Evaluation of Loop-mediated isothermal DNA Amplification as a Diagnostic Tool For Malaria in Reactive Case Detection in Namibia

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Munyaradzi Tambo

200969251

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Supervisors: Dr. Davis R Mumbengegwi

Dr. Ronnie Bock

Dr. Hugh Sturrock

Abstact

Malaria, the disease, is a clinical diagnosis that is caused by *Plasmodium* parasites and is spread through the bites by infected female *Anopheles* mosquitoes. Malaria is a health concern in temperate tropical areas, but a scale up of control interventions resulted in reduction and elimination of the disease in developed countries. In 2010 Namibia adopted a strategy to eliminate malaria within its borders by the year 2020 as a result of the reduction in malaria cases. However, as malaria case incidences are reduced the number of low parasite density sub-patent infections increases posing a challenge for diagnosis. Therefore, in Namibia which is a low prevalence setting, malaria cases could be going undetected due to difficulty in detection of low parasite density infections with the routinely used Rapid Diagnostic Tests (RDTs). This study evaluated the use of reactive case detection to trace malaria cases, symptomatic and asymptomatic and compared the routinely used RDTs with a highly sensitive molecular tool, Loop-mediated isothermal amplification (LAMP). All reported cases in the Engela Health District of Namibia were traced back to their place of residence and everyone in the same household as the reported case and the four surrounding households was tested for malaria with RDTs. In addition, Dry Blood Spots (DBS) were also collected from all persons tested. RDT and DBS samples were collected from 2790 individuals. DNA was extracted from all the DBS and RDT samples and was used to test for malaria with LAMP. All positive Pan-LAMP samples and 10% of the negative LAMP samples were tested by nested PCR (nPCR) as the reference technique. In addition, all positive Pan-

LAMP samples were tested with Pf-LAMP specific kits in order to determine the presence of *P. falciparum* and cytochrome B digestion was done on all n-PCR positive samples for species determination. RDTs detected a total of 37 malaria infections; only 2.7% were from control neighbourhoods with a sensitivity of 56.06% at 95% CI. LAMP detected a total of 66 malaria infections; only 3% of the infections were from control neighbourhoods with a sensitivity of 100% at 95% CI. A total of 64 of the LAMP positive samples were also nPCR positive and all LAMP negative samples were also nPCR negative. N-PCR, the reference standard, had a sensitivity of 96.97% at 95% CI. Both RDTs and LAMP determined that all the malaria infections were caused by *P. falciparum* and this was confirmed by n-PCR. The number of malaria infections detected doubled with the use of LAMP as compared to RDTs. In addition, LAMP with n-PCR as a reference, detected 4 times more secondary cases than RDTs. The majority of the malaria infections, 97%, were from case neighbourhoods. This indicates that individuals in proximity to malaria infections are more likely to be infected by malaria. Therefore, reactive case detection is an important surveillance tool in order to detect all cases around reported cases that are usually asymptomatic as a step towards malaria elimination. LAMP detected 4 times more secondary cases than RDTs with n-PCR as the reference. This shows that RDTs have short comings in the detection of low parasite density infections and a highly sensitive tool such as LAMP is required to detect all malaria cases as a step towards malaria elimination.

Contents

List of figures

List of tables

List of conferences and posters

1. Tambo, M., Mwinga, M & Mumbengegwi, D. Multidisciplinary Research Centre, Namibia. The use of Loop-mediated isothermal amplification (LAMP) and Polymerase chain reaction (PCR) as quality assurance tools for malaria diagnosis using Rapid Diagnostics Tests (RDT) in Northern Namibia. Monday 7 October, 6th Multi-lateral Initiative on Malaria (MIM) Conference. Durban, South Africa.
2. Davis Mumbengegwi, Joyce Auala, Munyaradzi Tambo, Erastus Haindongo (2014). Molecular techniques and findings from malaria elimination research in Ohangwena and Omusati. Windhoek, Namibia.
3. Tambo M, Mumbengegwi D.R, Bock R.A, Sturrock H.J (2014). LAMP as a diagnostic tool for detection of sub-patent a symptomatic malaria infection in pre-elimination settings in Northern Namibia. New Orleans, Louisiana, USA
4. Tambo M, Mumbengegwi D.R (2013). Evaluating stored Rapid Diagnostic Tests as a source of DNA for molecular analysis of genetic diversity and drug resistance markers in Plasmodia. Windhoek, Namibia.

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My Family that is always loving, supportive and encouraging in all areas of life. The joy of our Lord is our strength.

Declaration

I, Munyaradzi Tambo, declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

..... Date.....

Dedication

I dedicate this work to all those that have been a part of my journey, contributing positively to my life.

Pane ziya pane dovi, namatira ipapo sechikwekwe!

List of Abbreviations

ACD	-	Active Case Detectection
ACT	-	Artemisinin Combination Therapy
BIP	-	Backward Inner Primer
Bst	-	*Bacillus stearothermophillus*
CI	-	Confidence Interval
DBS	-	Dry Blood Spots
DDT	-	Dichloro-diphenyl-tricthloroethane
DNA	-	Deoxy-ribonucleic acid
dNTP	-	Deoxy-nucleotide-tri phosphate
EA	-	Enumeration areas
FIP	-	Forward Inner Primer
GPS	-	Geographical Positioning System
HIV	-	Human Immuno-deficiency Virus
HRP II	-	Histidine Rich Protein II
IEC	-	Information, Education and Communication
IRS	-	Indoor Residual Spraying
LAMP	-	Loop-mediated isothermal DNA Amplification

LLINs - Long Lasting Insecticide treated Nets

LPB - Loop Primer Backward

LPF - Loop Primer Forward

MoHSS- Ministry of Health and Social Services

nPCR - nested Polymerase Chain Reaction

NVDCP- National Vector-borne Disease Control Program

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

pLDH - plasmodium Lactate dehydrogenase

RACD - ReActive Case Detection

rDNA - ribosomal Deoxy-nucleic acid

RDTs - Rapid Diagnostic Tests

RNA - Ribonucleic acid

SARS - Severe Acute Respiratory Syndrome

TB - Tuberculosis

UV - Ultra-Violet light

WHO - World Health Organisation

Chapter 1: Introduction

Orientation of the study

Malaria has remained one of the most important arthropod-borne diseases although there has been a global reduction in the estimated incidents of malaria infections (Rosas-Aguirre et al., 2013). Malaria affects an estimated 219 million people annually worldwide and the bulk of the burden of malaria mortality (90%) in 2012 was in Africa with children under the age of five being the most vulnerable (World Health Organisation, 2012). It is caused by five *Plasmodium* parasites, *P.falciparum, P.malariae, P.ovale, P.vivax and P.knowlesi* (Su, 2010; Sabbatani, Fiorino, & Manfredi, 2010). *Plasmodium falciparum* is the most virulent of the five species (Greenwood et al., 2008). The malaria parasites are spread through bites by an *Anopheles* mosquito; the parasites require a human host in order to complete their life cycle as shown in figure 1.

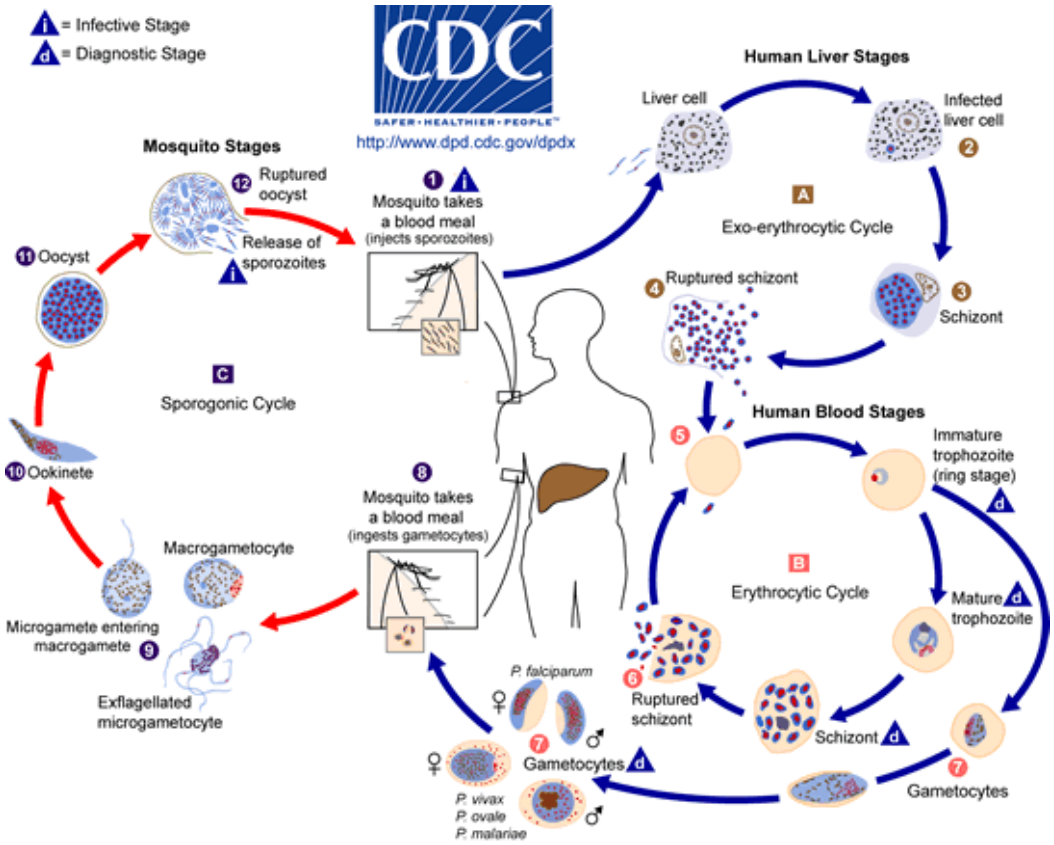

Figure 1: Plasmodium parasite life cycle in the mosquito and human host. Source: http://www.dpd.cdc.gov/dpdx

Malaria can be treated and prevented but it still remains a public health concern in Namibia (*Malaria Indicator Survey*, 2009). Malaria transmission is confined to the eastern and central parts of Northern Namibia where it is endemic. However, malaria is on the decline, in the year 2000 there were 477 000 cases as compared to 2013 (up to August) where only 1546 malaria cases were reported, a reduction of 96.76%. Figure 2 shows the low malaria prevalence in Namibia in 2013. The National Vector-borne Disease Control Program (NVDCP) at the MoHSS adopted a strategy in 2010 to eliminate malaria from Namibia by the year 2020 (*Nat. Mal. M&E Plan*, 2010).

Figure 2: A map showing low malaria prevalence in Namibia in 2013 (WHO, 2014)

As countries like Namibia go for the elimination of malaria it will be important to implement interventions that identify and treat all the malaria infections both symptomatic and asymptomatic cases (Moonen et al., 2010). Recently, a case management study estimated that in very low prevalence settings, sub-patent infections comprise 70-80% of all malaria infections (Okell et al., 2012). Therefore in a low transmission setting like Namibia, there could be a significant number of sub-patent infections. These sub-patent infections are infections with low parasite density (<50parasites/µL) that cannot be detected by the conventional diagnostic tools, microscopy and RDTs due to poor sensitivity at low parasite density with a detection threshold of 100parasites/µL. The sub-patent infections are normally asymptomatic individuals (do not show symptoms of malaria) (Bousema et al., 2012). Therefore, passive detection of malaria cases at health facilities might not be sufficient as Namibia goes for malaria elimination. The World Health Organization (WHO) now recommends the testing and treating of malaria in defined risk populations regardless of them being positive or negative in low transmission settings (WHO, 2012); this type of intervention is referred to as Active Case Detection (ACD). A form of ACD that is normally

used for low transmission settings is Reactive Case Detection (RACD) which involves testing and treating individuals in close proximity to reported cases because malaria cases can be geographically clustered (WHO, 2010; Bejon et al., 2010). This can therefore be a positive step in ensuring that all malaria cases are followed up and treated in order to interrupt malaria transmission in Namibia.

Malaria diagnosis currently relies on rapid diagnostic tests (RDTs) or microscopy that have a detection limit of 50 – 100pasites/µL to identify infected individuals (Mosha et al, 2013). There is a growing body of evidence that these diagnostic tests miss a substantial proportion of malaria infections in endemic areas when compared to tests performed by the Polymerase Chain Reaction (PCR) with a detection limit of 1 – 2 parasites/µL (Okell et al., 2009; Okell et al., 2012). This is primarily due to the fact that RDTs and microscopy do not effectively detect low parasite density infections (van den Broek et al., 2006). Without tools to detect such low parasite density infections, they are left as reservoirs that could perpetuate the spread of malaria as these low density infections although asymptomatic, are still able to infect mosquitoes with *plasmodium* parasites. These infected mosquitoes then spread malaria to individuals in proximity to the low density infections. Consequently, this could impede the efforts to eliminate malaria in Namibia.

Molecular methods have potential as diagnostic tools for malaria in low transmission settings (Murray et al., 2008). The detection and amplification of deoxy-ribonucleic acid (DNA) by the Polymerase Chain Reaction (PCR) can be a useful tool for diagnosis of malaria when conventional techniques are giving false negative results, especially since PCR allows accurate species identification and can detect low level parasitaemia (Murray et al., 2003). However PCR diagnosis is expensive to setup, has a long turnaround time, requires highly skilled personnel and is limited to reference laboratories in developing countries like

Namibia. Therefore PCR is not appropriate for use in most areas where malaria is endemic. Loop mediated isothermal DNA amplification (LAMP) is a powerful novel and innovative gene amplification technique that is emerging as a simple rapid diagnostic tool for early detection and identification of microbial diseases (Parida et al., 2008). LAMP does not require highly skilled personnel, individuals without molecular biology training can be trained to perform LAMP and it is relatively cheap compared to PCR, not requiring expensive equipment (Hopkins et al., 2013). LAMP is a novel technique hence there are limited reports on its usefulness as a tool in detection of malaria in clinical samples. In this study, LAMP will be assessed as a tool for malaria diagnosis in reactive case detection on clinical samples in Namibia compared to RDTs.

1.2. Statement of the problem.

In low prevalence malaria settings like Namibia, sub-patent infections comprise 70-80% of all malaria infections (Okell et al., 2012) and there is evidence showing that the routinely used diagnostic tools, RDTs and microscopy, do not detect low density parasitaemia (<50 – 100 parasites/µL) (Van der Broek et al., 2006; Mosha et al., 2013). The low parasite density, undetectable cases can still transmit malaria if bitten by mosquitoes and perpetuate the spread of malaria thus hindering efforts to eliminate malaria. In addition, persistence of antigens in the blood circulation of the patient after parasite clearance can generate false-positive with RDTs (Endeshaw et al., 2008). RDTs as a result have short-comings as the main diagnostic tool for low transmission settings. Therefore it is necessary to evaluate alternative rapid techniques that are specific, sensitive and have a short turn-around time for results such as LAMP if elimination and effective control are to be achieved.

1.3. Research objectives

- To assess the appropriateness of LAMP for detection of *Plasmodium* in clinical samples from RDTs and DBS in a low transmission setting.
- To assess the reliability of LAMP as a diagnostic tool for malaria compared to n-PCR.
- To compare the suitability of LAMP to RDTs as a diagnostic tool for malaria surveillance during reactive case detection.

1.4. Hypothesis

LAMP is a suitable tool for diagnosis of all malaria cases (including asymptomatic infections) in reactive case detection in Namibia.

1.5. Significance of study

In 2010 Namibia adopted a strategy to eliminate malaria within its borders by the year 2020(*Nat. Mal. M&E Plan*, 2010). In Namibia, a low prevalence setting, malaria cases could be going undetected due to difficulty in detection of low parasite density infections (Van der Broek et al., 2006). Therefore, to eliminate malaria, all asymptomatic reservoirs of malaria that could perpetuate the spread of malaria need to be traced by reactive case detection to test and treat all the individuals at risk as opposed to passive detection at health facilities that only focuses on reported symptomatic cases. In combination with reactive case detection, highly sensitive molecular diagnostic tools are required in order to detect all malaria infections. Use of LAMP (highly sensitive molecular tool) in reactive case detection to detect *Plasmodium* will enable detection of all reported malaria cases and their surrounding

(secondary) malaria cases. It will be a positive step towards malaria elimination. The study will influence Namibia's policy regarding the surveillance and diagnosis of malaria to ensure that all cases are identified and treated.

1.6. Limitations of the study.

The study focused on one health district, it was a pilot and may not be representative of national scenario in terms of sample quality, collection, preparation and storage. In addition, the study was retrospective; some samples were stored longer than others and some samples especially DBS differ in blood volume. N-PCR was not run on all of the samples. Therefore only a subset of the samples were tested using the reference technique, the data from PCR was generalised to all samples.

