plowe *et al*., 1995). The final DNA elution was carried out in 50 µl volumes. A third fraction was made using the reconstituted pellet fraction in a small overlaying volume of supernatant (to make a 10X concentrated suspension). 450ml of supernatant was discarded (Figure 5).

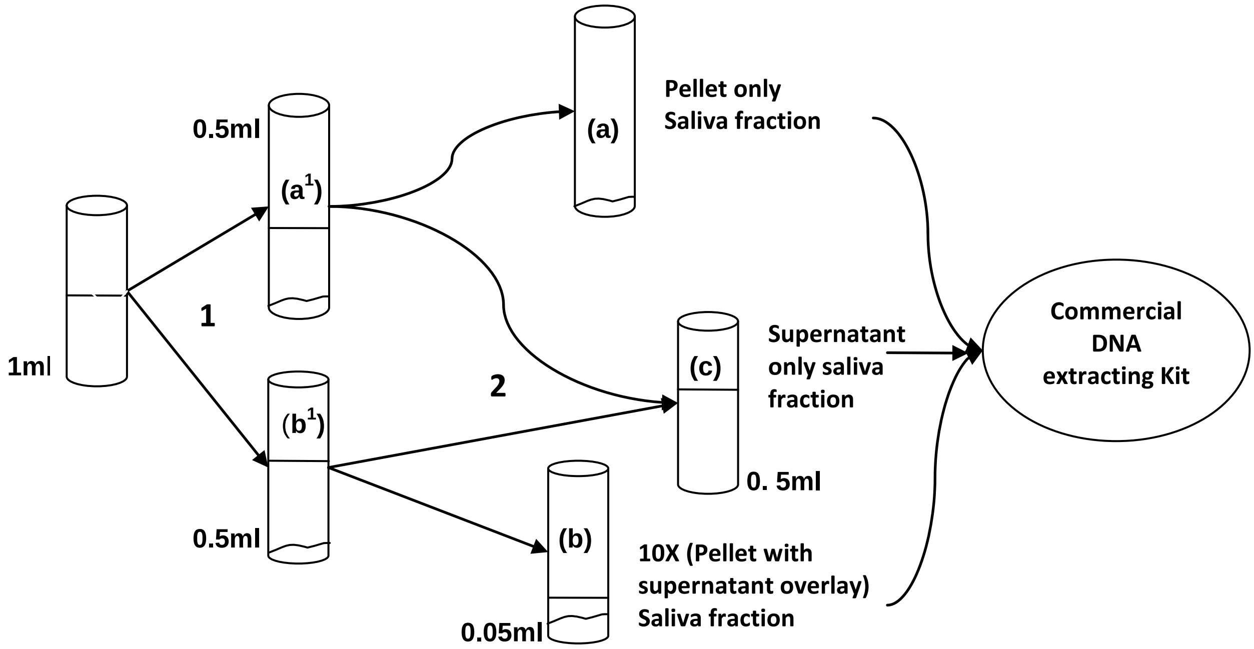

Figure 5 Saliva fractioning flowchart. The diagram show how different fractions; the Pellet only (a), Pellet with supernatant overlay (b) and Supernatant only (c) saliva fractions where prepared for DNA extraction.

Blood and saliva samples from subjects that tested positive for malaria by microscopy were collected and stored (-20°C) at the MIAM Molecular laboratory. Following methods used by Mharakurwa *et al.*, (2006), DNA extracted from blood spots was subjected to nested PCR, primers on the DHFR domain, using genus and species-specific FM/4 and U-primers (Table 1). The PCR protocol and PCR primers were adopted from Mharakurwa *et al*., (2006). Nested PCR was conducted on the saliva extracts and on the DNA extracted from blood spots. Primary and secondary PCR reactions were 15 μl volumes, comprising of 1.2 μl template, 0.25 μM primers, 25 mM magnesium, 10 μM dNTP's, 10× PCR Buffer and 5 U/μl of Taq DNA polymerase.

All PCR amplifications in this study consisted of initial denaturation at 94°C for two minutes, followed by 25 cycles of denaturation at 94°C for 45 seconds, annealing at 43.4°C for 45 seconds and extension at 65°C for one minute. The final extension step was at 65°C for two minutes. All PCR runs had two negative controls and one positive laboratory standard. The Amplified DNA bands were resolved by electrophoresis on 1.5% agarose gel (5 μl of sample per lane) and visualised by UV transillumination.

2.5.4. Specific Aim 4: The optimisation of a PCR based assay for the detection of malaria in human saliva and to assess PCR efficacy using the optimised assay with different extraction kits.

i. Rationale

This investigation aimed at optimising and enhancing the sensitivity and specificity of *P. falciparum* detection by PCR on saliva extract, relative to blood extracts. The data would introduce an adoptable PCR assay for malaria detection using epidemiological research and surveillance programme that is not reliant on blood for accurate diagnosis.

ii. Procedure

DNA extractions were conducted on the pellet fraction of the saliva with a supernatant overlay (as described in Section 2.5.3) using 88 samples including laboratory standard controls. DNA was extracted using the Crude cell lysate protocol and Buccal cells and swab protocol, using the Qiagen DNeasy® purification kit and Zymo Quick-gDNA™ MiniPrep (see Appendix A.3. for methodology) respectively. The samples were subjected to nested PCR, DHFR primers, using the short amplicon primer sets (U1/U2, U3/U4). In order to maximise PCR amplification, the PCR recipe was altered and optimised (Table 2). The recommended PCR protocol was modified by increasing the DNA template and *Taq* polymerase volumes. The saliva extracts were comparatively subjected to nested PCR using the recommended and the modified protocol respectively, with blood DNA as the gold standard (2006).

Table 2 Protocols used in PCR optimisation

	Recommended Protocol	**Modified Protocol**
H2O	10.605 µl	8.625 µl
µM Primers	0.375 µl	0.375 µl
25 mM Mg^{+2}	0.9 µl	0.9 µl
10 mM dNTP	0.3 µl	0.3 µl
10 × PCR Buffer	1.5 µl	1.5 µl
5 µl/1U Taq	0.12µl	0.3µl
Template	1.2 µl	3 µl
Total volume	**15 µl**	**15 µl**

Data from amplicons on the saliva extracts were then compared to that obtained from corresponding blood samples of each individual. The amplified DNA PCR products were resolved by electrophoresis on 1.5% ethidium bromide agarose gels (5 µl of sample per lane) and visualised by UV transillumination on a 1D Kodak (EDAS 290) imaging system (Eastman Kodak Company, USA).

2.6 Data analysis

- PCR results on saliva were cross-tabulated against microscopy and and PCR blood extracts readings, which served as gold or reference standard.
- Diagnostic performance was measured by calculating sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), and receiver operating characteristic (ROC) area under the curve (AUC) calculated using MedCalc ® version 9.6.2.0 nonparametric tests (Hanley and McNeil, 1983).
- Pearson's chi-square test of association was used to evaluate the strength of association between various tests. SPPS was used for this statistical analysis. Inter-rater agreement (**K**, Kappa) (Cohen, 1960) was also used to measure agreement, the strength of agreement was interpreted as follows: poor (<0.20), fair (0.21-0.40) moderate (0.41-0.60), good (0.61-0.80) and very good (0.81-1) **K** is calculated with 95% confidence interval (Altman, 1991).