Chapter 2: Literature review

2.1The malaria reduction trend

Malaria is a clinical diagnosis that is caused by the *Plasmodium* parasites and it is spread through the bites by infected *Anopheles* mosquitoes (Cibulskis et al., 2011). Malaria is a health concern in temperate areas, but economic development and public health measures resulted in reduction and elimination of the disease in developed countries(Greenwood et al., 2008). There was a call for complete eradication of malaria to be the main goal for the fight against malaria in 2007 by Bill and Melinda Gates which saw many research groups and governments employing this strategy (Feachem & Sabot, 2008). As a result of the different interventions the incidence of malaria infections has fallen significantly as shown in figure 3.

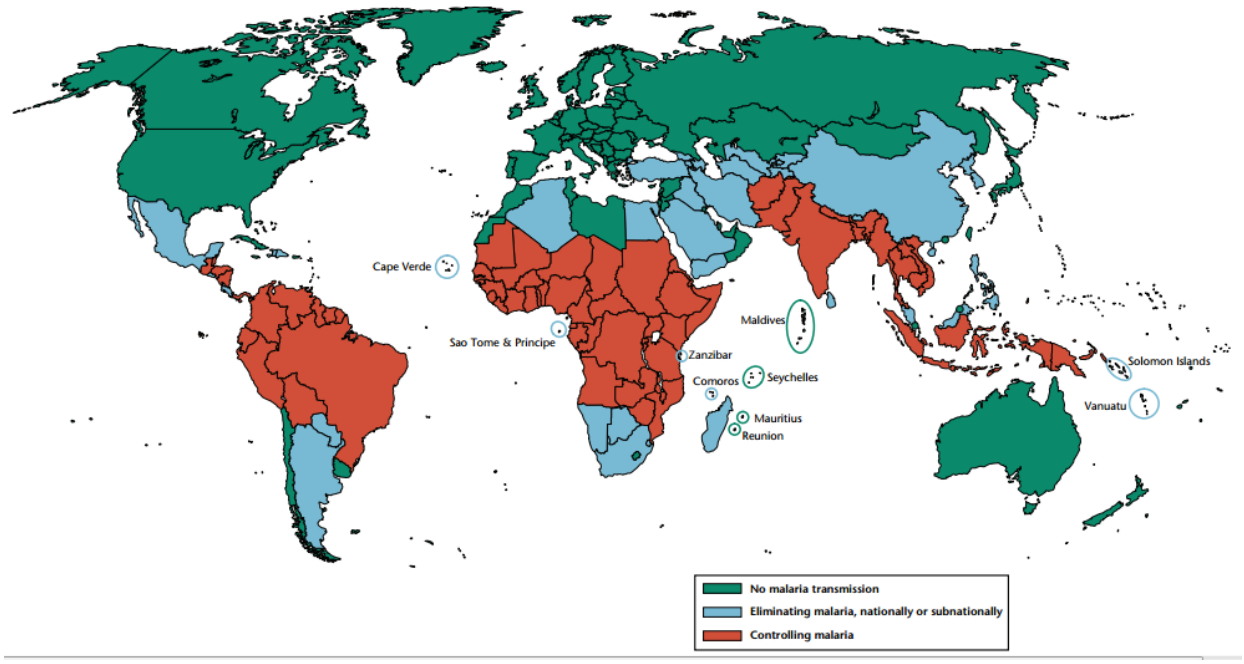

Figure 3: The malaria distribution map showing the global effort to eradicate malaria (Feachem et al., 2009).

The fight against malaria has four defined phases; Control, Pre-elimination, elimination and certification and verification as shown in figure 4.

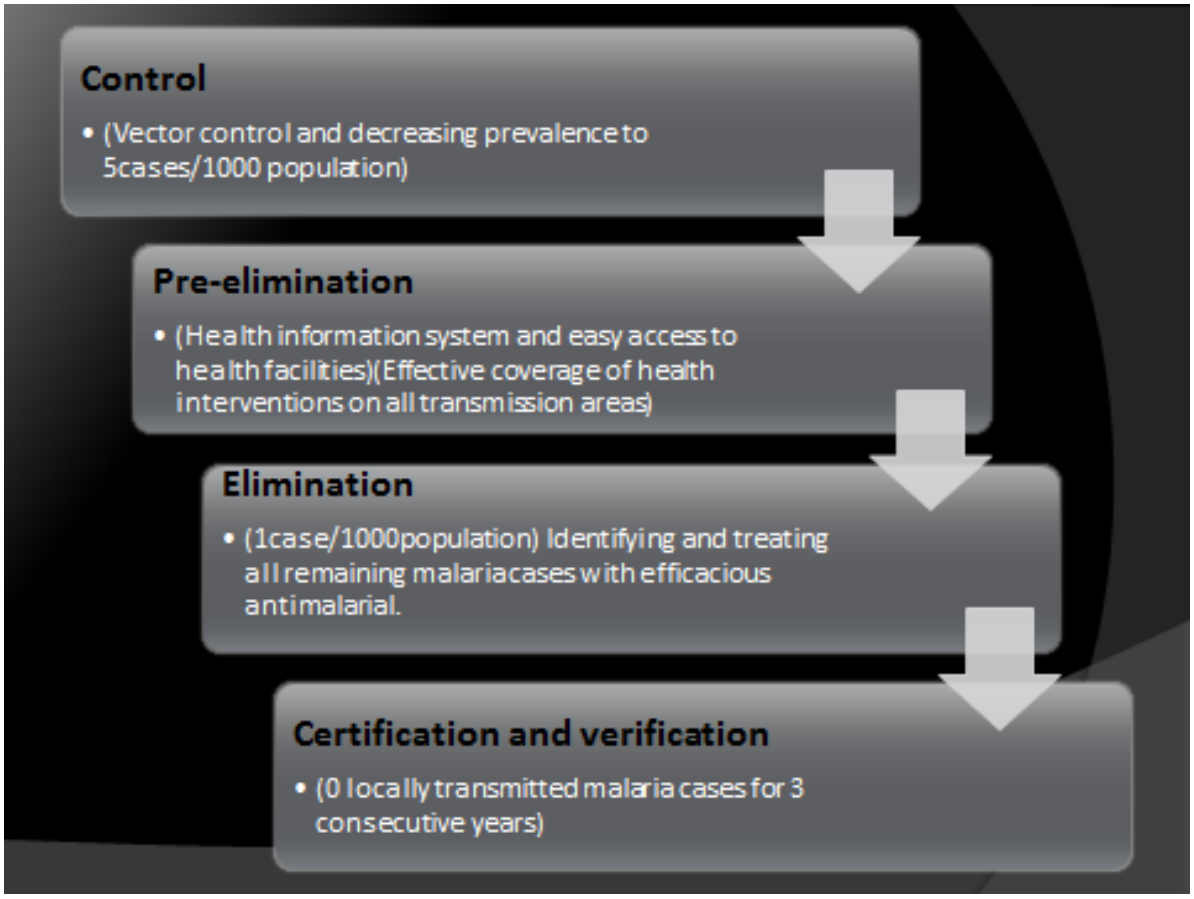

Figure 4: Phases in the fight against malaria

The control phase involves vector control and decreasing prevalence to 5cases/1000population. In Southern Africa, Angola and Zambia are examples of countries in the control phase (WHO, 2012). Indoor Residual Spraying (IRS) is amongst the major intervention tools currently used for malaria prevention and it has been shown to be highly effective for vector control (Kleinschimdt et al., 2009; Mabaso et al., 2004), shown in figure 5. In Namibia, IRS (as shown in the figure below) with Dichloro-diphenyl-tricthloroethane (DDT) 75% WP and deltamenthrine 250 WG is used with an annual coverage above 80% (MoHSS, 2010c). This extensive coverage with IRS has also contributed to the reduction in the number of malaria cases in Namibia. Long Lasting

Insecticide treated Nets (LLINs) are designed as an effective vector control measure for malaria and they are mostly used in combination with IRS in order to achieve greater health benefits (Okumu et al., 2013). LLINs have a dual effect on the malaria vector, the *Anopheles* mosquito, they act as a physical barrier to prevent mosquito bites and they have insecticidal action (Russell et al., 2010). In Namibia, more than 500 000 LLINs were distributed with pregnant women and children under the age of 5 being the focal points as they are the most vulnerable (MoHss, 2010a). The combination of LLINs and IRS is wide spread in malaria endemic regions in Namibia as an effort to keep reducing the number of malaria cases.

Figure 5: Indoor Residual Spraying of a house in a village for the prevention of malaria. Source: http://ccp.jhu.edu

In the pre-elimination phase, health information systems, easy access to health facilities and effective coverage of health interventions on all transmission areas are the main interventions.

Information, education and communication for any disease plays a key role in encouraging implementation and adherence to the interventions put in place to fight against the disease (Ahmed et al., 2014; Samba & Kakoko, 2012). Studies have shown that the more knowledge the community has about malaria, they are more likely to adhere to the interventions in order to prevent themselves from getting the disease (WHO, 2010; Minja et al., 2001). Therefore, in Namibia, IEC is used by the Ministry of Health and Social Services(MoHSS) to supplement activities to fight against malaria and it has contributed to the decline in the number of reported malaria cases (MoHSS, 2010a; MoHSS, 2010c).

During the elimination phase, identifying and treating all remaining malaria cases with efficacious antimalarial drugs are the focal points. Namibia and Swaziland are some of the countries in Southern Africa that are going for malaria elimination. Ensuring that there are zero locally transmitted cases in three consecutive years is the goal for certification and verification (WHO, 2010). To date, there are no countries in Southern Africa that are in the certification and verification phase. Malaria elimination is defined as the interruption of malaria transmission in a defined geographical area, with zero reported local cases (Feachem et al., 2009). The reduction of malaria cases has seen Namibia officially declaring efforts to eliminate (1 case/1000population) malaria by the year 2020(*Nat. Mal. M&E Plan*, 2010).

To date, there are on-going investigations of malaria treatment because there is no effective vaccine and the *Plasmodium* parasites are developing resistance against drugs like amodiaquine (Willcox et al., 2011). Following this wide spread drug resistance, Artemisinin-based combination therapy (ACT) has been adopted as the most effective treatment option against malaria (Watsierah & Ouma, 2014). This is because ACTs, shown in table 1, are a combination

of different drugs, making it difficult to develop resistance. Appropriate and fast treatment upon diagnosis is important before the malaria develops into complicated malaria.

Table 1: WHO recommended ACTs

ACT	Commercial name
Artemether-lumefantrine	Coartem®
Artesunate plus amodiaquine	Arsuamoon®
Artesunate plus sulfadoxine-pyrimethamine	Sulfamon plus 500
Artesunate plus mefloquine	Artequin™

In order to move towards elimination, improved diagnostic and surveillance strategies are required (Alonso et al., 2011). In most malaria endemic countries, malaria is monitored by passive surveillance. Passive surveillance involves the reporting of cases at health facilities and it is limited by incomplete reporting, individuals preferring private health care systems and poor diagnostic capacity. These challenges are particularly amplified in low transmission settings where health workers are not normally exposed to malaria cases (Hsiang et al., 2012). Some of the challenges posed by passive detection can be overcome by implementing active surveillance. Active surveillance involves cross sectional surveys of defined sample populations with the proportions of individuals infected with malaria being used as malaria indicators (Hay et al., 2008). Reactive case detection is a form of active case surveillance that is preferred for low malaria transmission settings (Sturrock et al., 2013). Reactive case detection is when reported malaria cases confirmed with RDTs at health facilities (Passively detected cases) are tracked back to their place of residence and all the individuals around the index (reported) case are also

tested and treated for malaria if found positive (Sturrock et al, 2013). This enables the detection of asymptomatic cases that further the spread of malaria. In Swaziland, a low transmission setting, reactive case detection was carried out in a cross sectional survey and additional malaria infections were detected with more efficiency with pooled PCR than with RDTs (Hsiang et al., 2012). Reliance on RDTs for reactive case detection in low transmission settings has been shown to be inadequate as the RDTs miss cases due to poor sensitivity.

2.2. Malaria diagnosis in low transmission settings.

Malaria remains an important cause of mortality and morbidity in many parts of the world and it could have adverse impact on the population, both from health and socio-economic attitudes (Zonghi et al., 2012). Recent reductions in malaria coupled with increased funding have resulted in a renewed focus on malaria eradication (Mendis et al., 2012). World Health Organization (WHO) recognizes 17 countries including Namibia as having pre-elimination or elimination programs (Feachem et al., 2010). During the elimination phase, programmes become more focused on reducing malaria transmission in a few specific regions. At this stage, interventions focus on detecting (diagnosing) all malaria cases, preventing onward transmission, managing malaria foci, and managing imported malaria cases (WHO, 2013). Most deaths caused by Malaria are because of wrong, late and or unavailable diagnosis (Wiwanitkit, 2009). Rapid and accurate diagnosis is the key to effective management of malaria cases in order to reduce morbidity and mortality caused by delayed or poor management of patients (Hawkes et al., 2014). Diagnosis becomes a key factor in a low transmission setting like Namibia where there could possibly be asymptomatic infections acting as reservoirs of the malaria parasites that can potentially spread the disease. Currently, the diagnostic tools available are clinical diagnosis, microscopy, Rapid Diagnostic Tests (RDTs), ELISA and molecular tools; the Polymerase Chain

Reaction (PCR) and Loop-mediated isothermal amplification (LAMP) (Wongsrichanalai et al., 2007; Mosha et al., 2013). Clinical diagnosis although still used in countries with a high malaria burden as it malaria is the most probable cause for fever, is no longer encouraged in low transmission settings as it is based on the symptoms of malaria which overlap with a number of other diseases. Microscopy and RDTs have been reported to perform poorly at low parasite density (Van der Broek et al., 2006). Therefore, asymptomatic infections are not treated as they could be missed by RDTs and microscopy; these cases pose a risk of furthering the spread of malaria and becoming symptomatic attack (Harris et al, 2010).

In order to eliminate malaria in Namibia, more sensitive and specific techniques should be employed on a large scale. Loop-mediated isothermal amplification (LAMP) may offer a practical alternative. The malaria LAMP kit to be used in this study in this study achieved accuracy comparable to that of nested-PCR in a United Kingdom reference laboratory (Polley et al., 2013). A study reported in Uganda demonstrated similar sensitivity when performed in a remote clinic in a malaria-endemic area (Hopkins et al., 2013). Like PCR, LAMP is a molecular technique that amplifies nucleic acids, but uses simpler equipment and is less time-intensive (Hopkins et al., 2013).

2.3. Diagnosis of malaria using microscopy (Giemsa stain)

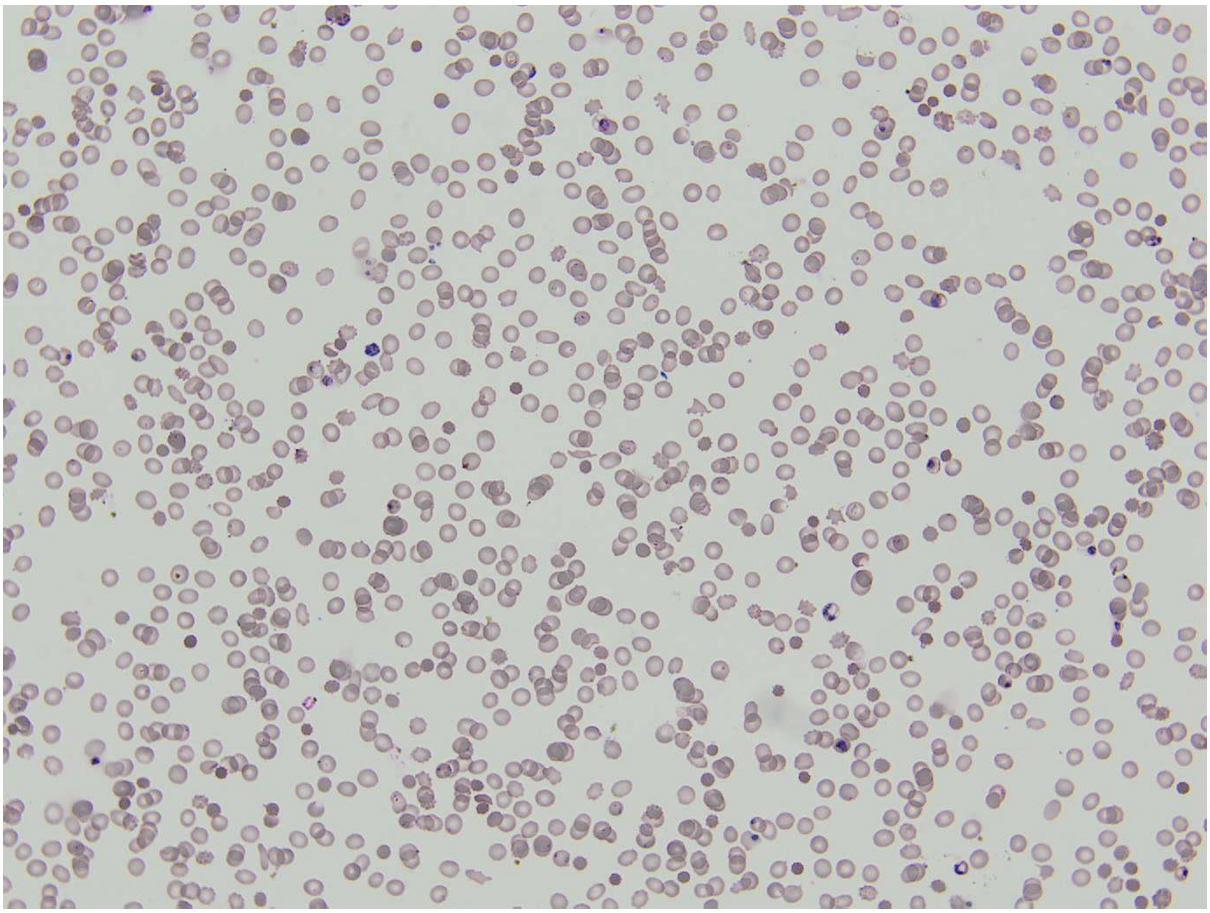

Figure 6: A micrograph of a stained blood smear slide under a microscope. The dark purple cells are infected with Plasmodium parasites.

Conventional malaria diagnosis based on the examination of stained blood smears under light microscope remains the gold standard for malaria diagnosis because clinical tests based on symptoms such as fever can be misleading (Shillcut et al., 2008). Malaria microscopy requires the examination of both thin and thick blood smears from the same individual. Microscopy can routinely detect parasitaemia levels as low as 40parasites/µl, and experienced microscopists can detect as low as 10 parasites/µl of blood. Microscopy also has the advantage of distinguishing the different *plasmodium* parasites, low cost and differentiating the parasite stages (WHO, 2000; Kiggundu et al., 2011). However, it is labor-intensive, time-consuming, requires technical expertise and the availability of a good quality microscope (Batwala et al., 2011). In addition, microscopic slide examination varies significantly across species and geographic locations and it

performs poorly at low parasite density (Alemu et al., 2014). In Namibia, Slide positivity rate is also very low, that is the number of positive slides/1000 and the infrequency of positives can make microscopy tedious as you rarely get a positive at low prevalence. The microscopists will eventually stop looking carefully on each slide after getting so many negative slides.The use of microscopy in a low transmission setting like Namibia could possibly lead to a number of cases being missed; therefore alternative diagnostic tools are required.

2.4. Diagnosis of malaria using immunochromatographic techniques

Malaria RDTs are WHO recommended immunochromatographic tests that detect parasite antigens in whole blood samples (McMorrow et al., 2011). Currently, immunochromatographic tests shown in figure 7 can target the histidine-rich protein 2 of *P. falciparum*, a pan-malarial *Plasmodium* aldolase, and the parasite specific lactate dehydrogenase (McMorrow et al., 2011). These tests are fast, easy to perform and do not require electricity or specific equipment (Hopkins et al., 2008; Barber et al., 2013). As a result, RDTs have improved access to malaria tests even in resource poor settings. Rapid diagnostic tests (RDTs) for malaria are increasingly being used for management of patients and different studies have shown significant failure rate of RDTs, especially in children and in areas of low malaria transmission (Waitumbi et al., 2010).The tests can detect >100 parasites/µl but with lower parasitaemia their sensitivity decreases, making these tests unsuitable for patients with low numbers of parasites (Okell et al., 2012). Another drawback is the reported persistence of antigens, in particular histidine-rich protein II (HRP-II), in the blood circulation of the patient after parasite clearance, generating false-positive results when microscopy is used as a reference test and LDH is unstable at high temperatures making diagnosis with RDTs unreliable (Endeshaw et al., 2008; McMorrow et al., 2011)). In addition to this, there have been reports of extensive levels of sequence diversity due

to mutations and deletions in the HRP-II gene in South America and Africa (Kurma & Sharma, 2012; Maltha et al., 2013). As a consequence of the sequence diversity and deletions in the HRP-II gene, RDTs are prone to give false negative results even when there is a malaria infection. RDTs are therefore inadequate for diagnosis in surveillance of low parasites density infections.

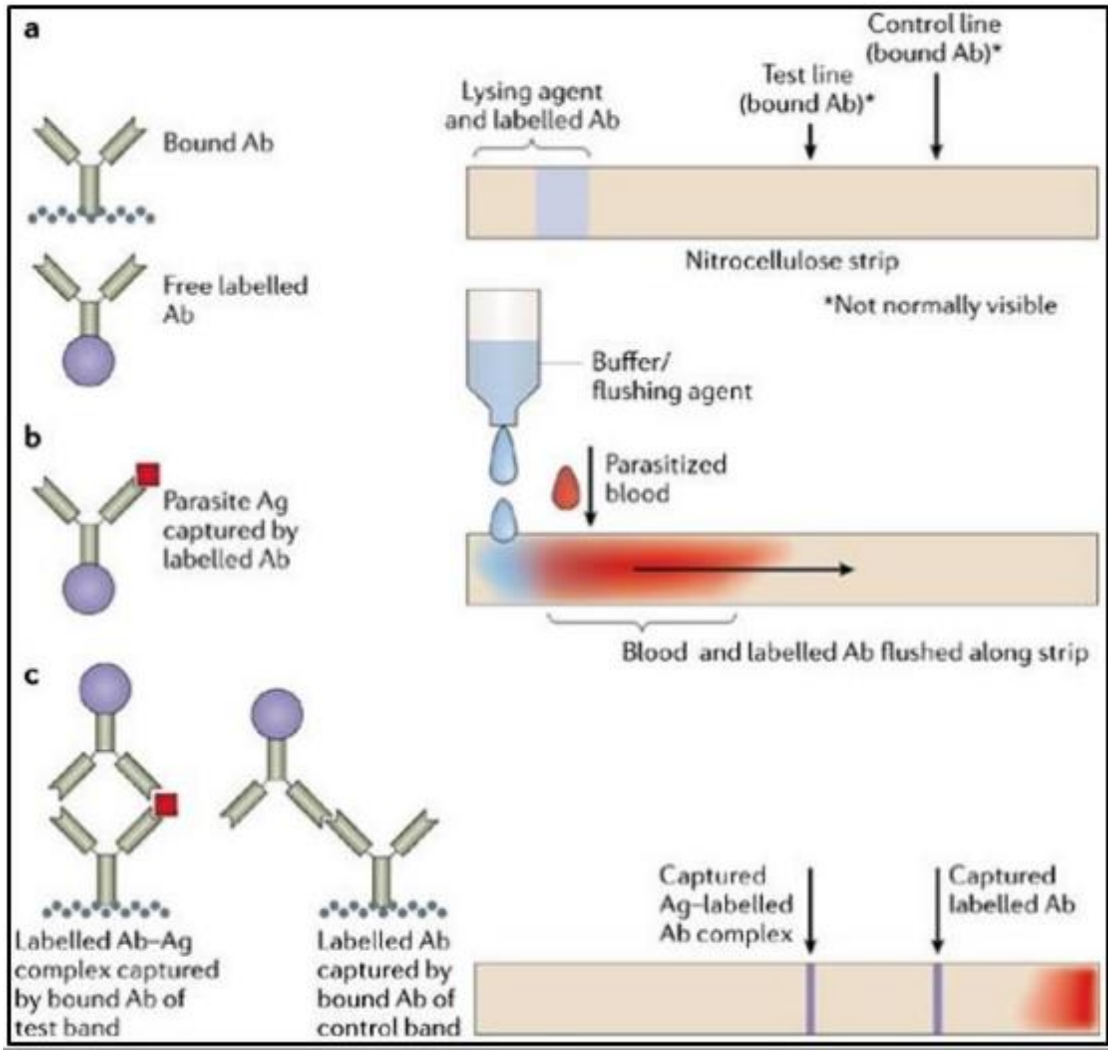

Figure 7: Rapid Diagnostic Test mechanism. Bell et al., 2006

2.5. Molecular diagnosis of malaria

In low transmission areas, a number of studies have reported that the majority of infections are asymptomatic with low parasite density that are frequently below the detection limit of microscopy and RDTs (Mosha et al, 2013; Moonen et al., 2010). These individuals are a risk for further transmission of malaria (Okell et al., 2012). Currently, the most sensitive diagnostic tools for malaria are based on the molecular detection of parasite DNA or RNA (Morris et al., 2013). The detection of malaria parasites with high sensitivity by molecular method is based on DNA extracted from fresh and dry blood samples (Moonen et al., 2010).

2.5.1 Diagnosis of malaria using Loop mediated isothermal amplification (LAMP)

LAMP (shown in figure 8) is a novel approach to nucleic acid amplification which uses a single temperature incubation thereby obviating the need for expensive thermal cyclers (Mohon et al, 2014). In LAMP there is strand displacement DNA synthesis performed with *Bst* (*Bacillus strearothemorphilus*) DNA polymerase from therefore isothermal conditions are used since there is no need for denaturation (Notomi et al., 2000). LAMP also makes use of a robust polymerase enzyme that is not as sensitive to PCR inhibitors thereby making it more suitable for use under field settings (Mori et al., 2013). LAMP is a novel molecular technique that has a lot of potential, it is highly sensitive and specific, gives results faster than PCR, requires minimal processing and instrumentation, and allows result detection with the naked eye (Gonzalez et al., 2011; Surabattula et al., 2013). The LAMP assay has a high specificity and sensitivity because amplification occurs when 6 separate regions of target

DNA are recognized and it targets mitochondrial DNA that has a high copy number, it has a detection limit of 1 – 2 parasites/µL (Hsiang et al., 2014; Polley et al., 2010; Gonzalez et al., 2011). Detection of amplification product can be by photometry for turbidity caused by increasing quantity of Magnesium pyrophosphate in solution or with addition of SYBR green, a color change can be seen without equipment (Mori et al., 2001). Also in-tube detection of DNA amplification is possible using manganese loaded calcein which starts fluorescing upon complexation of manganese by pyrophosphate during in vitro DNA synthesis (Tomita et al., 2008), therefore results can be seen on a table top under Ultra Violet (UV) light.

LAMP's high sensitivity and specificity make it a potential diagnostic tool in a low malaria transmission setting like Namibia and other eliminating countries where there are sub-patent infections being potentially missed. In Uganda, LAMP was shown to detect low parasite density infections in clinical samples, with the same efficiency as Nested PCR in a reference lab in the United Kingdom (Polley et al., 2013). LAMP is not limited to the diagnosis of malaria, there are kits available for the diagnosis of tuberculosis (TB), Human Immunodeficiency Virus (HIV), Severe Acute Respiratory Syndrome (SARS) and other pathogens like *Salmonella spp* (Han et al., 2007; Hsiang et al, 2014).In this study LAMP will be evaluated as a tool for reactive case detection in Namibia as the country is going for elimination of malaria. This is to ensure that asymptomatic cases around reported cases are treated in order to remove all reservoirs of malaria parasites and this will be a step towards achieving malaria elimination.

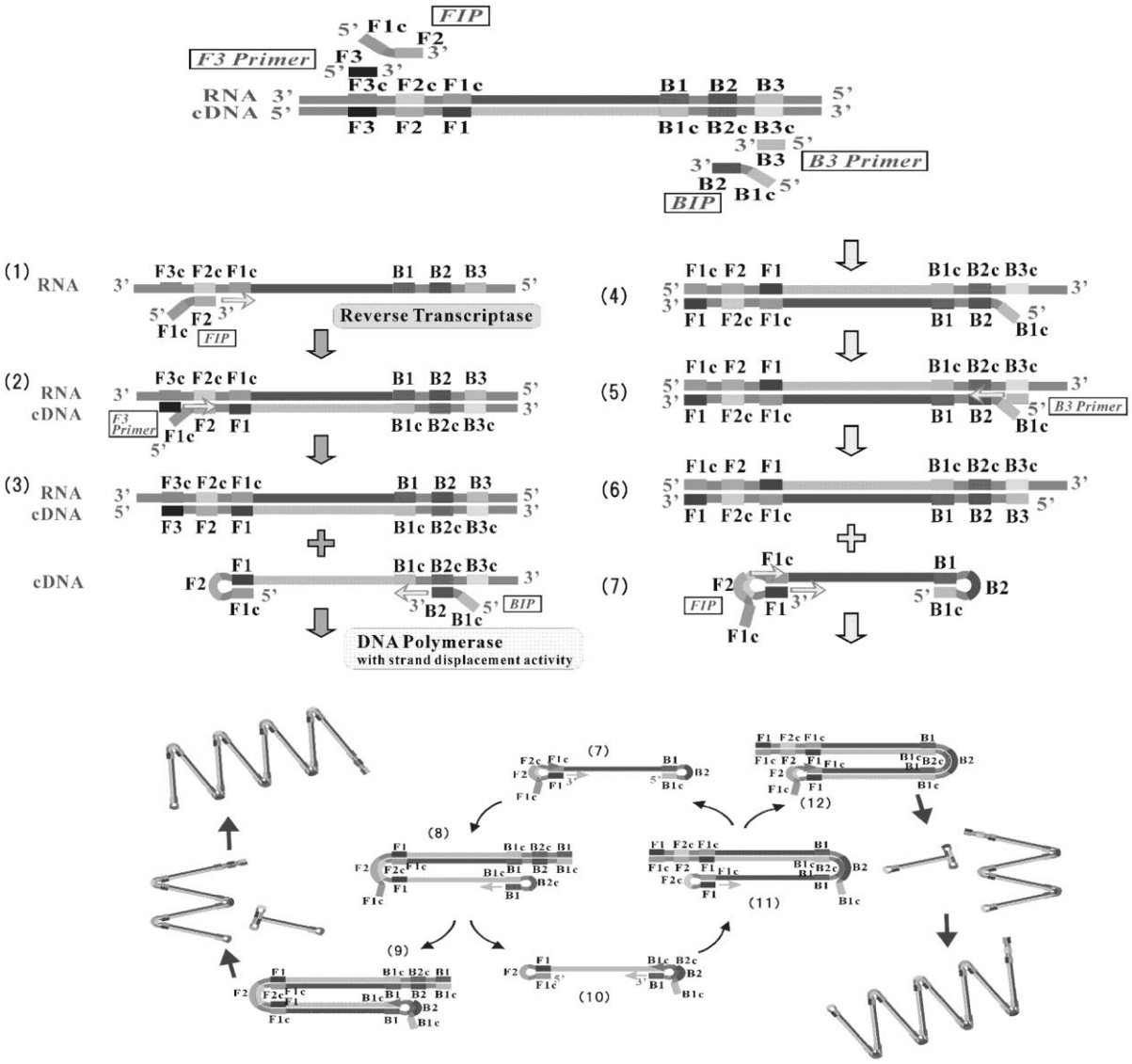

Figure 8: Step by step amplification with LAMP. Source: Poon et al., 2006

2.5.2 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) can be a useful tool for diagnosis of malaria when the results of conventional techniques are negative; it allows accurate species identification and can detect low level parasitaemia (1 – 2 parasite/µL) (Harris et al., 2010). PCR has a sensitivity and specificity of 100 per cent with a detection limit of just 1 *P. falciparum* or 3 *P. Vivax* parasites/µl of blood when compared with the microscopy (Morassin et al., 2003). PCR has also

been credited to have been able to detect mixed infections with ease in many studies and it achieves this by using primers specific for the different species and amplification of parasite DNA for improved sensitivity (Mens et al., 2007). However, PCR, is a highly sophisticated technique, requires infrastructural support, expensive to set up and more time consuming than microscopy and immunochromatography (Iglesias et al, 2014). As a result, in developing countries like Namibia, PCR is restricted to reference-level laboratories and requires considerable training to perform. Therefore, in most resource poor settings where malaria is endemic, PCR cannot be used routinely. In this study PCR was used as a reference as it is already a validated technique.

Chapter 3: Materials and Methods

3.1. Research design

This study employed both qualitative and quantitative research approaches. Qualitative design involved the interpretation of the results from RDTs, nested-PCR and LAMP as either positive or negative. Quantitative design involved comparison of the number of positives between LAMP and RDTs in order to determine if there is a significant difference between the two in detecting malaria infections in a low transmission setting. Nested-PCR was used as a quality control tool for LAMP results as it is a validated technique with high sensitivity. The findings from this study can be used as evidence by policy makers when deciding on which tools to use for diagnosis in reactive case detection of malaria moving towards elimination.

3.2. Methods

3.2.1. Population

The target population was all households from which cases were reported at a health facility and the four surrounding households, as well as control households; all were from the Engela Health district, in the Ohangwena region of Namibia shown in figure 9 below.