2.7 Ethics

The study was approved by University of Zambia research ethics committee and University of Zululand ethics committee. Study permission was sought from local chiefs and headmen in whose area the study was conducted. Patient participation was obtained through the consent of the patients themselves or guardians, where minors were concerned (see Appendix C).

Chapter 3

Results

3.1 Subject enrollment and sample processing

A total of 86 samples (blood and saliva) were collected and analyzed from willing individuals who participated in this study, 35 (40, 7 %) were males and 51 (59, 3 %) were females. The age range was from 3 months to 99 years old (mean=29.6 years; median=18 years). None of the examined volunteers had a body temperature reading above 38°C nor was there any below 35°C on the day of examination. However of all the patients' that were tested, 20 participants reported previous history (48 hours prior to testing) of fever and after diagnosis only 10 of those patients were actually malaria infected. Of the total enrolled in this study 51 were *Plasmodium falciparum* positive either by PCR on blood or by microscopy (Table 3). A total of 25 cases of general malaria infection symptoms were reported, 14 of these participations were malaria positive (Table 3).

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Fever is a clinical symptom frequently associated with malaria in malaria endemic areas. However, in this study fever displayed a poor association, χ^2=0.998 (p=0.2475). Similarly diagnosis based on general malaria infection symptoms was not statistical significant, χ^2=0.998 (p=0.3178).

Table 3 Fever and headache frequency table

		Plasmodium falciparum Positives		**Total**
		Positive	Negative	
Fever	Present	10	10	**20**
	Absent	42	26	**68**
Total		**52**	**36**	
General malaria infection symptoms	Present	14	11	**25**
	Absent	38	25	**63**
Total		**52**	**36**	

Fever and headache symptoms could not be correlated to the presence of malaria. The age range of these samples was distributed between 3months and 99 years. A total of 15.9% of the participants displayed these malaria-related symptoms and of these, 40% (4/10) of fevers were observed in the 5-14 years age group. A low percentage of microscopy positive subjects were symptomatic.

3.2 Assessment of the sensitivity of detecting malaria using short amplicon primers in nested PCR reactions to amplify *pfDHFR* gene from blood extract

Data published by Mharakurwa *et al*., (2006), showed that *Plasmodium falciparum* amplification could be influenced by the PCR primer set used for amplification. This experiments sought to further validate if *P. falciparum* DNA amplified by short amplicon primer sets could enhance detection sensitivity. Two primer sets, the short amplicon (U1/U2, U3/U4) primer set and the long amplicon (M1/M3, F/M4) primer set were used to investigate amplification yield (Table 1). DNA samples were extracted from blood spots collected on filter papers from all participants of this study. The DNA extracts were then subjected to nested PCR using the two primer sets on separate batches, alongside standard positive and negative laboratory controls respectively. DNA amplifications were visualised using an electrophoresis gel, the presence of the 229 bp DNA band corresponding to the positive control amplicon DNA was regarded as a positive for *P. falciparum* infection whereas the absence of the 229 bp DNA fragment was regarded as negative for all samples (Figure 6). Positive infections that could not be identified by microscopy but were positive by PCR were regarded as submicroscopic infections.

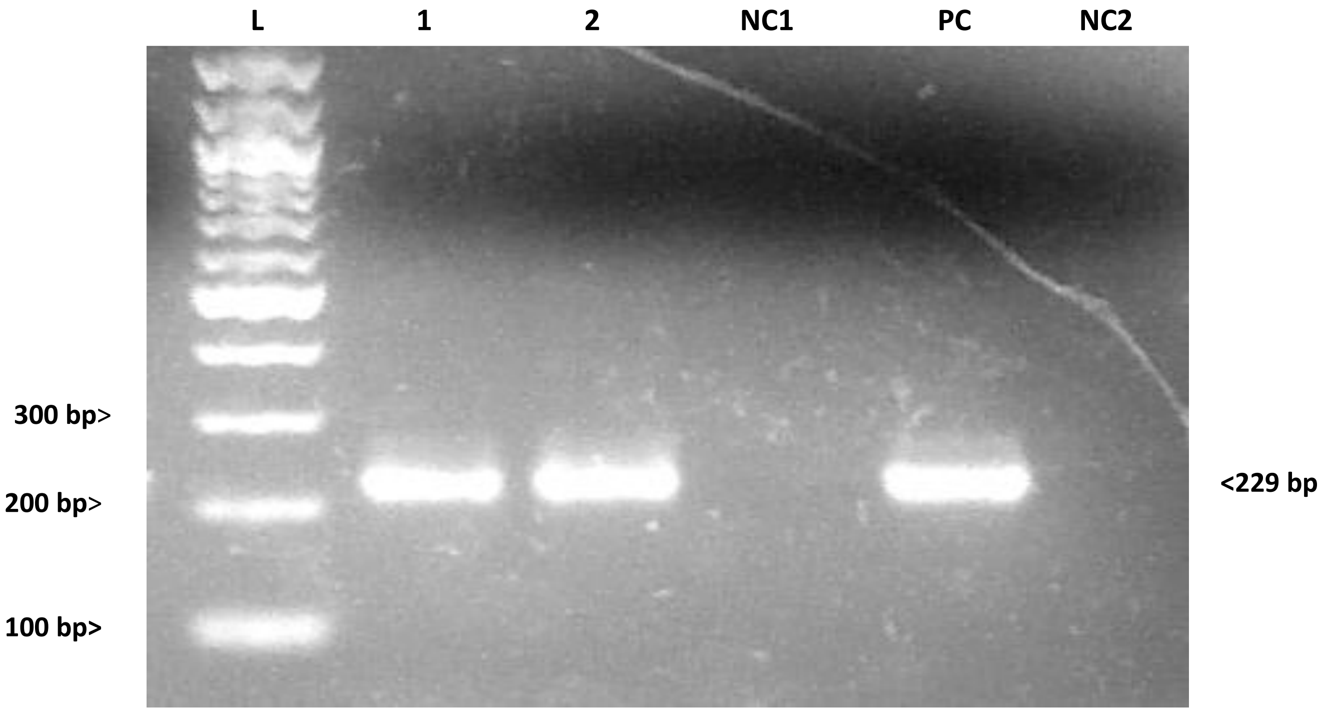

Figure 6 Amplification of *Plasmodium falciparum* DNA fragment. The extracted DNA from blood samples collected on filter paper from two thick positive patients (Lanes 1 and 2), were subjected to PCR amplification. Lane PC, represents the positive laboratory standard (229 bp). NC1 and NC2 are negative laboratory controls. Lane L, is the DNA molecular weight marker.

When the blood extracts were subjected to PCR amplification with the long amplicon primers, a total 33 (37.5%) samples were confirmed to be *P. falciparum* positive. As could be expected PCR had a higher detection sensitivity than microscopy which confirmed only 13 (14.7%) positives. However, short amplicon primers were able to detected 51 (57.95%) *P. falciparum* infections, 31 (35.23%) were submicroscopic and 18 (20.45%) were below long amplicon primer detection limit.