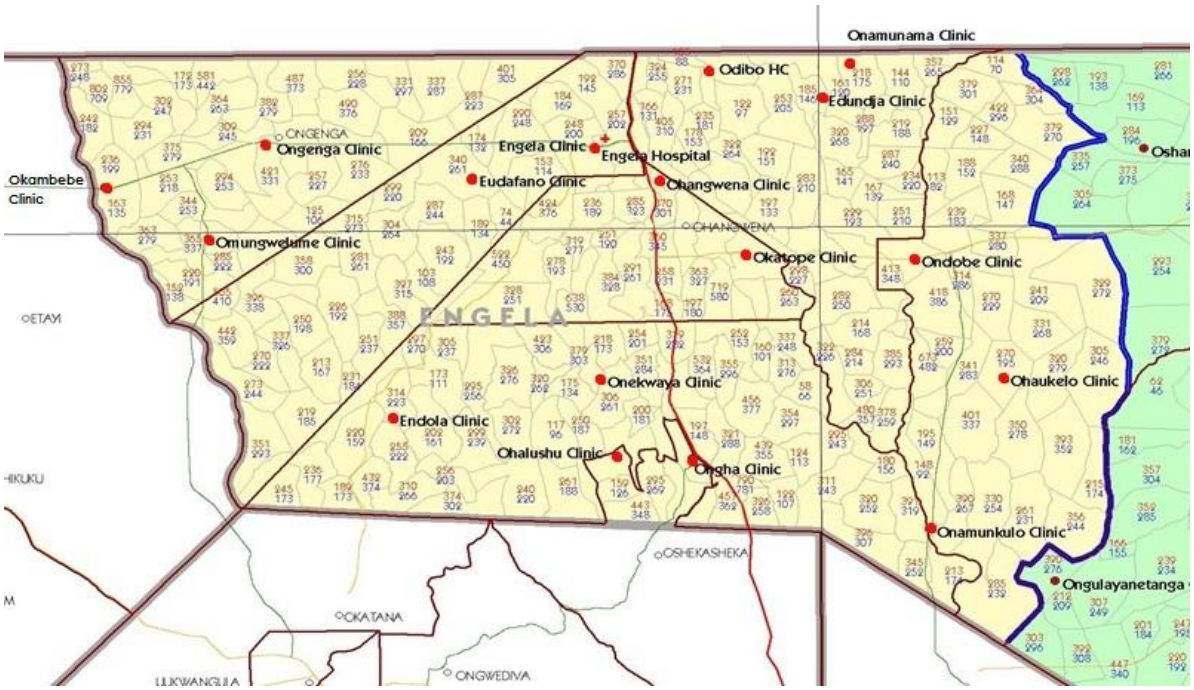

Figure 9: A map showing the health facilities in the Engela district.

3.2.2 Neighbourhood structure

In this study, a neighbourhood was defined as a targeted (index) household and its four surrounding households within a 500 meter radius for both cases and controls as shown in figure 10.

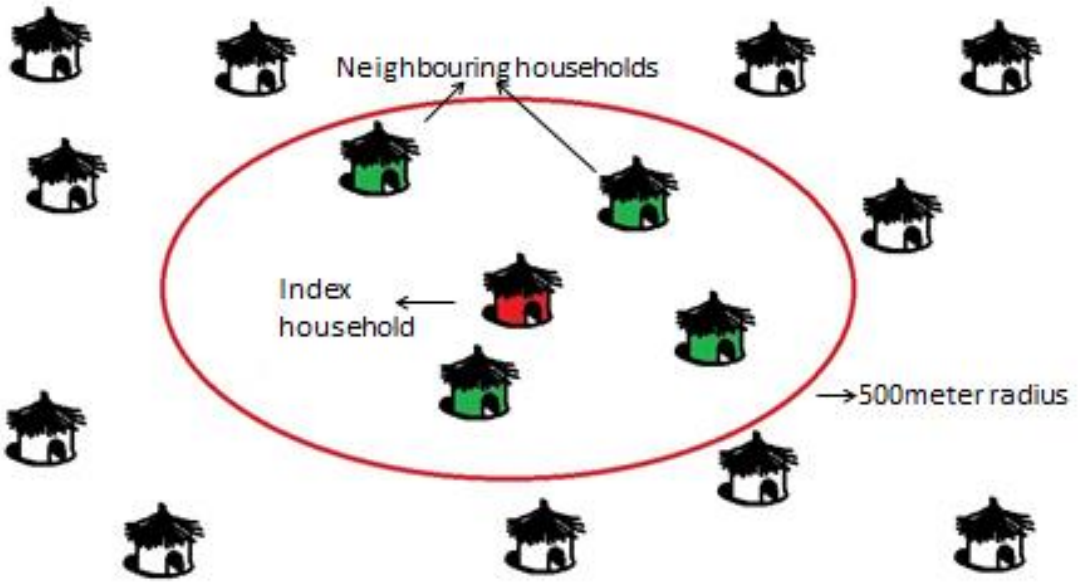

Figure 10: A diagram showing how a typical neighbourhood is structured

3.2.3 Sample collection

The study was part of a broader malaria epidemiology study in the North Central parts of Northern Namibia focusing on malaria risk factors. In order to aid in achieving Namibia's elimination goals, Reactive Case Detection (RACD) of malaria was employed in this study whereby all RDT confirmed cases that were willing to participate in the study, from the 17 clinics in the Engela District were followed up; all the individuals in the same household as the index case and the 4 surrounding households (neighbourhood) were tested and treated for malaria if found positive. The same procedure was followed for the control neighbourhoods. Controls were randomly chosen neighbourhoods in the Engela district where the individuals in the targeted household had not tested positive to malaria within 1 week prior to recruitment. Controls were selected from randomly selected enumeration areas (EA) from the full census list provided by the Namibia Bureau of Statistics; Households were randomly selected from

Geographical Positioning System (GPS) coordinates of the total number of households present in the EA. At each household, permission to conduct the study had to be given by the head of the household who also gave consent for individuals under the age of 18 years to participate in the study. After consent was given, every individual was interviewed, tested and treated for malaria if positive. Rapid diagnostic tests were performed by adding ≈5μL of blood in addition to 2 drops of buffer to the RDT and blood was collected from each individual according to the manufacturer's protocol as shown in figure 11. In addition, from the same finger prick (using a sterile lancet) used for the collection of blood for the RDT, blood was collected on filter paper and left to air dry for 20 minutes to make 4 Dried Blood Spots (DBS). All the RDTs and DBS were labeled with unique identifiers. The same unique identifier was placed on the RDT and DBS used for the same individual. The RDT results were recorded within 20 minutes of performing the test. The RDT and DBS samples from each neighbourhood were then stored at -20°C in a dry and sealed plastic bag with a desiccant to absorb moisture. These collected RDTs and DBS samples were transported to the Malaria lab at the University of Namibia for laboratory analysis with LAMP and nested-PCR. A total of 5580RDT and DBS samples were collected from 2790 individuals.

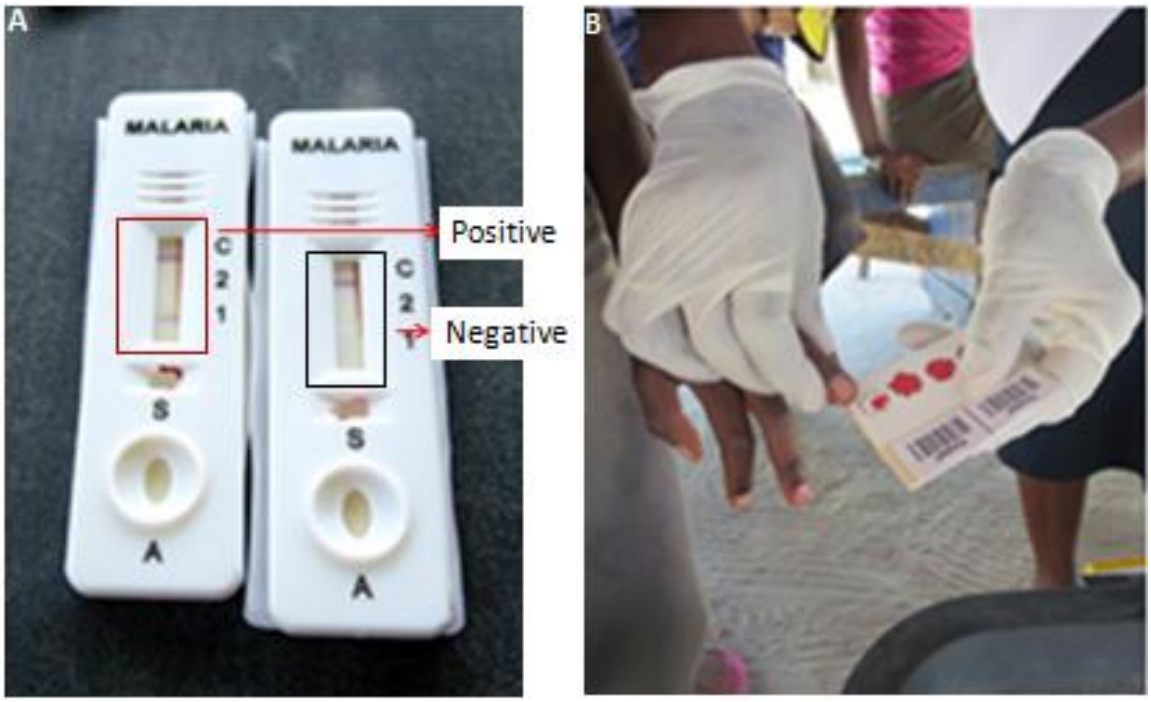

Figure 11: Collection of blood for testing for Plasmodium. Panel A is showing RDT samples after the test was performed, the one on the left was positive and the one the right side was negative. Panel B is showing the collection of blood from a finger prick on filter paper that made DBS samples.

3.2.3Chelex DNA extraction

DNA was extracted from the collected RDT and DBS samples using the chelex extraction method. In order to collect blood from RDTs, the RDT cassette was opened with a surgical blade. A total of 4 pieces of similar size were cut from the nitrocellulose strip inside the RDT cassette with a surgical blade that was sterilized with ethanol after each use as shown in figure 12. The DNA is concentrated between the control line and the blood loading point and the 4 pieces were cut from this section. There were 4 dried blood spots on each DBS sample; a small circular piece (≈5mm) in diameter was cut from one of the four dried blood spots on the filter paper using a surgical blade that was sterilized with ethanol between samples.

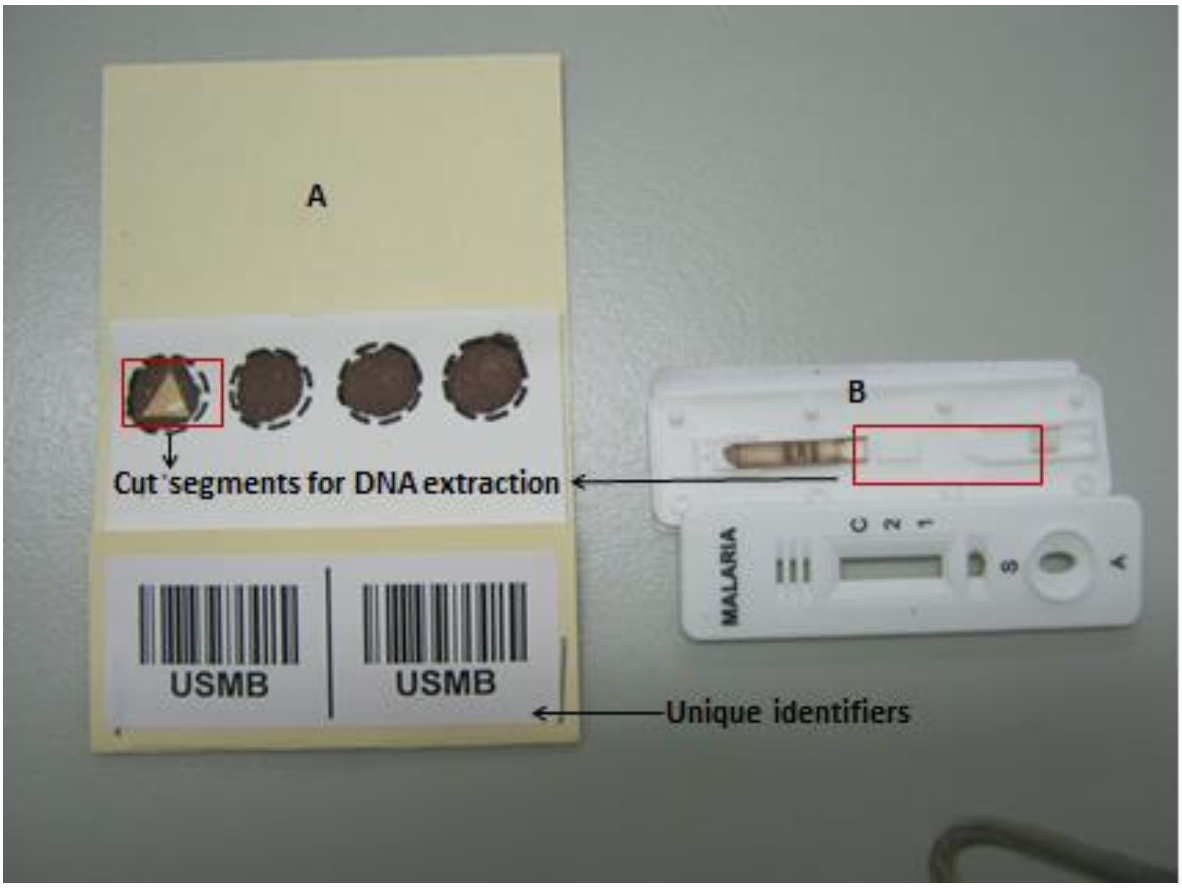

Figure 12: Showing RDT (B) and DBS (A) samples with cut segments used for DNA extraction highlighted in red

The DNA extraction steps that followed were the same for both RDTs and DBS after the cutting. The cut segments from both RDTs and DBS were inserted in clearly labeled 1.5mL microfuge tubes. In addition, to each sample contained in a microfuge tube, 50µl of saponin and 1000µl of Phosphate Buffered Saline (PBS) were added and the tubes were vortexed for 5 seconds. The tubes were then incubated overnight at 4ºC. This was followed by aspirating the supernatant (PBS and saponin) which was discarded and a wash step of the samples with 1000µl of PBS for 30 minutes at 4°C in order to remove remaining traces of saponin. After the wash step, the PBS was aspirated and discarded, 100µl of water and 50µl of 20% chelex solution were added to the samples. The samples were then heated at 98°C using a heat block for 10 minutes, to lyse the

cells in the solution containing chelex beads; the chelex beads bind to the metals that act as PCR inhibitors.

After the first 2 minutes of heating, the tubes were opened briefly, closed and vortexed to avoid pressure build up in the tubes that could result in spillage of the samples. The tubes were then centrifuged for 5 minutes at 8000rpm and the supernatant transferred to a second set of labeled tubes in order to remove the chelex beads. The second set of tubes was centrifuged at 8000rpm for 10 minutes and the supernatant was transferred to a third set of labeled tubes to ensure all the chelex beads were removed. The extracted DNA was stored then at -20°C. Figure 13 shows a flow chart with the chelex DNA extraction procedure.