To determine the efficacy of using short amplicon primers, thick film microscopy results were used as reference standard to evaluate the sensitivity and specificity of each primer. Short amplicon primers were more sensitive (95%) as compared to long amplicon primers (52.63%) in amplifying *P falciparum* DNA. A poor PPV was observed for long and short primer sets used, 30.30% and 37.25% respectively (Table 4). The area under the curve and ROC curve analysis was used to display the visual index of the accuracy for the two primer sets with microscopy results as the gold standard.

Table 4 Diagnostic test results on blood samples amplified with long amplicon and short amplicon primers.

	PCR using Long amplicon primers	PCR using with Short amplicon primers
Sensitivity	52.63%	95.00%
Specificity	66.18%	52.94%
Positive Predictive Value	30.30%	37.25%
Negative Predictive Value	83.33%	97.30%

The further the curve lies above the diagonal reference line, the greater the accuracy of the primer set used, the short amplicon primer set curve lies the furthest from the reference line as compared to the long amplicon primer set curve (Figure 7). Short amplicon primers had a greater AUC were significantly accurate when compared to long amplicon primers; 0.740, p=0.0005 and 0.594, p=0.2176 respectively as compared to long amplicon primers for the 88 blood samples observed in this study (Table 5).

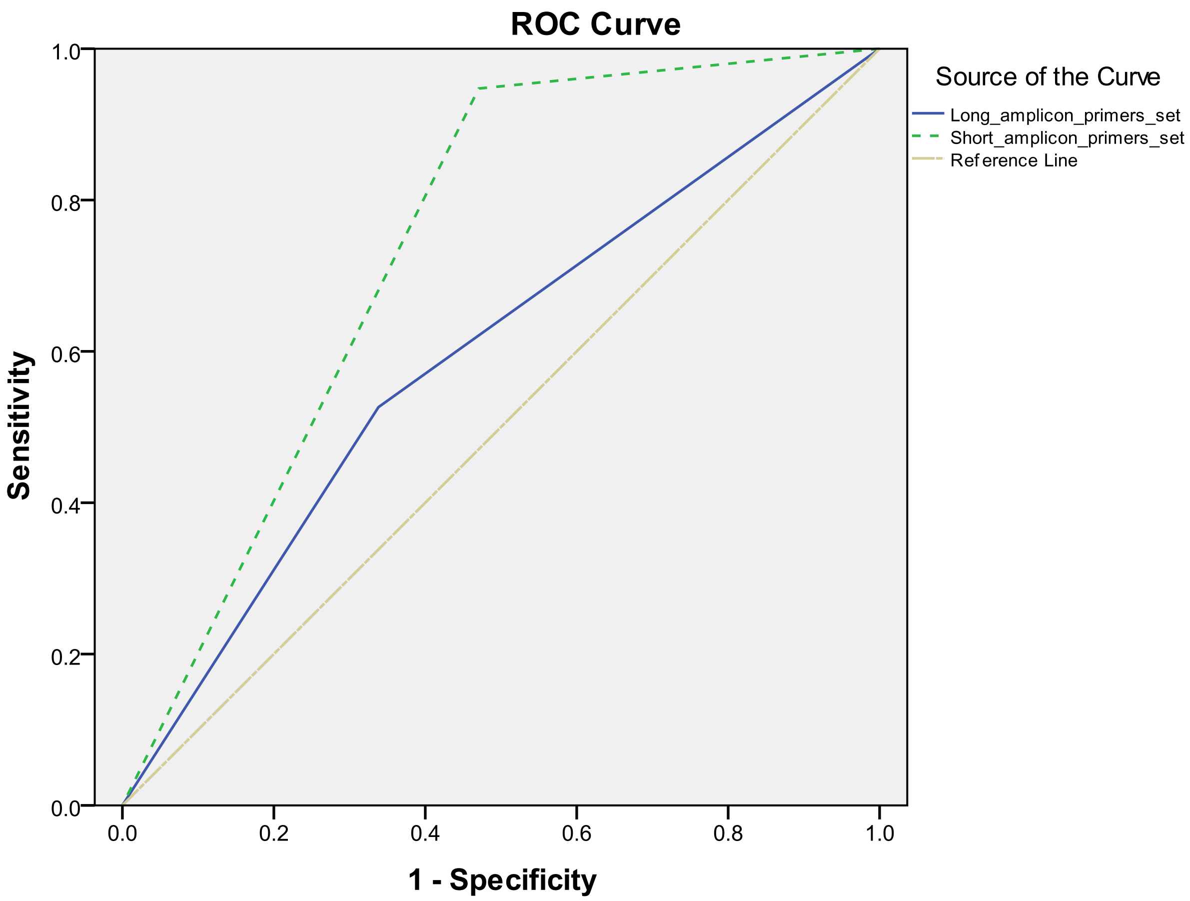

Figure 7 ROC curve on long amplicon and short amplicon primers. The curves in the graph were generated from nested PCR amplifications with long (Blue) and short (green) amplicon primers and the diagonal reference line (Brown)

Table 5 AUC for long amplicon and short amplicon primer set

Area Under the Curve			
Test Result Variables	**Area**	**Std. Error**	**P-value**
Long amplicon primers	0.594	0.0763	0.2176
Short amplicon primers	0.740	0.0688	0.0005

3.3 The fraction of saliva containing PCR amplifiable *P. falciparum* DNA

Human saliva can be used as a source of amplifiable malaria parasite DNA in infected patients, as noted by previous investigators (Mharakurwa *et. al.,* 2006). PCR was conducted on both blood and saliva samples. Below is a gel picture of DNA amplicons obtained from three thick film positive patients who donated saliva and blood samples (Figure 8). The saliva and blood extracts were subjected to nested PCR using the same protocol, all the tested samples amplified the 229 bp DNA fragment as envisaged and none of the negative controls gave false positive results.

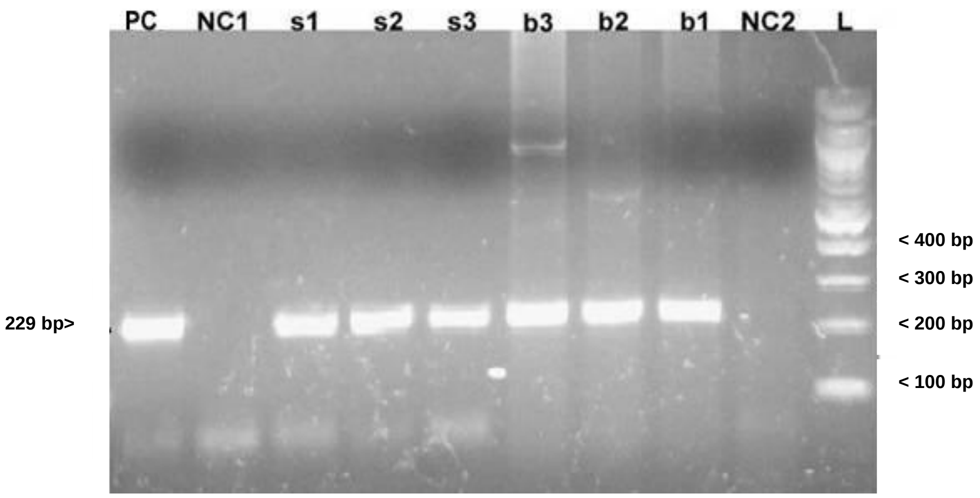

Figure 8 Amplified *Plasmodium falciparum* DNA extracted from saliva and blood DNA samples
Saliva and blood DNA extracts from three individuals, subjected to nested PCR amplification. Amplicons for DNA extracted from saliva sample (s1, s2 and s3) and blood (b1, b2 and b3) extracts. Lane PC represents positive laboratory control (229 bp), lane L represents the DNA molecular weight marker the DNA mole. NC1 and NC2, represents negative controls.

To determine the fraction of saliva harbouring the malaria amplifiable DNA, saliva samples obtained from the 42 *Plasmodium falciparum* positive human subjects and three negative subjects, were separated into three fraction saliva fractions, the pellet only fraction, supernatant only fraction and the pellet fraction reconstituted in a small overlaying volume of supernatant (to make a 10X concentrated suspension). After DNA extractions the samples were further subjected to PCR in separate batches and the DNA amplicons were resolved on a gel electrophoresis. The short amplicon primers were used for all PCR amplification. DNA amplified from the pellet only fraction had a highest (Table 6) amplification with PCR amplification on blood as the reference standard.

Table 6 Comparison of Nested PCR amplicons extracted from saliva fractions.

Nested PCR on blood samples amplified using short amplicon primers		
	Positive (n=42)	Negative (n=3)
Supernatant only fraction		
Positive	26	1
Negative	16	2
Pellet only fraction		
Positive	31	0
Negative	11	3
10X fraction		
Positive	29	1
Negative	13	2

All three saliva fractions were more sensitive than conventional microscopy. The sensitivity and specificity of the pellet fraction, 73.81% and 100% respectively was the most accurate of the three DNA fraction sources (Table 7). The positive predictive value for all three test DNA sources were high (>96%), as opposed negative predictive value which was relatively low (<22%), possibly due to the low number of negative samples in the study. An inter-rater agreement value (κ, Kappa) (Cohen, 1960) was calculated with 95% confidence interval. According to the Altman (1991) interpretation, DNA amplified from the supernatant only and pellet with supernatant overlay had a poor (κ<0.20) amplification agreement, whereas DNA extracts amplified from the pellet only saliva fraction had fair agreement, of κ=0.273 (Table 7).

Table 7 Diagnostic performance test for amplicons yields obtained from different saliva fractions

	Supernatant only	**Pellet only**	**Pellet with supernatant overlay**
Sensitivity	61.90%	73.81%	69.05%
Specificity	66.67%	100.00%	66.67%
Positive Predictive Value	96.30%	100.00%	96.67%
Negative Predictive Value	11.11%	21.43%	13.33%
Kappa (K)	0.086	0.273	0.125

The DNA extracts prepared from the insoluble fraction of saliva and the pellet with supernatant overlay fractions were subjected to PCR and the results from these amplifications were not statistically significant, χ^2=0.134 (p=0.7144) and χ^2=0.402 (p=0.5262). However DNA amplification from the pellet fraction displayed the highest contingency coefficient value=0.289 and χ^2=4.09, p=0.0431 (Table 8). This result therefore suggests there is a significant relationship in the amplification of malarial DNA from the saliva pellet fraction, relative to amplification done on blood samples using the recommended nested PCR protocol by Mharakurwa *et al*. (2006). The results further propose that the pellet may be responsible for the introduction of malarial DNA in saliva.

Table 8 Pearson's chi-square test for different saliva fractions amplicons subjected to nested PCR using short amplicon primers.

Chi-square test				
	Value	Df	Significance level	Contingency co-efficient
Supernatant only amplicon	0.134	1	p= 0.7144	0.054
Pellet only amplicon	4.09	1	p=0.0431	0.289
Pellet with supernatant amplicon	0.402	1	p=0.5262	0.094

The AUC and ROC curve analysis were used to display the visual index of the accuracy for the two primers with microscopy results as gold standard (Figure 9). The further the curve lays above the diagonal/reference line, the more accurate the test. Short amplicon primers used to amplify *P. falciparum* DNA saliva samples was significantly accurate. ROC curve analysis confirmed that DNA amplified from the pellet fraction of the saliva was significantly accurate (p=0.034) as compared to DNA amplicons extracted from the supernatant only (p=0.413) and the pellet with supernatant overlay (p=0.306) fraction of the saliva.

Table 9 AUC reading for amplicons yields obtained from different saliva fractions

Area Under the Curve			
Test Result Variables	Area	Std. Error	P-value
Supernatant only	0.643	0.165	0.413
Pellet only	0.869	0.062	0.034
Pellet with supernatant overlay	0.679	0.164	0.306