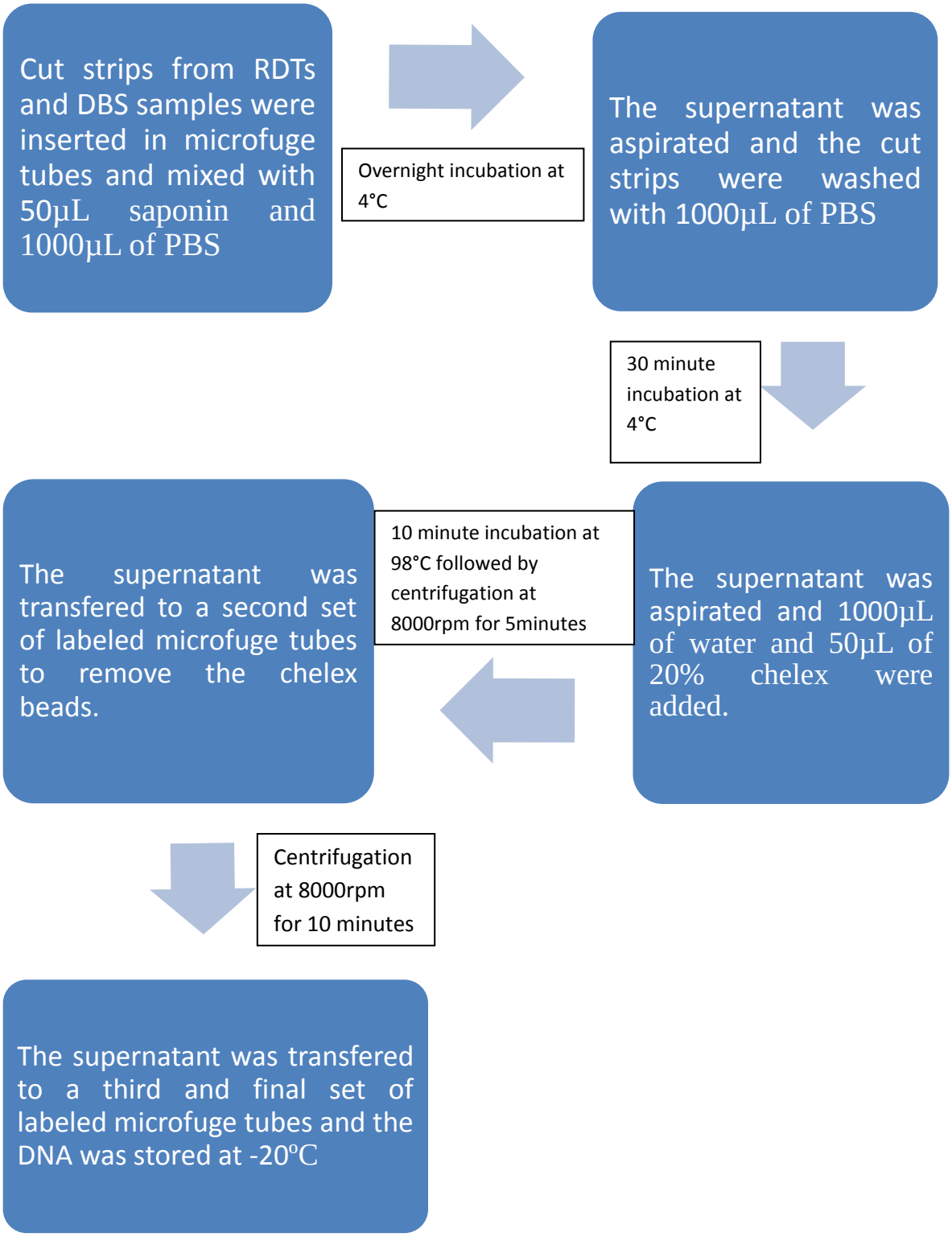

Figure 13: Flow chart showing the chelex DNA extraction procedure

3.2.4. Loop-mediated isothermal DNA Amplification (LAMP)

In this procedure, Pan-LAMP tubes which are capable of detecting 4 species of Plasmodium; *P. falciparum, P. vivax, P. malariae and P .ovale* were used. In addition P. f-LAMP kits that specifically amplify *P. falciparum* were used to determine the presence or absence of *P. falciparum.* Both kits were used according to the manufactures protocol (LMC 562, Eiken Chemical Co., Ltd. Tokyo, Japan). LAMP was performed to determine the presence of *Plasmodium* parasites in the blood samples based on the presence or absence of *Plasmodium* DNA. A volume of 15µl of DNA from the stored samples and 15µl of nuclease free water was added to Pan-LAMP tubes. The Pan-Lamp tubes were then inverted up-side down for 2 minutes to mix the DNA and nuclease free water with primers shown in figure 14 (FIP and BIP comprising F1, F2 and B1, B2 priming sites, correspondingly and two "Displacement primers" F3 and B3) and the *Bst* polymerase (from *Bacillus stearothermophilus*) that come vacuum dried in the Pan-LAMP tube cap (Notomi et al, 2000). After 2 minutes the tubes were inverted 5 times to ensure that the solution was thoroughly mixed with the LAMP reagents. The tubes were then put in a thermo-cycler that was used to amplify the DNA at 65°C for 45 minutes. Visualization of the results was achieved by observing the change in turbidity and fluorescence under Ultra Violet (UV) light at 366 nm. The results of the LAMP reaction were immediately entered into a lab book and into an excel spreadsheet as raw data as shown in figure 6.

All the positive Pan-LAMP samples were then run with P. f-LAMP kits to determine the presence of *P. falciparum.* The protocol for Pan-LAMP kits and P. f-LAMP kits was the same as shown in figure 15. Therefore the same method was used as well to visualize the results. The results of the reaction were immediately entered into a lab book and into an excel spreadsheet.

REGION	PRIMER SEQUENCE (5' to 3')
F1P (F1C+F2)	AGCTGGAATTACCGCGGCTGGGTTCCTAGAGAAACAATTGG
B1P (B1+B2C)	TGTTGCAGTTAAAACGTTCGTAGCCCAAACCAGTTTAAATGAAAC
F3	TGTAATTGGAATGATAGGAATTTA
B3C	GAAAACCTTATTTTGAACAAAGC
LPF	GCACCAGACTTGCCCT
LPB	TTGAATATTAAAGAA

Figure 14: Primer sequences for LAMP amplification.

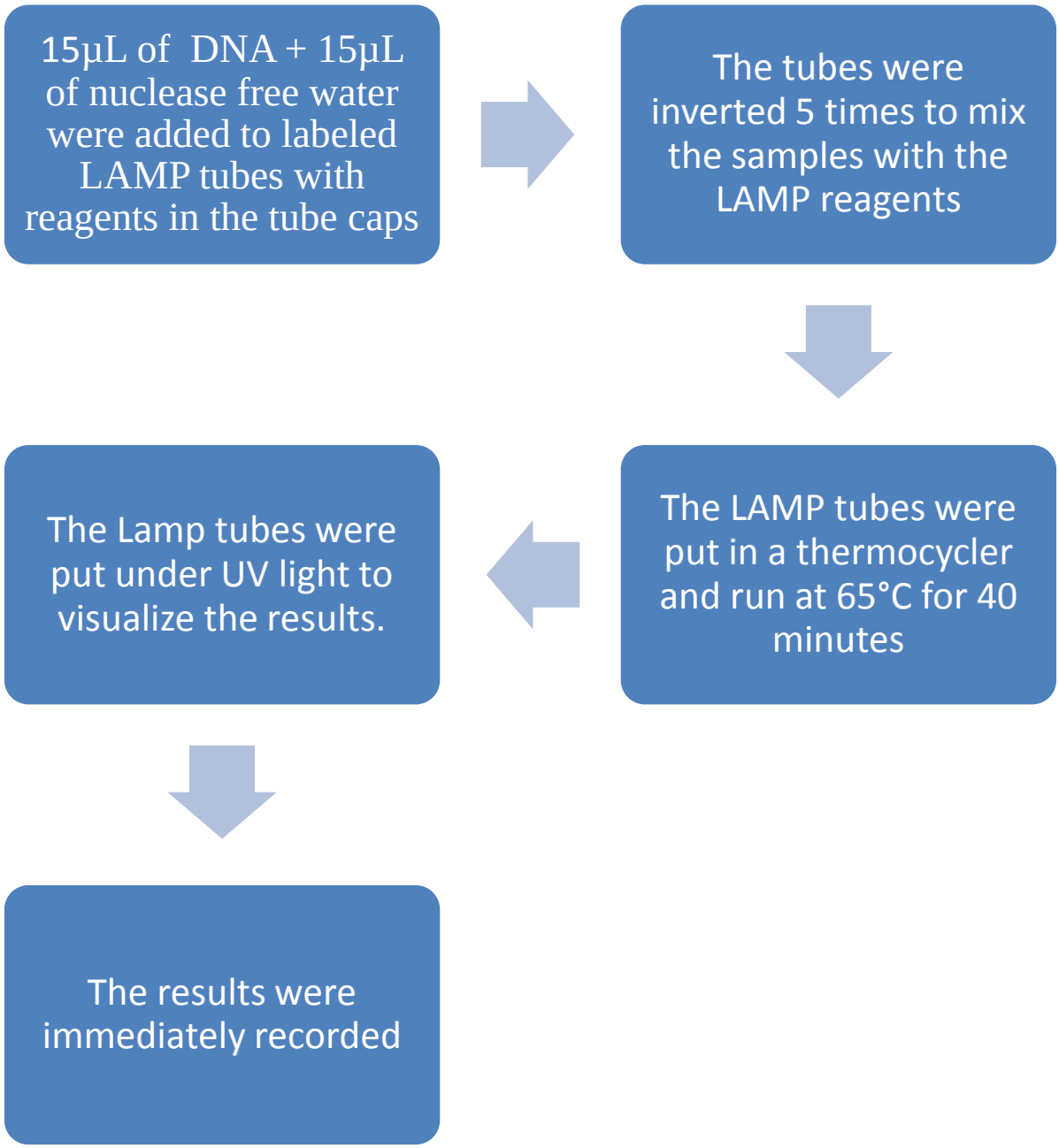

Figure 15: A flow chart showing the LAMP procedure

3.2.5 Polymerase Chain Reaction (PCR)

Nested-PCR was used as a reference diagnostic tool for LAMP as it is a validated technique. Nested-PCR was performed to amplify *Plasmodium* DNA in order to determine if there were malaria infections based on the presence or absence of *Plasmodium* DNA. Nested-PCR was run on every positive RDT and LAMP sample and on every 10^{th} negative sample as a reference for quality assurance of DNA isolation of positive and negative results. The nested-PCR was run using the primers CB1 and CB2 for the primary round and the product from this primary round was diluted by a factor of 1:5. The primers NCB1 and NCB2 were used for the nested round as shown in figure 6 with the diluted product from the first round being used as a template. The master mix for the nested-PCR reaction was prepared according to the protocol shown in Table 2 and the PCR conditions are shown in figure 17. After the nested round of PCR, 5µL of the PCR product were mixed with 2µL of loading dye. These samples were then loaded onto a 2% polyacrylamide gel and run for 110 minutes at 90volts. After running the gel, it was placed in a gel documenting system that was connected to a desktop computer to visualize the results. The results were saved as a picture on the computer, entered into a lab book and also entered into an excel spread sheet. All PCR positive samples with bands present on the gel were used for species determination and for this, a restriction enzyme *AlluI* was used. The products from the nested round of PCR were incubated with the *AlluI* enzyme for 4hours and then mixed with 2µL of loading dye. These samples were loaded onto a 2.5% Polyacrylamide gel and run at 90volts for 70minutes. The gel was then placed in a gel documenting system to visualize the results. The restriction band patterns that indicated the *Plasmodium* species present from the gel were saved as pictures on the computer, the species were entered in a lab book and an excel spreadsheet.

Primary Round:

Primer Name	Sequence
CB1	5' TTTAGCAAGTCGATATACACCAGA
CB2	5' CTTTAACTTGCCAACTCCCTATCA

Nested Round:

Primer Name	Sequence
NCB1	5' GAG AAT TAT GGA GTG GAT GGT G
NCB2	5' TGGTAATTGACATCCAATCC

Figure 16: Primer sequences for first and second round PCR

Table 2: Master Mix composition for primary and secondary PCR

Round 1	Stock Conc.	Reaction Conc.	1x (µL)	Multiple (µL)	Final Volume (µL)
Water	… …	… …	8.1		
Primer – CB1	10µM	1µM	2.5		
Primer – CB2	10µM	1µM	2.5		
10x Buffer	10x	1x	2.5	X__	
dNTP	2mM each	200µM each	2.5		
$MgCl_2$	50mM	3.0mM	1.25		
Taq(Invitrogen Regular)	5U/µL	2U/µL	0.4		
Master Mix per Well		20			
Template DNA		5			

Round 2	Stock Conc.	Reaction Conc.	1x (µL)	Multiple (µL)	Final Volume (µL)
Water	… …	… ….	8.35		
Primer – NCB1	10µM	1µM	2.5		
Primer – NCB2	10µM	1µM	2.5		
10xBuffer	10x	1x	2.5	X____	
dNTP	2mM each	200µM each	2.5		
$MgCl_2$	50µM	2.5µM	1.25		
Taq (InvitrogenRegular)	5U/µL	2U/µL	0.4		
Master Mix per Well			20		
Template DNA			5		

1)**Species, Primary Round PCR Program: 3 hrs**
2)Initial denaturation
3)94°C x 5m
4)PCR
5)40 cycles of 94°C x 30s, 52.5°C x 90s, 68°C x 90s
6)Final Elongation
7)68°C x 10 m
8)Hold @ 4 °C

Species, Nested Round PCR Program: 3 hrs
Initial denaturation
94°C x 5m
PCR
40 cycles of 94°C x 30s, 60°C x 90s, 72°C x 90s
Final Elongation
72°C x 10m
Hold @ 4 °C

Figure 17: Cycling conditions for amplification of the PCR primary and secondary round

3.2.6 Sterilization

LAMP is very sensitive and prone to contamination due to its high amplification efficiency; the following measures were taken to avoid contamination.

A lab-coat designated for LAMP was worn for the purposes of setting up LAMP reactions, the lab-coat was washed routinely and if any contaminant came into contact with it, it was immediately changed. The bench top was first sterilized with 10% bleach; the bleach was allowed to dry and then wiped down twice with water before and after running LAMP and nested-PCR reactions. All the pipettes were sterilized with 10% bleach and left to air dry before they were rinsed twice with distilled water and dried thoroughly with clean tissue wipes. A new

pair of gloves was then worn and the bench top was covered with a bench coat. The LAMP reaction was then set up after following the mentioned steps.

After running LAMP, the LAMP tubes were immediately disposed of by incineration. The Bench coat was also disposed in the same bag as the LAMP tubes. The bench top and pipettes were sterilized with 10% bleach, allowed to dry and then wiped down with distilled water. After following these steps, the bench space was suitable for the next reactions.

The above protocol also applied to the DNA extraction phase and PCR. In addition to this protocol, separate DNA extraction and amplification (LAMP and nested-PCR) stations were allocated and there was no cross transfer of equipment and reagents between stations (Extraction and amplification area).

3.2.7 Data analysis

The quantitative data, number of positive and negative samples from each diagnostic tool were analysed statistically.The Medcalc® statistical software was used to do the diagnostic test evaluation and relative risk test at 95% Confidence Interval (CI). The diagnostic evaluation test calculates 5 parameters which are sensitivity, specificity, disease prevalence, positive predictive value and the negative predictive value of 0.05 level of significance.

3.2.8 Research ethics

The study was given ethical clearance by, the Ministry of Health and Social Services Biomedical Research Ethics Committee in Namibia, London School of Hygiene and Tropical Medicine and University of California at San Francisco Institutional Review Board. Consent forms were issued

out to individuals before blood was taken from them, for individuals below the age of 18 a parent or guardian gave consent. Participation was voluntary.

Chapter 4: Results

4.1 Sample processing

DNA was extracted and amplified from 65 control neighbourhoods (1132 individuals) and 68 case neighbourhoods (1658 individuals), making up a total of 2790 individuals. A total of 5580 Dried Blood Spot (DBS) and Rapid Diagnostic Test (RDT) samples were collected; 1 DBS and 1 RDT sample per individual. All the RDT results were recorded during the collection of the samples.

4.2 Detection of malaria infections by RDTs, LAMP and nPCR

There were a total of 37 RDT positive samples from a total of 2790 individuals. A total of 36 of the RDT positive samples were from case neighbourhoods and 1 RDT positive sample was from a control neighbourhood. This shows that only 2.7% of the malaria infections were from control neighbourhoods as shown in figure 18. Figure 18 shows an example of an RDT positive sample.