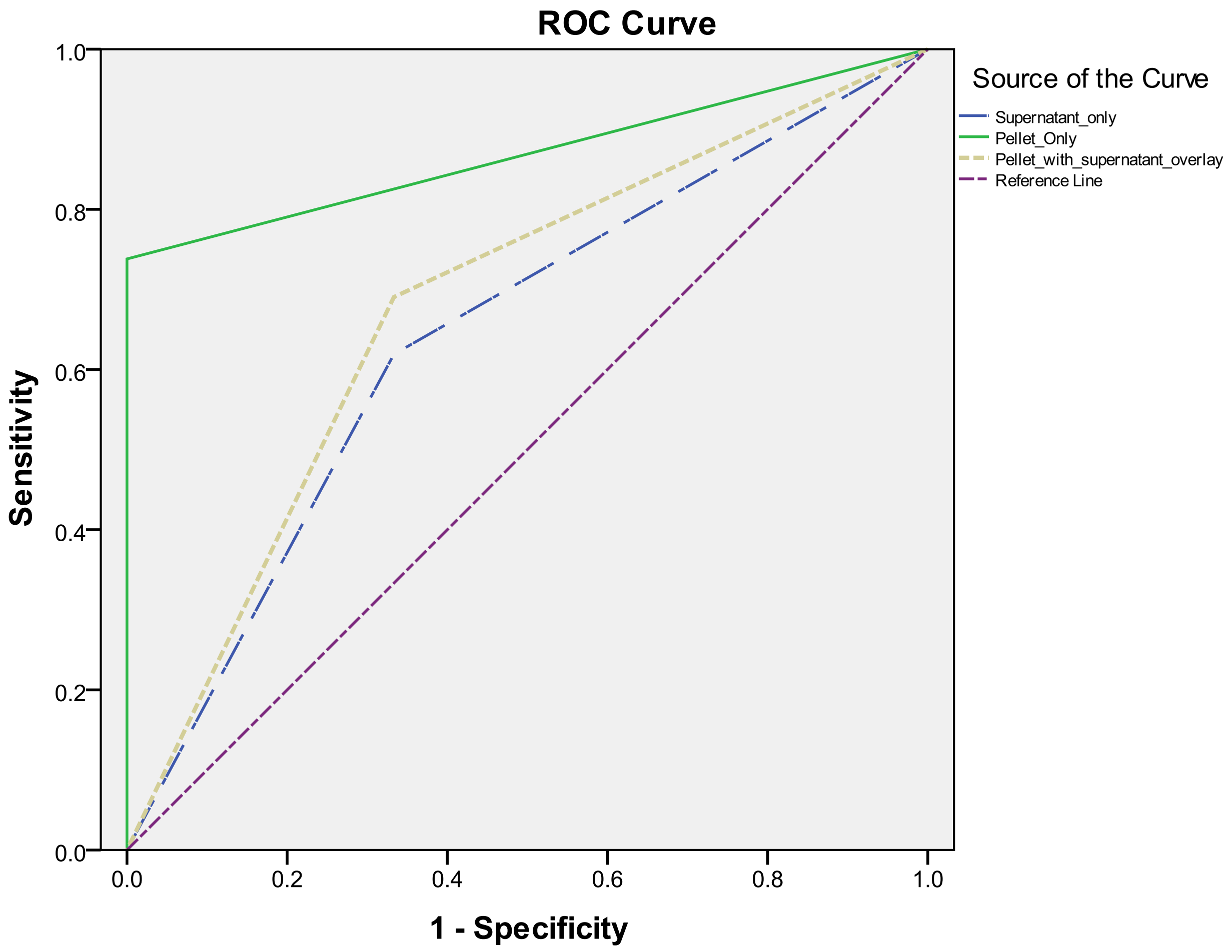

Figure 9 ROC curve analysis for PCR amplicons from different saliva fraction extracts. A graphical representation for ROC curve analysis for DNA samples extracted from different fractions of saliva. DNA samples subjected to nested PCR, the results

3.4 Nested PCR optimisation and the effects of amplification using different extraction kits

The primary objective of this study was to assess if saliva could potentially be used as a non-invasive DNA source used in malaria detection. Furthermore the study sought to enhance and optimise the sensitivity and specificity of *P. falciparum* detection in human saliva samples relative to blood extracts. Different DNA extraction kits were employed to establish the best method for DNA recovery. Nested PCR amplification was conducted on DNA derived from 88 saliva samples, after rigorous PCR protocol optimisation had been undertaken. The modified PCR protocol showed a greater sensitivity and specificity relative to blood extracts using short amplicon primers. DNA extracted from the pellet fraction covered in a small overlaying volume of supernatant (making a 10X concentrated suspension), was used for nested PCR amplification using the optimised protocol. When compared to blood, 48 true positive samples were confirmed in both saliva and blood DNA samples and 36 true negatives were identified (Table 10).

Table 10 PCR amplification cross tab count for DNA derived from saliva DNA samples extracted with different extraction kits.

		Nested PCR on blood samples		**Total**
		Negative	Positive	
Qiagen Kit extracts	Negative	36	3	**39**
	Positive	1	48	**49**
Total		**37**	**51**	**88**
Zymo kit extracts	Negative	35	18	**53**
	Positive	1	31	**32**
Total		**36**	**49**	**85**

To assess the effects of amplification for different DNA extraction kits, DNA was purified from the same saliva samples using the Zymo kit. The DNA extracts were also subjected to nested PCR using the optimised protocol, relative to blood. Thirty-one true positive samples were properly amplified in saliva as in blood and 35 true negatives were identified, lower than DNA amplified from the Qiagen kit. DNA amplified from the Zymo kit also incorrectly identified 1 negative as a positive sample (Table 11). DNA extracts from saliva samples obtained using the Qiagen kit displayed a sensitivity of 94.12%, specificity 97.3%, the PPV was 97.96 and the NPV was 92.31% when subjected to PCR, DNA obtained from blood samples were used as the gold standard. DNA samples extracted with the Zymo kit were also subjected to PCR using the optimised protocol. DNA extracted with the Zymo kit were less sensitive (63.27%) when compared to DNA amplified from samples extracted with the Qiagen kit. DNA amplification on the Zymo extracts displayed a specificity of 97.22%, PPV was 96.87% and the NPV 66.04% against blood samples as the gold standard (Table 11). The Cohen's kappa test displayed a very good (κ=0.907) agreement value for saliva DNA extracted with the Qiagen kit when compared to PCR on blood extracts. The kappa test κ=0.569 agreement for amplicons for samples extracted with the Zymo kit as compared to amplicons extracted with the Qiagen kit. These results seem to suggest that DNA purification is enhanced by the extraction kit used.

Table 11 Diagnostic performance comparing DNA extraction kits. Nested PCR results from blood samples are used as the reference standard.

	Qiagen-kit	**Zymo-Kit**
Sensitivity	94.12 %	63.27 %
Specificity	97.3 %	97.22 %
Positive Predictive Value	97.96 %	96.87 %
Negative Predictive Value	92.31 %	66.04 %
Kappa Value (κ)	0.907	0.569

The AUC represents the probability that the assay result for a randomly chosen positive case exceed the result for a randomly chosen negative case. Below is a Table 12 with AUC test result for the following variables; microscopy, saliva DNA extracted with the Qiagen kit and saliva samples extracted with the Zymo kit. All test variables were statistically significant (p-value ≤ 0.05). The Qiagen kit, however, displayed the highest AUC value of all test variables (0.969) and microscopy had the lowest AUC value (Table 12). The ROC curve is a visual index for the accuracy of the saliva assay in malaria diagnosis, a ROC curve was generated using the DNA amplification results. Higher sensitivity was achieved using DNA extracted by the Qiagen kit as compared to the same test conducted using DNA purified with the Zymo kit (Figure 10).

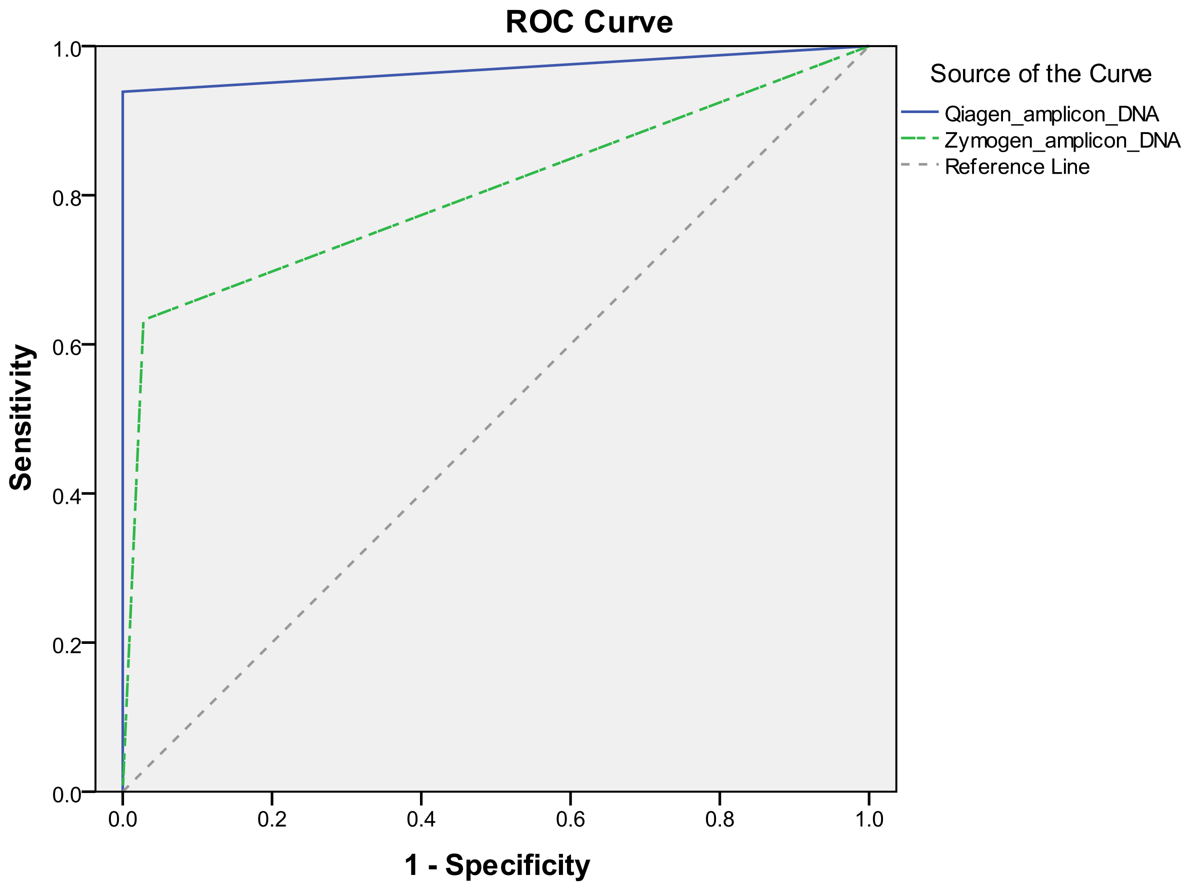

Figure 10 ROC curve analysis for DNA amplified from saliva samples extracted using different kits. DNA samples were purified from saliva samples using the Qiagen kit and with the Zymo kit. The samples were then subjected to DNA amplification to using PCR and the result were used to plot the ROC curve**.**

Table 12 AUC results for saliva DNA amplicons extracted using different kits.

Area Under the Curve			
	Area	Std. Error	p-value
Qiagen Kit amplicon	0.969	0.020	0.000
Zymo Kit amplicon	0.802	0.048	0.000

The statistical significance of the difference between the two areas under ROC curves were tested using MedCalc statistical AUC comparative test. The calculated difference between the AUCs was 0.167, (p-value=0.0013) which revealed that two ROC curves were significantly different.

Chapter 4

Discussion

PCR is expected to generally have an elevated sensitivity when compared to microscopy especially in detecting parasitemia at levels undetectable to microscopy parasite (Snounou *et al*., 1993). Results obtained from PCR amplification on blood samples were superior to those obtained by thick film microscopy; the majority of samples that were thick film negative were identified to be positive by PCR for *P. falciparum* infection. A total of 22.7% *P. falciparum* cases could be identified using microscopy, 35.23% were below microscopy detection levels. A similar finding was reported where microscopy detected a total of 350 *P. falciparum* infections while PCR detected a further 331 *P. falciparum* submicroscopic infections (Zurovac *et al*., 2006). According to Bejon *et al*. (2006) true parasite counts are underestimated by microscopy due to the staining procedure, hence the poor microscopy performance. In order for endemic countries to effectively maintain malaria interventions, asymptomatic infection and latent disease reservoirs need to be closely monitored so as to avoid potential disease resurgences. Hence PCR can be a very useful diagnostic method applied for the accurate identification of individuals carrying parasites below the threshold for slide microscopy is necessary to determine the remaining potential for malaria transmission in a population.

As previously noted the probability of PCR amplification is influenced by the PCR primer set used from a given infection (Mharakurwa *et al.,* 2006). Short amplicon primers used in this study had a higher amplification rate as compared to long amplicon primers and consequently, the former were more reliable for detecting latent infections. These results obtained in these study further support claims previously presented by Mharakurwa *et al.,* (2006) that short amplicon primers enhance the sensitivity for *P. falciparum* DNA amplification. It might be possible that at low level of parasite density, long amplicon primers were less reliable than short amplicon primers because of the poor recovery of good quality parasite DNA. Possible DNA degradation in saliva during storage and transportation (O'Brien, 2002; Kaufman and Lamster, 2002) may be responsible for the low amplification sensitivity when using long amplicon primers.

This study for the first time presented results that attempt to identify the fraction of saliva that harbours amplifiable malarial DNA. According to the findings of this study, DNA extracted from the pellet fraction was a more reliable template for the PCR test. Histological experiments conducted in this study failed to detect *P. falciparum* parasite cells from the saliva samples examined. This then tends to suggest that *P. falciparum* DNA is introduced into human saliva through an intracellular component; probably through ruptured RBC's or DNA from parasites trapped in macrophages (Kaufman and Lamster, 2002). The DNA amplified in the supernatant portion of saliva is presumably due to reduced accidental loss of pellet material during pipetting or DNA extraction or during sample collection. However, further study is still required to clarify how malarial DNA is transported to saliva of malaria infected patients.

Through optimising the nested PCR recipe on saliva DNA extracts, this study was able to achieve high amplicon yields than previously reported (Buppan *et al.*, 2010), relative to DNA purified from blood samples. For example, DNA derived from saliva using the Qiagen extraction kit and blood, a 94, 1% sensitivity and 97% specificity was observed on saliva samples, with PCR on blood as the gold standard. Buppan *et al*. (2010) further suggests that saliva samples preserved in ethanol yielded superior positive PCR results when compared to samples kept on ice. However, the absence of ethanol preservation in this study does not appear to have negatively affected PCR amplification. A very good agreement (κ=0.907) was observed for DNA derived from saliva using the Qiagen extraction kit relative to blood extracts, this study further supports the accuracy of saliva as an alternative source for malaria infection diagnosis which may be adopted for large scale malaria screenings (Breman *et al*., 2004).

In this study, only 15.9% of the volunteers had a history of malaria-related symptoms. Under normal circumstances the 84.1% asymptomatic patients would not have gone for malaria testing, this could have led to a delay of the correct diagnosis and could compromise early disease management. Malaria control requires the effective identification of asymptomatic parasite carriers, who are often a source of perpetual disease transmission (WHO, 2008). This study further confirms diagnosis of malaria in saliva as a non-invasive method for diagnosing malaria that could be readily adopted to monitor asymptomatic latent malaria.

PCR amplification is also greatly influenced by the extraction kit. The Qiagen DNeasy® purification kit showed a greater accuracy when compared to the Zymo Quick-gDNA™ MiniPrep. Although the Qiagen extraction kit increases amplification probabilities it is expensive which presents an obstacle for its use in endemic countries. Future studies should be directed at development of cheaper DNA extraction methods. Nevertheless, this study presents prospects to avoid the use of needles or sharps in the drawing of blood. The data also helped to add new knowledge on possible mechanisms by which detectable malaria parasite material enters into saliva of infected human hosts. The improvement of this method is of primary importance before saliva and possibly urine samples can be reliably applied in alternative diagnosis of malaria epidemiological and vaccine efficacy studies.