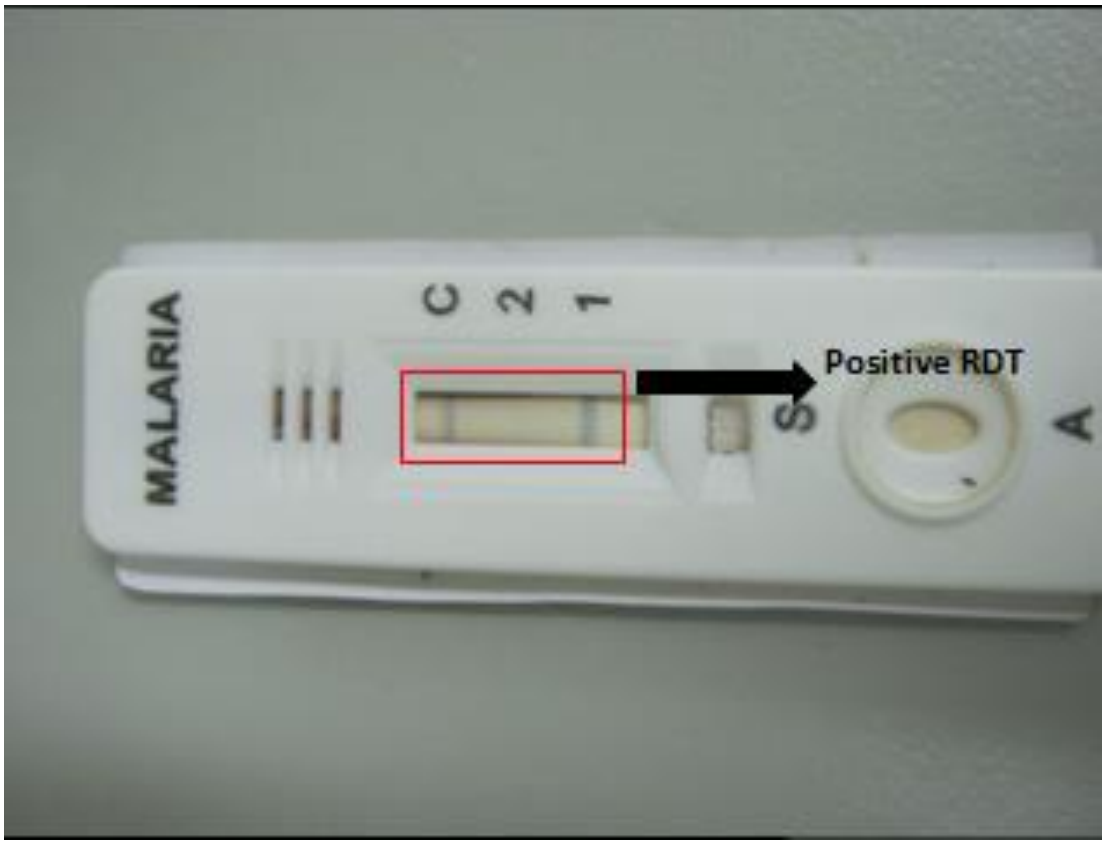

Figure 18: Showing a positive RDT indicated by 2 or more lines, there were 37 positive RDTs from 2790 individuals

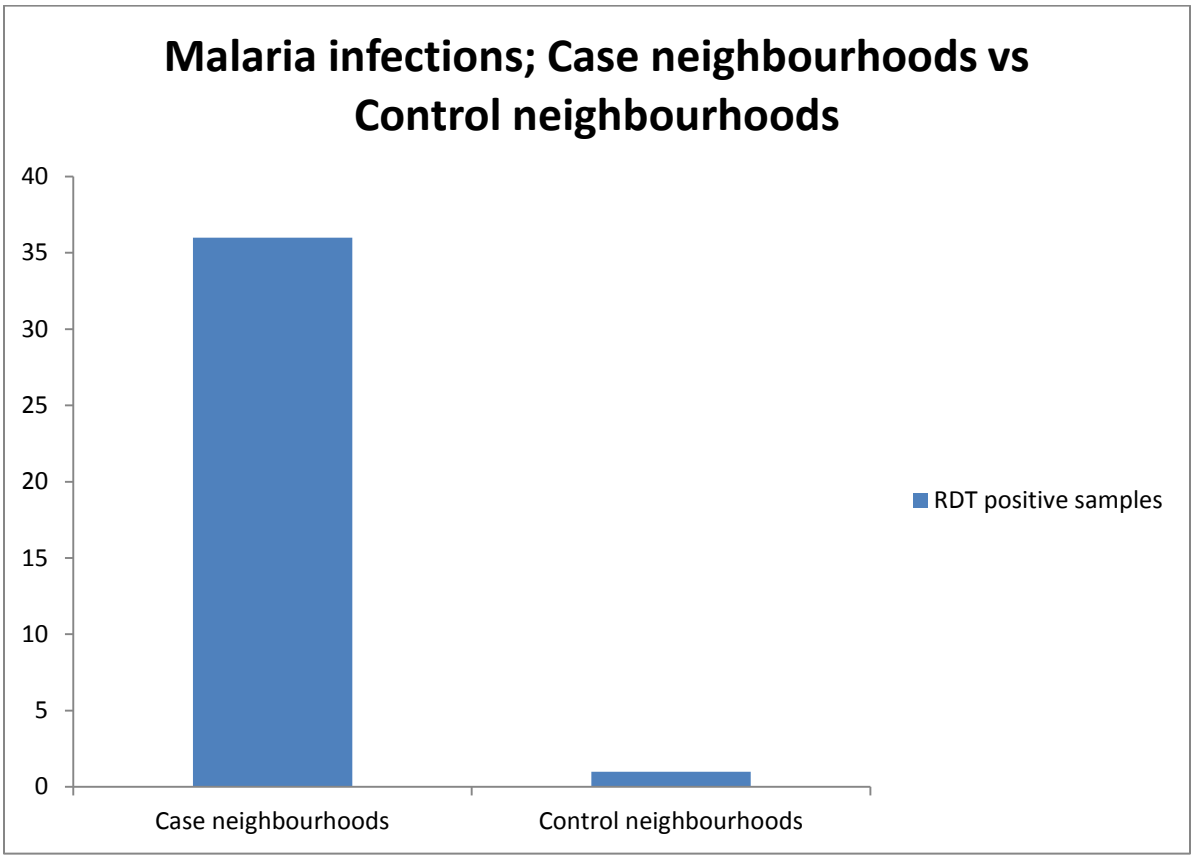

Figure 19: Only 2.7% of the malaria infections were from control neighbourhoods

There were 66 malaria positive samples with LAMP, 1.78times more positives with LAMP than with RDTs. A total of 64 of the LAMP positive samples were from case neighbourhoods and the other 2 LAMP positive samples, 3% of malaria infections were from control neighbourhoods as shown in figure 18 above. Figure 19 shows LAMP positive samples indicating the presence of a malaria infection. There were 3 positive RDTs that were negative with LAMP.

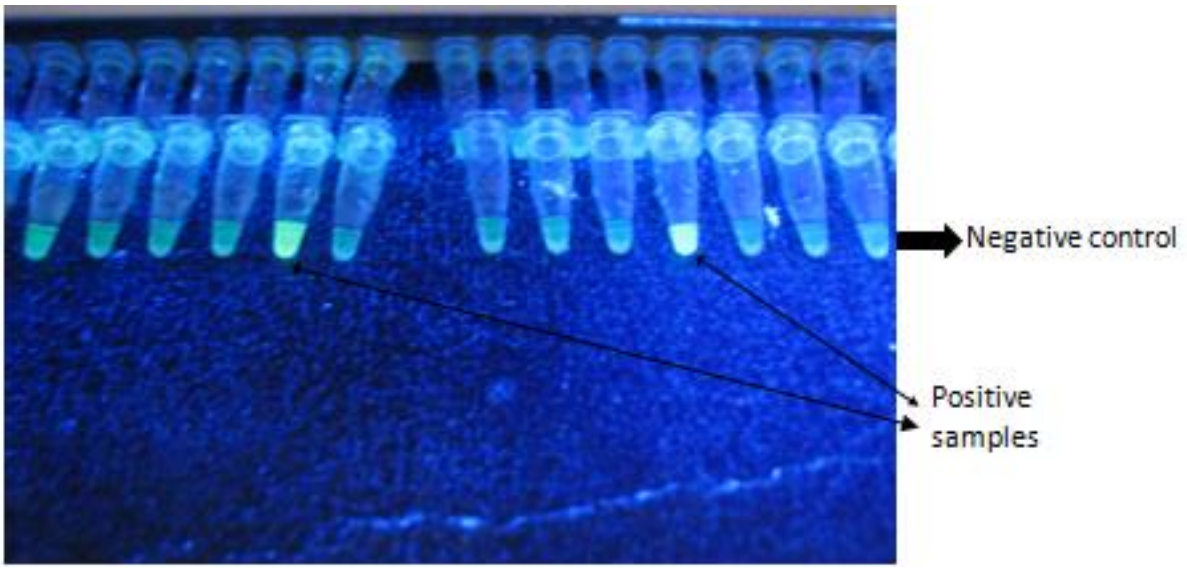

Figure 20: Showing 2 fluorescing LAMP tubes indicating presence of a malaria infection

There were 64 positive samples with nested PCR, 1.72 times more positives with nested-PCR than with RDTs. LAMP has 0.03 times more malaria positive samples than nested-PCR. A total of 62 of the nested-PCR positive samples were from case neighbourhoods and the other 2 nested-PCR positive samples, 3%, were from control neighbourhoods as shown in figure 19. Figure 20 shows a gel with the nested-PCR positive samples. There were 3 positive RDTs that were nested-PCR negative as shown in figure 21 below.

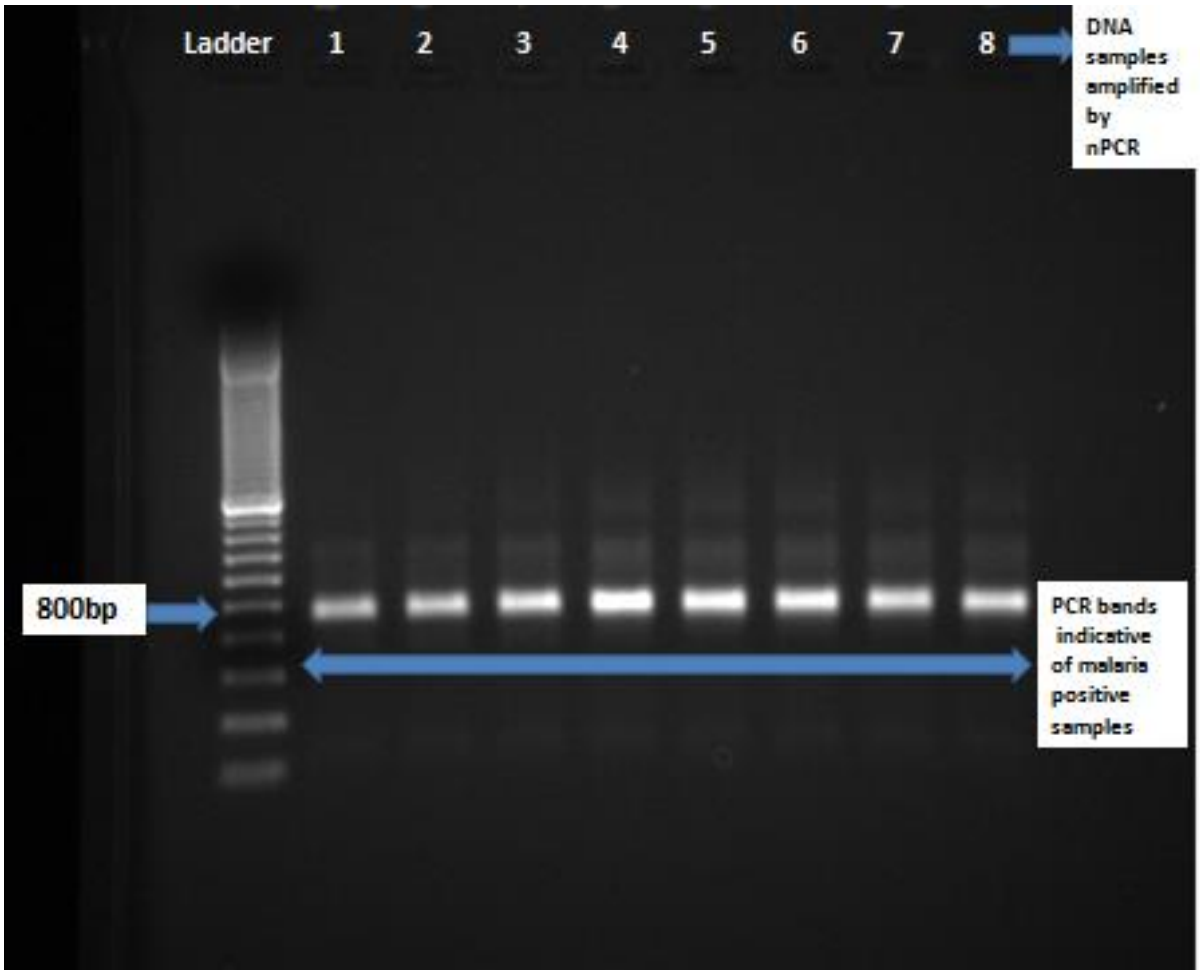

Figure 21: A gel showing PCR positive samples. First lane – 50bp Ladder, Lane 1 - Positive control, Lanes 2, 3, 4, 5, 6, 7 and 8 - Positive samples indicating the presence of malaria infections.

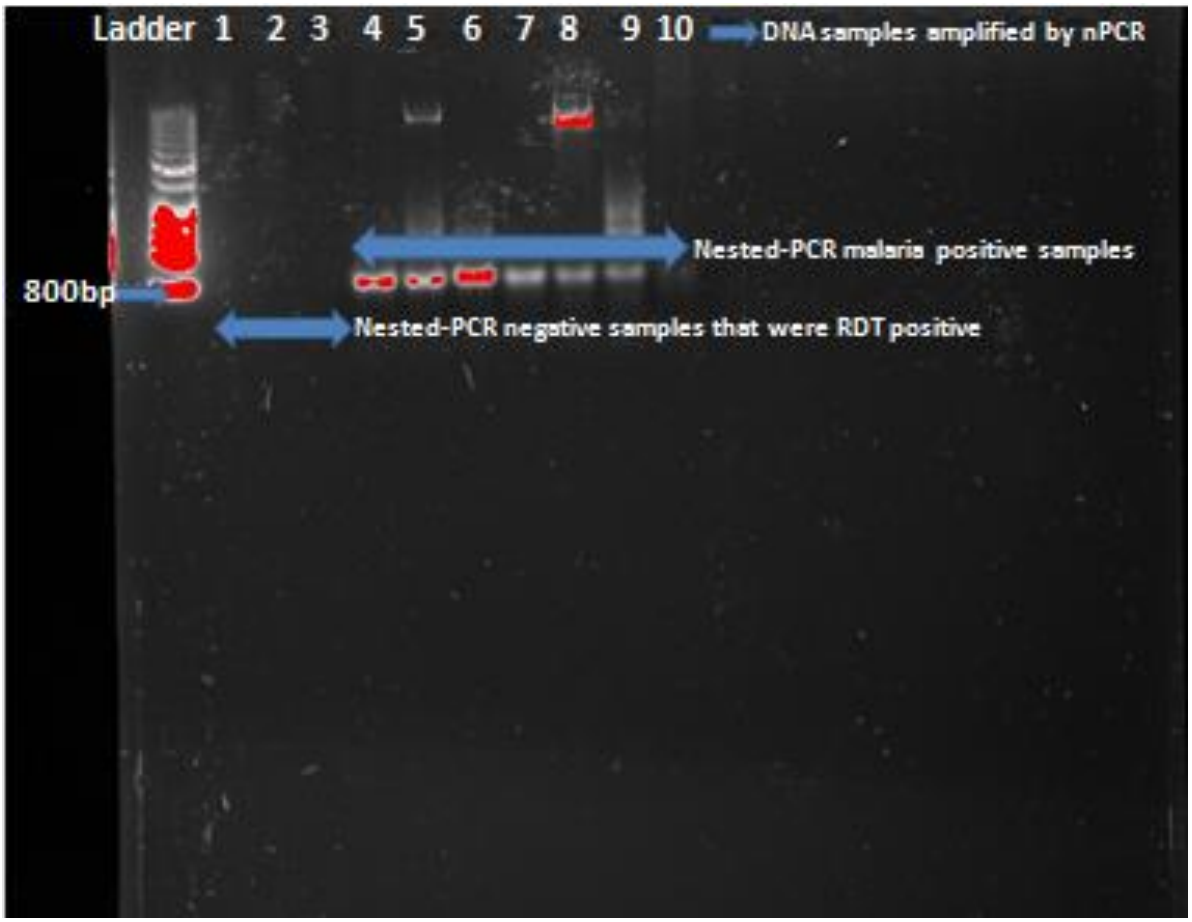

Figure 22: A gel showing PCR positive samples. First lane – 50bp Ladder, Lanes 1, 2 and 3 – PCR negative samples that were RDT positive, Lanes, 4, 5, 6, 7, 8, 9 and 10– Positive malaria samples with PCR

4.3. A comparison of the detection of malaria infections in case samples using LAMP, nested-PCR and RDTs

From 1658 case samples 64 samples were positive with LAMP, 62 samples were positive with nested PCR and 36 of these samples were RDT positive as shown in figure 24. The number of positive samples doubled with LAMP and nested-PCR compared to RDTs. Three positive RDT samples were negative with LAMP and nested-PCR.