Chapter 5

Conclusion and suggestions for future studies

This study further confirms that saliva samples are a reliable non-invasive alternative to blood for the PCR detection of malaria, as previously reported. The source of DNA was primarily in the pellet fraction though; further refinement is still needed to identify the exact source of DNA in that fraction. Future work could involve finding cheaper non-commercial DNA extraction methods to alleviate the financial cost, saliva processing with the chelex 100 extraction which has proved to be effective in preventing PCR inhibition for saliva samples (Matto *et al.*, 1998). The execution of nested PCR in one reaction vessel could also be investigated to further aid this PCR diagnostic assay against cross-contaminations. More work could also be based on optimisation of this assay to make it cheaper and more robust for use in malaria surveillance. The recently described loop-mediated isothermal amplification by Han *et al*. (2007), yielded comparative sensitivity and a specificity results to those obtained by nested PCR on blood samples may also be investigated for amplification using saliva extracts for malaria detection, could also be a useful tool in malaria surveillance as its not strictly laboratory based.

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Appendix A

General Experimental Procedure

A.1. Giemsa staining on thick blood films

The specimen usually consists of fresh whole blood collected by finger puncture on microscopic slides. The slides were allowed to air-dry thoroughly, and were not fixed. Stain with diluted 5% Giemsa solution for 30 min. Rinse gently under running water. The slides were left to air dry in a vertical position and later examined using a light microscope.

A.2. Agarose gel electrophoresis

DNA was visualized on 1.5 % agarose, the require amount of agarose was suspended in 0.5 x TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0). The agarose was heated in a microwave for approximately 60 – 90 seconds to dissolve. The agarose gel was then cooled to about 55˚C before adding ethidium bromide to a final concentration of 0.5 µg/ml. The agrose gel was then poured into a casting tank in the presence of a comb for marking the gel wells. The comb was removed, after the gel had set. The gel was placed into an electrophoresis tank and 0.5 x TBE buffer added to a level sufficient to cover the gel. Air bubbles trapped in gel wells were removed, following which the DNA was loaded. The electrophoresis tank was connected to a power source ensuring that the DNA moves towards the anode. Voltage was adjusted to 100 volts and the electrophoresis run was allowed to proceed until the gel front had travelled for about ¾ of the gel length. The DNA was then visualized by UV transillumination on a 1D Kodak (EDAS 290) imaging system.

A.3. DNA preparation from filter paper blood spots

For each sample that was processed, 180 ,ul of a 5% (wt/vol) Chelex-100 solution (Bio-Rad, catalog no. 1422832) was added to a 1.5-ml microcentrifuge tube and placed in a heating block at 100°C for 5 min. Each filter paper sample (approximately 1 cm in diameter) was excised with a new, clean razor blade and added to the hot Chelex solution. The tube was capped, gently vortexed for 30 s, and returned to the heat block for 10 min. The samples were centrifuged (12,000 x g for 1.5 min), and the supernatant was removed to a new microcentrifuge tube and spun again (12,000 x g for 1.5 min). The supernatant was removed to a new microcentrifuge tube and either used immediately in an amplification reaction or stored at 4°C until required.

A.4. DNA preparation from saliva samples

i. The Qiagen DNeasy® Blood & Tissue Kit (cat. no. 69504 or 69506) was used for DNA extraction. The purification of genomic DNA from crude lysate protocol was used to extract DNA from saliva samples, according to the manufactures instruction with modifications on the final DNA elution volume from the recommended 200 μl to 50 μl. The extraction was conducted as follows:

- The 500 μl saliva sample was lysed in 200 μl sample-specific lysis buffer sample.
- Proteinase K (20 μl), followed by 200 μl Buffer AL was added to the sample, and thoroughly mixed by vortexing.
- Absolute ethanol (200 μl) was added and thoroughly vortexed.
- The mixture was then added into the DNeasy Mini spin column placed in a 2 ml collection tube then centrifuge at 8000 rpm for 1 min.

- The flow-through and collection tube was discarded.The DNeasy mini spin column was added into a new 2 ml collection tube, 500 µl.
- Buffer AW1 was added and centrifuge for 1 min at 8000 rpm. The flow-through and collection tube were then discarded.
- The DNeasy Mini spin column was added into a new provided collection tube,
- A 500 µl of AW2 buffer was added and centrifuge for 3 min at 14,000 rpm to dry the DNeasy membrane. The flow-through and collection tube was discarded.
- Following the centrifugation step, the DNeasy Mini spin column was removed and placed into a new 1.5 ml microcentrifuge tube.
- A volume 200 µl Buffer AE directly onto the DNeasy membrane, incubated at room temperature for 1 min, and then centrifuged for 1 min at 8000 rpm.
- The DNeasy membrane was discarded and the remaining DNA elution was immediately for PCR amplification or stored below -20°C later use.

ii. The Zymo research quick-gDNA™ MiniPrep (cat. no D3006) was also used to purify DNA from saliva samples using the buccal cells and swabs protocol. DNA was extracted according to the manufactures instruction as follows:

- A volume of 500 µl Genomic lysis buffer, was added onto 500 µl of saliva sample vortexed for 6 seconds, and then left to stand at room temperature for 10 minutes.
- The mixture was transferred to the Zymo-Spin™ to the provided column with collection tube and centrifuged at 10,000 x g for one minute.

- The collection tube was discarded with the flow through the place Zymo-Spin™ column to a new collection tube.
- DNA pre-wash buffer (200 µl) was added to the spin column and the centrifuged at 10,000 x g for one minute.
- A 500 µl volume of g-DNA Wash Buffer was added to the spin column. Centrifuge at 10,000 x g for one minute. The spin column was then transferred to a clean microcentrifuge tube.
- The DNA was eluted in 50 µl of the manufactures elution buffer. The buffer was added into the spin column and Incubated for 4 minutes at room temperature and then centrifuge at 14000 rpm for 30 seconds
- The eluted DNA was immediately for PCR amplification or stored below -20°C later use.

➢ Phosphate buffer saline (PBS) composition

Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4 and 0.24 g KH2PO4 in 800 ml of distilled H2O. Adjust the pH to 7.4 with HCl. Add H2O to 1 liter. Sterilize by autoclave

Appendix B

Supplementary Data

Table 8 Data sample sheet table.

			Blood sample			Saliva samples				
						Qiagen Extracts			Optimised PCR Protocol	
Sample ID	**AGE**	**Gender**	**Thick film Microscopy**	**Long amplicon primers**	**Sort amplicon primer**	**S.O**	**P.O**	**10 X**	**Qia 10X**	**10 X Zymo**
H10-26	14	M	+	+	+	-	+	-	+	+
H10-56	8	F	+	-	+	+	+	-	-	+
H10-52	12	F	-	+	+	+	+	+	+	+
A9-75	4	M	+	-	+	-	+	+	+	-
H10-6	14	M	+	+	+	+	+	-	+	-
A5-2	66	M	-	+	+	-	+	+	+	+
A5-18	11	M	+	-	+	+	+	+	+	+
A5-87	ND	M	-	+	+	-	-	+	+	+
A9-45	0.24	F	-	+	+	+	+	+	+	+
A9-7	5	F	-	+	+	+	+	+	+	+
B5-86	7	F	+	-	+	+	+	+	+	-
C10-23	13	F	+	+	+	+	-	+	+	+
C10-34	6	F	+	-	+	+	-	+	+	-
F1-6	62	F	-	+	+	+	-	-	+	+
F1-50	6	M	+	-	+	+	-	-	+	+
A4-52	13	M	+	-	+	-	+	-	+	+
A5-1	26	M	-	+	+	-	+	+	+	-
A5-4	30	M	-	+	+	+	-	-	+	+
A5-52	35	F	+	+	+	+	+	+	+	-
D4-9	6	F	+	+	+	-	+	-	+	+
E9-1	64	M	-	+	+	-	+	+	+	+
E9-2	58	F	-	+	+	-	+	-	+	+
E9-3	56	F	-	+	+	+	-	-	+	+
E9-4	78	F	-	+	+	-	+	+	+	+
E9-5	5	F	-	+	+	-	+	+	+	-
E9-6	5	F	-	+	+	-	-	+	+	+
E9-7	5	M	-	+	+	+	+	+	+	+
E9-8	5	M	-	+	+	-	+	+	+	+
E9-9	7	M	-	+	+	+	+	+	+	-
E9-10	6	M	-	+	+	+	+	+	+	-
H10-47	11	M	+	+	+	-	-	-	+	-
A10-43	44	F	+	-	+	+	+	+	+	+
D10-9	11	F	-	+	+	+	+	+	+	+
F1-3	36	F	-	+	+	+	+	-	+	-
F1-163	6	F	+	+	+	+	+	+	+	-
F1-137	9	M	+	+	+	+	-	+	+	+
A9-11	26	M	-	+	+	+	+	+	+	-
B5-42	10	F	+	+	+	-	+	+	+	+
A5-47	10	F	+	ND	+	+	+	-	+	+
G10-10	18	F	+	-	-	+	-	+	+	ND
A9-27	38	F	-	+	+	-	+	+	+	ND

A9-6	37	M	-	+	+	+	-	+	+	ND
NC1			+	+	+	+	+	+	+	+
PC			-	-	-	-	-	-	-	-
NC2	23	M	-	-	-	-	-	-	-	-
A5-6	23	F	-	-	-				-	-
A5-9	99	F	-	-	-				-	-
B5-7	10	M	-	-	-				-	-
B5-2	61	M	-	-	-				-	-
A5_5	53	F	-	-	-				-	-
A5-3	70	M	-	-	-				-	-
C10-1	23	M	-	-	-				-	-
C10-2	31	M	-	-	-				-	-
C10-3	64	M	-	-	+				+	+
C10-4	18	F	-	-	+				-	-
C10-5	17	F	-	-	+				+	+
C10-6	25	F	-	-	+				+	-
C10-7	42	F	-	-	-				-	-
C10-9	57	F	-	-	-				-	-
E9-1	57	F	-	-	-				-	-
E9-2	18	M	-	-	-				-	-
E9-3	54	F	-	-	-				-	-
E9-4	9	F	-	-	-				-	-
E9-5	41	F	-	-	-				-	-
E9-6	65	M	-	-	-				-	-
E9-7	10	M	-	-	-				-	-
E9-8	9	F	-	-	-				-	-
E9-9	31	F	-	-	-				-	-
E9-10	59	F	-	-	-				-	-
A10-2	69	M	-	-	-				-	-
A10-3	49	F	-	-	+				+	-
A9-4	30	F	-	-	-				-	-
A9-10	18	F	-	-	+				+	-
C10-9	57	F	-	-	+				+	+
B5-4	55	M	-	-	+				+	+
B5-2	73	M	-	-	-				-	-
B5-5	69	F	-	-	-				-	-
B5-7	18	F	-	-	-				-	-
B5-8	43	F	-	-	-				-	-
B5-9	12	F	-	-	-				-	-
D10-9	14	F	-	-	+				-	-
D10-2	70	F	-	-	-				-	-
D10-4	12	F	-	-	-				-	-
E9-42	6	F	-	-	-				-	+
F1-10	65	M	-	-	-				-	-
G10-7	13	M	-	-	-				-	-
G10-8	17	F	-	-	-				-	-
G10-9	18	M	-	-	-				-	-

The results recorded in this table were obtained from microscopy and PCR tests conducted on blood and saliva samples from participants of this study. Sample PC is a positive laboratory standard and sample NC1 and NC2 are standard laboratory negative samples. The gender of the participants was represented by "F" for females and "M" for males. The Qiagen kit was used to extract DNA from the supernatant only (S.O), pellet only (P.O) and pellet with supernatant (10 X) fraction of saliva, only 45 samples were analysed in this three saliva fractions. Positive and negative *P. falciparum* samples are represented by "+" and "–" symbols respectively, "ND" are sample which could not be analysed.

Appendix C

Ethical Approval

Ethics Committee
Faculty of Science and Agriculture
University of Zululand
C/O Mr L Vivier
Department of Zoology
University of Zululand
Private Bag 1001
KwaDlangezwa
3886
Tel: 035 – 902 6741
Email: lvivier@pan.uzulu.ac.za

10 June 2009

To whom it may concern

ETHICS EVALUATION OF RESEARCH PROJECT PROPOSAL

This letter serves to confirm that **Mr. O.J. Pooe** (**Student No 20053202**), registered for a MSc Degree in the Department of **Biochemistry and Microbiology** at the University of Zululand, in accordance with appropriate rules submitted a research project proposal to the Ethics Committee of the Faculty of Science and Agriculture at the University of Zululand. The research project will investigate: **Detection of *Plasmodium falciparum* in human saliva samples**. Based on the research protocol stipulated, and **because this study will be conducted in Zambia** and the necessary approval was granted by the University of Zambia Research Ethics Committee (see attached document) this committee could find no reason from an ethical standpoint to reject the proposed research.

Yours sincerely

H.L. JERLING

for. Mr L Vivier
Chairperson
Ethics Committee
Faculty of Science and Agriculture
University of Zululand

The Malaria Institute at Macha

P.O. Box 630166
Choma, Zambia
Email: admin@macha.org.zm
Tel: +260 213 261 022

May 29, 2009

A. Shonhai (PhD)

Lecturer

Department of Biochemistry & Microbiology

Zululand University

P Bag X1001

Kwadlangezwa, 3886

South Africa

Dear Dr. Shonhai,

RE: RESEARCH PROPOSAL FOR MR. OFENTSE POOE

I confirm herewith that the proposed research for Mr Ofentse Pooe, titled "Detection of *Plasmodium falciparum* in human saliva samples", falls under our main project at Macha that is approved by the University of Zambia Research Ethics Committee (UNZAREC). This project is currently ongoing work and Mr. Ofentse's study has IRB clearance to commence here under our supervision.

Sincerely,

Sungano Mharakurwa, PhD
Scientific Director

Malaria Institute at Macha
Office of Scientific Director
P.O. Box 630166
Choma
ZAMBIA