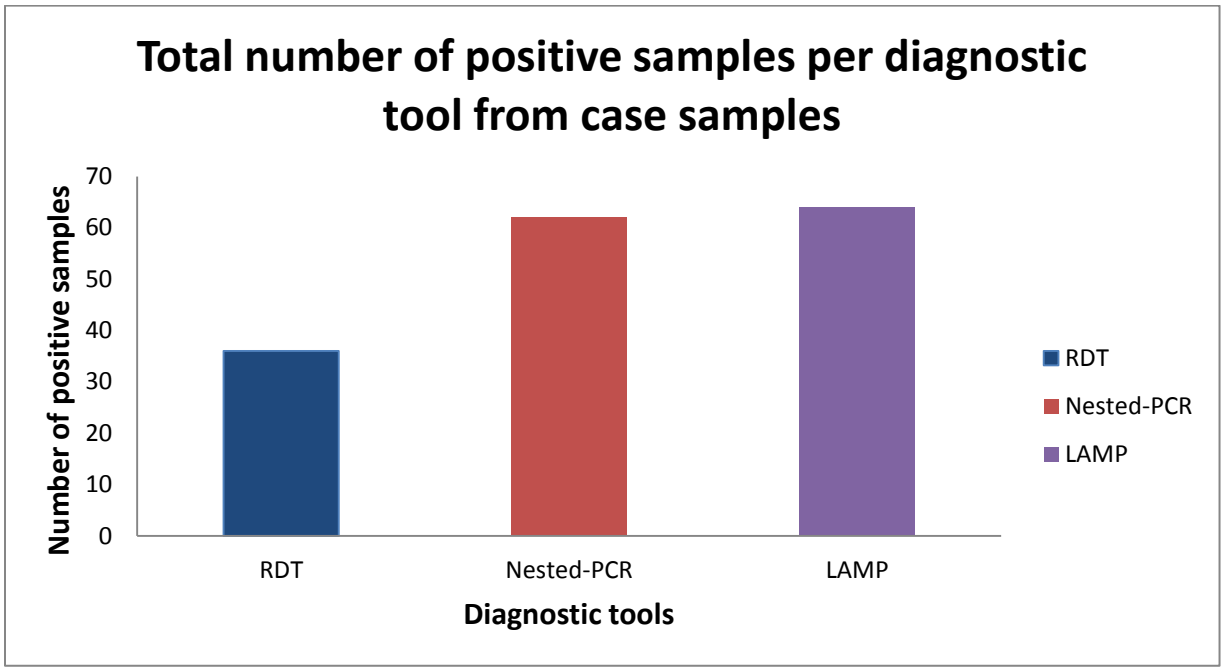

Figure 23: A graph showing the total number of case positive samples detected by each diagnostic tool

4.4. A comparison of the detection of malaria infections in control samples using LAMP, nested-PCR and RDTs

From a total of 1132 control samples, 2 samples were positive with LAMP and nested-PCR; one of these samples was RDT negative and the other one was RDT positive as shown in 25. The number of positive samples that was detected by RDTs doubled with LAMP and nested-PCR.

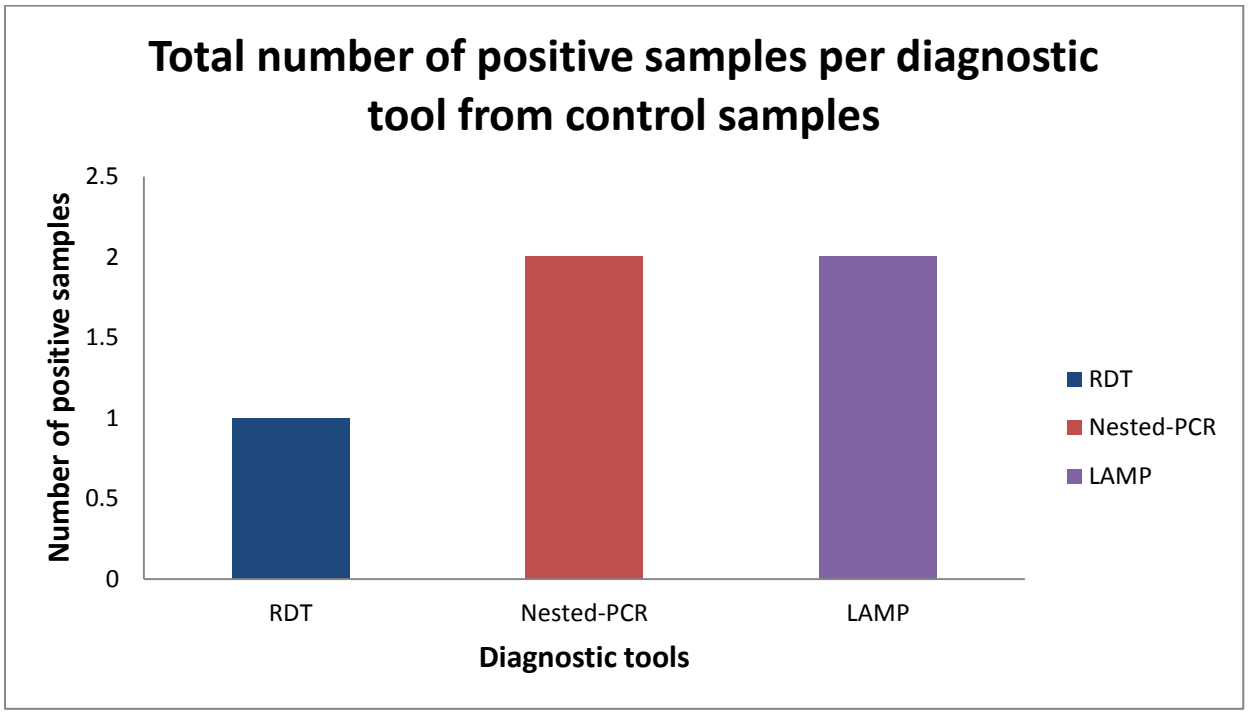

Figure 24: A graph showing the total number of control positive samples detected by each diagnostic tool

4.5 Summary of Secondary cases (Excluding index cases)

In the study, secondary cases were defined as all the additional malaria positive infections excluding the reported malaria case (Index case). There were a total of 37 secondary cases from 2970 individuals. LAMP detected 37 secondary cases, nested-PCR detected 35 secondary cases and RDTs detected 9 secondary cases as shown in figure 26 below. LAMP and nested-PCR detected 4 times more secondary cases than RDTs.

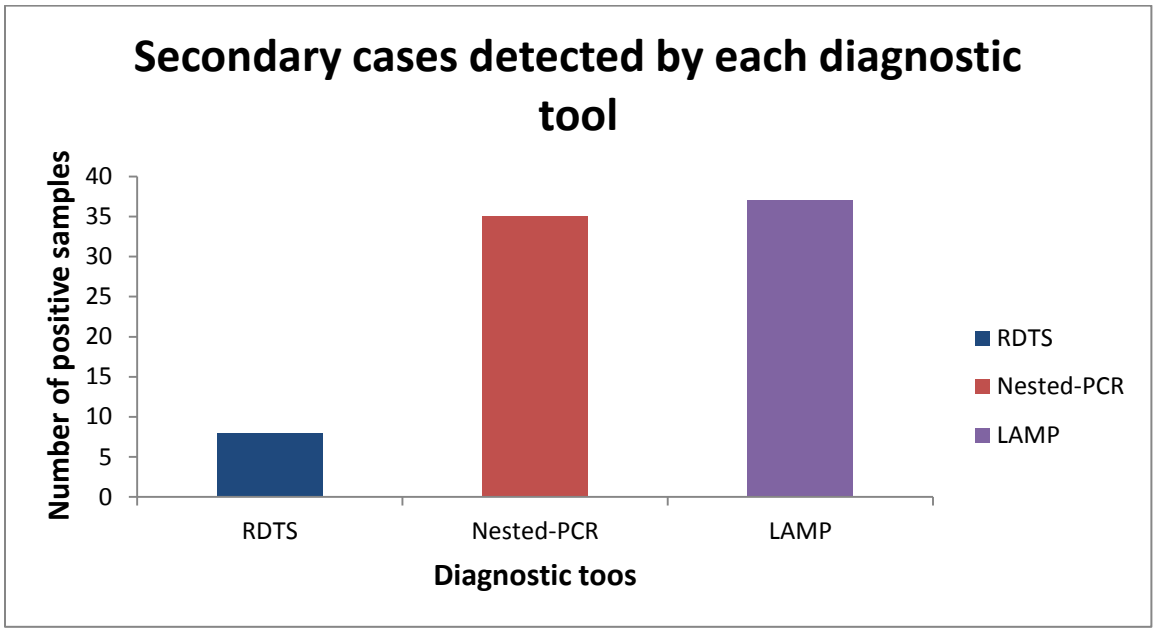

Figure 25: A graph showing the total number of secondary cases detected by each diagnostic tool

4.6 Secondary cases from case and control households

There were a total of 35 secondary cases from 68 index cases, 8 of these cases were detected by all three techniques; LAMP, nested-PCR and RDTs. A total of 25 of the secondary cases were detected by LAMP and nested-PCR only. The remaining 2 secondary cases were detected by LAMP only. LAMP and nested-PCR detected 3 times more secondary cases than RDTs.

There were 2 secondary cases from 65 control-household. The 2 secondary cases from control households were detected by both nested-PCR and LAMP. RDTs only detected one of the secondary cases. The number of secondary cases detected by RDTs doubled with LAMP and nested-PCR. Table 3 below shows the total number of secondary malaria cases detected by each diagnostic tool in case and control neighbourhoods.

Table 3: Summary of secondary cases detected by each diagnostic tool in case and control neighbourhoods

	RDT	PCR	LAMP
Case neighbourhood secondary positives	8	33	35
Control neighbourhood secondary positives	1	2	2

4.8 Species determination

All three techniques, RDTs, LAMP and nested-PCR were used to determine the *Plasmodium* species that were present for every infection that was detected. All positive RDTs indicated that the infections were caused by *Plasmodium falciparum*. *P. falciparum* specific LAMP kits were used to determine and confirm the presence of the species. These specific LAMP kits determined and confirmed that all the infections were caused by *P. falciparum*. Cytochrome B digestion with the Alu1 enzyme was also done to determine the *Plasmodium* species present in each infection. All the cytochrome B digestions indicated that all the infections were caused by *P. falciparum*. Representative pictures of the cytochrome B digestion results and LAMP species specific results are shown in figures 27 and 28.

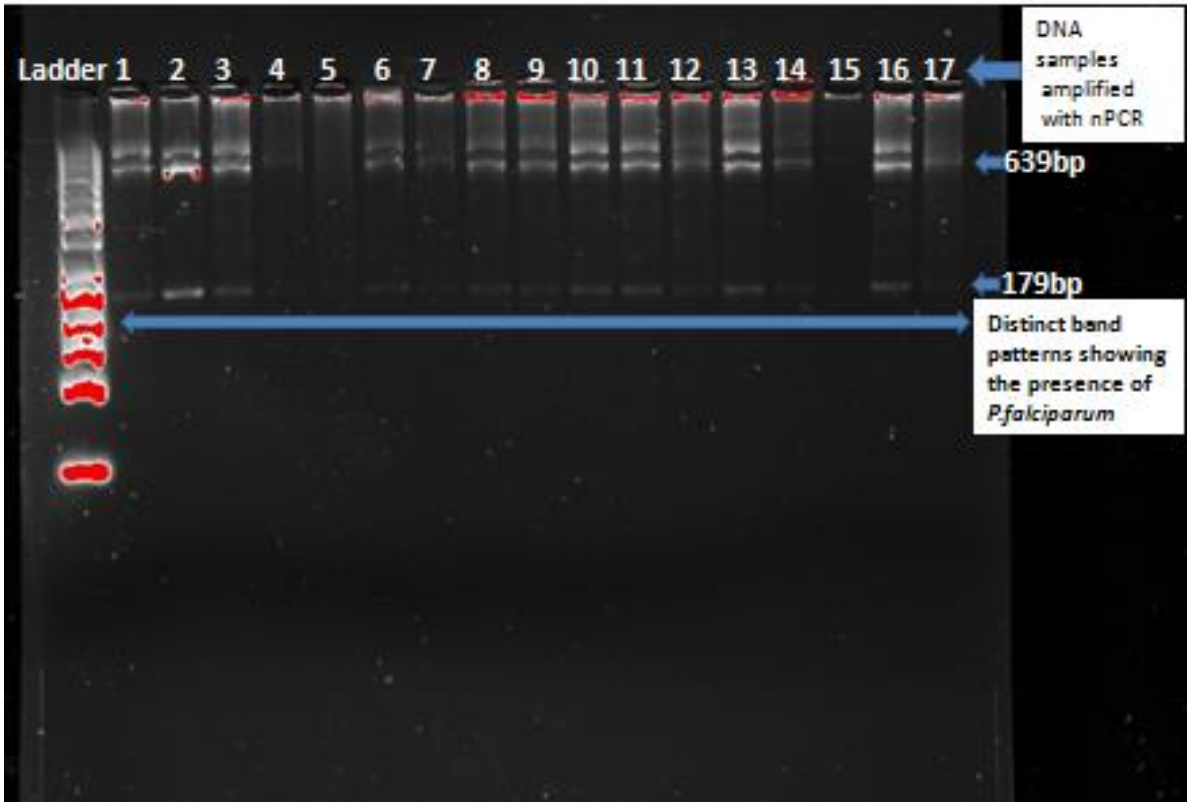

Figure 26: A gel showing digestion patterns that indicate the presence of P. falciparum. First lane – 50bp ladder, Lanes 1 to 17 positive samples with distinct P. falciparum band patterns.

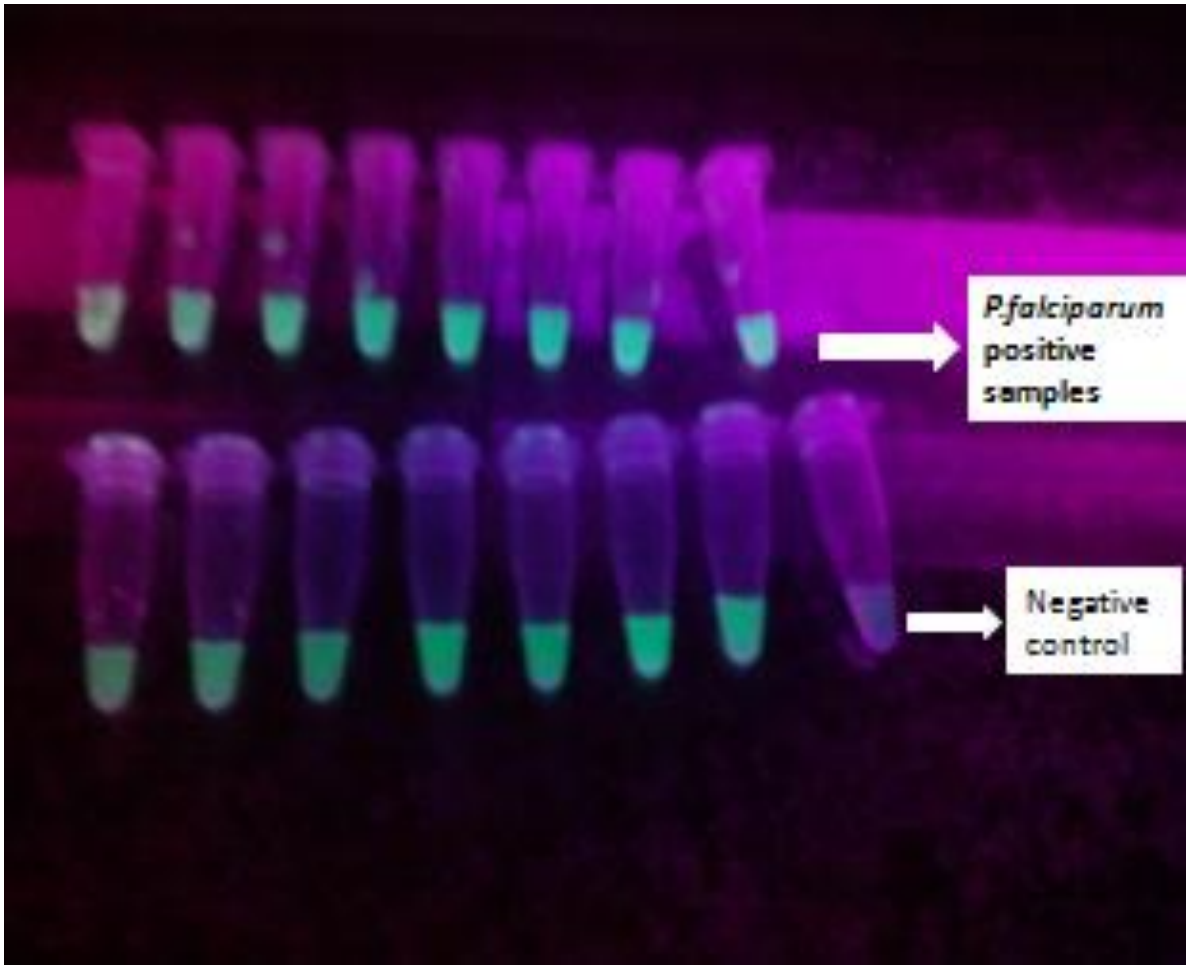

Figure 27: *Showing fluorescing LAMP tubes that are an indication of the presence of P.falciparum in all the infections.*

4.9. Statistical Analysis

Table 4: Diagnostic tool evaluation test

Diagnostic tool	RDTs (95% CI)	n-PCR (95% CI)	LAMP (95% CI)
Sensitivity	53.62% (41.21% - 65.72%)	96.97% (89.46% - 99.91%)	100% (94.51% - 100%)
Specificity	99.89% (99.89% - 99.98%)	100% (99.67%- 100%)	100% (99.86% - 100%)
Disease prevalence	2.44% (1.90% - 3.08%)	2.36% (1.83% - 2.99%)	2.37% (1.83% - 3%)
Positive predictive value	92.50% (79.59% -98.34%)	100% (91.34% - 100%)	100% (94.51% - 100%)
Negative predictive value	98.85% (98.38% - 99.29%)	99.82% (99.36% - 99.97%)	100% (99.86% - 100%)

The calculated relative risk was 21.07 with a 95% CI.

Chapter 5: Discussion

The need for more sensitive and specific diagnostic tools is becoming more undoubtedly important as there are reductions in the transmission of malaria and countries like Namibia are going towards elimination. It has been shown that as the transmission of malaria is reduced by the scaling up of the different interventions, the majority of the malaria cases will be sub-patent infections and these are missed by the commonly used conventional tools that are RDTs and microscopy (Sturrock et al., 2013). In order to effectively find and treat all the remaining cases of malaria, reactive case detection can be an important intervention as passive detection of malaria cases will not identify the majority of the cases that are sub-patent infections in low transmission settings (Hsiang et al., 2012). These sub-patent infections are important because they can act as reservoirs that perpetuate malaria transmission as they are able to infect the *anopheles* mosquito with the *plasmodium* parasites through mosquito bites. Therefore a combination of a highly sensitive molecular tool, LAMP and reactive case detection was used for this study in Northern Namibia with the RDTs and DBS being used as the template for DNA extraction.

Findings from previous studies have shown that the chelex method is a very effective technique for DNA extraction from filter paper and RDT in comparison with the other available techniques (Morris et al., 2013; Ishengoma et al., 2011). As a result, in this study, the Chelex DNA extraction method was the method of choice for DNA extraction from both RDT and DBS samples.

There were a total of 37 RDT, 66 LAMP and 64 n-PCR positive samples from the 2790 individuals that were tested for malaria in both control and case neighbourhoods. Only 1 of the RDT positive samples and 2 of the LAMP and n-PCR positive samples (≈2.9% of the total positive samples) were from control neighbourhoods, this suggests and shows that individuals that live closer to malaria infected people are at a higher risk of getting infected by malaria in the presence of the *Anopheles* mosquitoes as shown by the studies in Swaziland and Asia Pacific (Sturrock et al., 2013). The use of reactive case detection enabled detection of the asymptomatic cases around the passively detected positive malaria cases.

There were 36 RDT, 64 LAMP and 62 n-PCR positive samples from a total of 1658 individuals from case neighbourhoods. This shows a positivity rate of 2.17% by RDTs and 3.98% by LAMP in case neighbourhoods with n-PCR giving positivity rate of 3.86% as the reference technique. The number of infections detected by RDTs nearly doubled when LAMP was used with n-PCR as the reference. This was similar to the results from a study done in Zanzibar were the number of positive samples with LAMP doubled in comparison with RDTs (Cook et al., 2015). RDTs have a detection limit of about 100parasites/µL whilst LAMP has a detection limit of 1-2 parasites/µL which makes LAMP more suited for the detection of sub-patent infections (Schmidt et al., 2014). LAMP has a high sensitivity because like n-PCR, it amplifies the signal (DNA) for detection compared to RDTs that detect antigens in the blood. These results were consistent with

the results found in a study in Uganda, a low transmission setting, where LAMP had detection efficiency comparable to n-PCR (Hopkins et al., 2013).

LAMP also detected 2 more infections than n-PCR although both diagnostic tools amplify the signal (DNA) for detection. Similar results were found in Ethiopia in a study carried out by Sema and colleagues, where LAMP had one more positive than nPCR (Sema et al., 2015). This is because LAMP has primers that target mitochondrial DNA that has a higher copy number than the 18s rDNA targeted by the primers used in n-PCR. In addition to this, LAMP uses a more robust polymerase, *Bst*,which is not affected by most inhibitors that affect the reaction efficiency in n-PCR as it uses the *Taq* polymerase (Gonzalez et al., 2011; Schmidt et al., 2014; Hsiang et al., 2014).

There were 3 RDT positive samples that were negative by both LAMP and n-PCR. All three samples were the index cases; that is they were the cases that had been reported and treated at the health facilities. This is to be expected with RDTs as antigens can remain in the blood for up to a month after parasite clearance. In addition to this, poor storage of the RDT and improper use of the RDTs can give rise to false positives (Patel et al., 2014; Sturrock et al., 2013).

Secondary cases were defined as all the additional malaria positive infections excluding the reported malaria case (Index case) in this study and there were a total of 37 secondary cases from 2970 individuals. LAMP detected 37 secondary cases, nested-PCR detected 35 secondary cases and RDTs detected 9 secondary cases as shown in figure 27. LAMP, with nested-PCR as a reference detected 4 times more secondary cases than RDTs. This can be attributed to the high sensitivity of LAMP with a detection limit of 1-2 parasites/µL (Gonzalez et al., 2011). All of the secondary cases were asymptomatic, possibly as a result of low parasite density which caused most of them to be missed by the RDTs and be detected by LAMP. In addition to this, 94.59% of the secondary cases were detected in case neighbourhoods where malaria infections had been

reported. This is further evidence to show that an individual is at a high risk of getting malaria if they are close to an infected individual (Gueye et al., 2013; Hsiang et al., 2012) in any setting including Northern Namibia which is a low transmission setting. Therefore reactive case detection becomes very useful in low transmission settings as it allows following up, testing and treating the individuals that are most likely to be infected and also spread malaria; this is a positive step towards malaria elimination.

The diagnostic evaluation test gives an indication and comparison of how effective a diagnostic tool is compared to the other diagnostic tools. The parameters measured by the evaluation test are sensitivity, specificity, disease prevalence, positive predictive value and negative predictive value. Sensitivity is the probability that a test result will be positive when the disease is present (true positive rate). Specificity is the probability that a test result will be negative when the disease is not present. The positive predictive value (PPV)is the probability that the disease is present when the test is positive. The negative predictive value (NPV) is the probability that the disease is not present when the test is negative. LAMP had the highest sensitivity (100%), specificity (100%), PPV (100%) and NPV (100%) followed by n-PCR which had a sensitivity of 96.97%, specificity of 100%, PPV of 100% and a NPV of 99.82%. The RDTs had the lowest values with a sensitivity of 53.62%, specificity of 99.89%, a PPV of 92.50% and a NPV of 98.85%. These results show that the probability of detecting a malaria infection is doubled with the use of LAMP than with RDTs. Therefore the use of LAMP becomes important in reactive case detection in order to detect sub-patent infections that are missed by RDTs as a result of their poor sensitivity at low parasite density. LAMP had sensitivity higher than n-PCR which was used as the reference diagnostic tool. This is as result of the higher amplification efficiency and the use of a more robust polymerase enzyme by LAMP (Hsiang et al., 2014; Gonzalez et al.,

2011). The routine use of RDTs in Northern Namibia could be missing a significant number of malaria infections as shown by the results.

In addition to the high sensitivity of LAMP, it had a short turn-around time for results. The turn-around time for results for LAMP was an eighth of the turn-around time for n-PCR. LAMP preparations were done at room temperature on a bench-top unlike n-PCR preparations that were done on ice, LAMP has basic preparations that makes it an appropriate diagnostic tool even for low resource setting health facilities.

Human malaria, for the past 8 decades has been known to be caused by 4 plasmodium species, namely *P. falciparum, P. vivax, P. malariae and P. ovale*. A fifth species has been recently discovered to be a cause of human malaria, *P. knowlesi* (Kantele & Jokiranta 2011). The interventions employed to trace and treat the malaria infections are dependent on the species causing the infection (Sturrock et al., 2013) hence it is important to determine the species present. For example, *P. vivax* is normally maintained at low parasite density in infected individuals (Sturrock et al., 2013), therefore knowledge of the presence of *P. vivax* informs the clinicians that highly sensitive diagnostic tools have to be employed in order to detect all the infections. All the three diagnostic tools detected the presence of *P. falciparum* only. *P. falciparum* is the most dangerous of the species that cause human malaria. Knowledge of the presence of *P. falciparum* as mono-infections will be important in deciding on which interventions to employ in order to interrupt malaria transmission.

Chapter 6: Conclusion

The significantly high number of secondary cases found around reported cases show that reactive case detection has an important role in low transmission settings like Namibia as it enables the detection of asymptomatic malaria infections that are not reported in hospitals. RDTs have sensitivity (56.06%) significantly lower than that of LAMP (100%) and n-PCR (96.97%) in Northern Namibia, a low transmission setting. These findings draw attention to the need for more sensitive tools in order to detect all malaria infections including sub-Patent infections which make up the majority of infections in low transmission settings.

RDTs, in addition to the diagnosis of malaria are also an important source of DNA. As fewer cases are reported, it becomes more difficult to get blood samples for further research and the use of used RDTs could obviate this problem. Chelex DNA extraction from RDTs is a simple but sufficient technique for further molecular analysis with n-PCR and LAMP. LAMP is a highly sensitive molecular technique that is easy to use, requires minimum preparation since the reagents come vacuum dried in the tube caps. This makes LAMP an appropriate tool for a basic set up that can be used for malaria diagnosis in reactive case detection in Namibia and other low transmission settings even with limited resourses.

In addition, LAMP had very high sensitivity and specificity for both the symptomatic and asymptomatic infections which is important in low transmission settings. Therefore, LAMP has an important potential function in reactive case detection in low transmission settings including Namibia in order to detect all malaria infections and interrupt malaria transmission.

From the evidence presented in this study, it is therefore recommended that reactive case detection be employed as the main surveillance tool for malaria in combination with LAMP in order to detect and treat all reservoirs of malaria including asymptomatic infections that can spread malaria or become develop into symptomatic attacks if not treated in Namibia and possibly other low transmission settings. There is a need for quality assurance of the RDTs as the results show that they can give incorrect diagnosis as a result of parasite antigen persistence after treatment or low parasite density infections. In addition, these RDTs can be used as the source of DNA for further malaria research.

Chapter 7: References

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Chapter 8: Appendices

Appendix A: LAMP procedure by Eiken Japan

Instructions for Use

380201-A

For performance evaluation **REF LMP561**

Loopamp™ MALARIA Pan Detection Kit

INTENDED USE

The Loopamp™ MALARIA Pan Detection Kit is for qualitative detection of *Plasmodium* DNA extracted from human blood samples.

TEST PRINCIPLES

This product is based on the nucleic acid amplification method, LAMP (Loop-mediated Isothermal Amplification), developed by Eiken Chemical Co., Ltd.

The LAMP method has the following characteristics: (1) Only one polymerase enzyme is required and the amplification reaction proceeds under isothermal conditions;[1,2] (2) it has extremely high specificity because of the use of four primers recognizing six distinct regions on the target; (3) it has high amplification efficiency and can produce a high concentration of amplified product in a short time, which makes visual or automated detection possible.[3,4]

The Malaria Pan (genus)-specific primers provided with this product have been designed to detect the mitochondrial DNA of the four most widespread *Plasmodium* species causing malaria (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*). The targeted DNA sequences have been confirmed by alignment analysis to have well-conserved base sequence in all these *Plasmodium* species.

The test DNA solution extracted from blood samples is dispensed into a reaction tube. The strand displacement DNA polymerase, deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), calcein, reaction buffers and Malaria Pan (genus)-specific primers are stored in dried form in the cap of the reaction tube. These dried LAMP reagents (Malaria Pan detection reagents (dMAL Pan)) dissolve when the DNA solution is added. The reaction tube is then incubated at 65.0°C and the DNA is amplified by the strand displacement DNA polymerase in accordance with LAMP reaction.

The detection of amplified products is based on turbidimetric measurement of magnesium pyrophosphate (a white precipitate produced as a by-product of DNA amplification).[3] Alternatively, visual detection under ultraviolet light may be used. Before DNA amplification, calcein contained in the reagent is in the quenched state as it is bound to manganese ions. At the start of DNA amplification, the pyrophosphate ions that are generated out-compete the manganese ions for binding sites, and thus the calcein becomes fluorescent.[4]

CONTENTS OF THE KIT

Reagents are stable until the date on the label assuming the container remains unopened within a storage temperature of 1 – 30°C.

Malaria Pan detection reagent (dMAL Pan) ····· 10 X 48 tubes
The following reagents in dried form are contained in each reaction tube:
- *Bst* DNA polymerase*1
- Deoxynucleotide triphosphates
- Magnesium sulfate
- Calcein
- Manganese chloride
- Primers*2

Positive control Mal (PC Mal)*3 ····· 5 X 1.0 mL
Negative control Mal (NC Mal) ····· 20 X 0.5 mL

*1: *Bst* DNA polymerase derived from *Bacillus stearothermophilus* is a strand displacement DNA polymerase that lacks 5'→3' exonuclease activity.

*2: Primers designed for the mitochondria DNA of *Plasmodium* parasites, purified from synthesized oligonucleotides by HPLC.

*3: PC Mal contains a product resulting from *in vitro* amplification of an artificial gene designed from the mitochondrial DNA of *Plasmodium falciparum* (GenBank No.M76611).

The abbreviations of names of the following reagents, their Lot No. and the manufacturer (EKN), are printed on the containers.

Reagents	Labelling on the tube	Code on the cap
Positive control Mal	PC Mal Lot No., EKN	PC Mal
Negative control Mal	NC Mal Lot No., EKN	NC Mal

WARNINGS AND PRECAUTIONS

(1) For performance evaluation only. Do not use for other purposes including for any medical purpose such as patient diagnosis or patient care decisions.
(2) This product is designed only for detection of DNA of *P. falciparum*, *P. vivax*, *P. malariae and P. ovale* parasites in blood samples of human origin. Do not use for other purposes.
(3) When using this product, always follow this package insert.
(4) Do not freeze the reagents.
(5) Do not use any expired reagent.
(6) Do not mix reagents from different lots.
(7) Do not replenish any reagent.
(8) Performance of the Loopamp™ MALARIA Pan Detection Kit is dependent on operator proficiency and adherence to procedural directions. Testing should be performed by properly trained personnel strictly according to the instructions provided.
(9) Exposure to heat, humidity and light may cause deterioration of the dMAL Pan. Remove only the required number of reaction tubes (number of samples + number of controls) and re-seal the aluminium pouch immediately.
(10) Do not remove the desiccant from the aluminium pouch. High humidity can cause deterioration of the dried LAMP reagent in the reaction tubes.
(11) Read the instruction manual and ensure required equipment (turbidimeter or incubator) is available before commencing the procedure.
(12) Blood samples pose a potential risk for infection. Use universal precautions to minimize biohazard.[5]
(13) PC Mal and NC Mal both contain a small amount of sodium azide as preservative. Sodium azide is classified as toxic. Avoid any contact with eyes, mouth, or skin.
(14) In case of accidental contact of any reagent with eyes, mouth, or skin, immediately rinse the affected site with running water and seek medical advice.
(15) Do not dilute or add the PC Mal to the samples. Use the PC Mal only as described in this package insert in order to avoid DNA contamination.
(16) Store the PC Mal and any positive blood samples separately from the other kit reagents.
(17) The cap of each reaction tube contains dMAL Pan in dried form. Do not touch the inside of the cap.
(18) Before using the reaction tubes, check carefully to see if they have any cracks or scratches. Damaged tubes might give false results and lead to DNA contamination of the incubator and work area.
(19) Do not expose reaction tubes to UV light before the end of the LAMP reaction. Prolonged exposure to UV light might damage the tubes and lead to false results.
(20) When a UV lamp is used for visual fluorescence judgment, do not stare directly at UV light. Since UV light is harmful to the eyes, even watching for a short period may irritate eyes and cause symptoms similar to conjunctivitis. Use a glass screen or wear protective goggles/glasses/face shield whenever looking directly at the UV lamp.
(21) Refer to the manual of the incubator. When the LF-160 or the real-time turbidimeter is used, be careful when removing the reaction tubes from the incubator to avoid burns.

WASTE DISPOSAL

(1) Do not open the tubes after DNA amplification. Leave the cap closed and dispose of the used tubes as medical waste by incineration or after double bagging with sealable plastic bags.
(2) Never autoclave or re-use the reaction tubes. Amplified products will disperse and cause contamination.

(3) The main material for the reaction tubes and reagent tubes is polypropylene (PP); for the reaction tube tray, polyethylene (PET); for the aluminium pouch, aluminium; and for the kit case, paper.
(4) Dispose of any other reagent, container, or lab ware in accordance with regulations.

SPECIMEN COLLECTION

(1) Blood samples should be used immediately after collection.
(2) Collect blood in a separate room from the LAMP amplification room. Aerosols containing *Plasmodium* DNA can be generated during blood collection and may cause contamination.
(3) DO NOT USE EDTA and Citrate as anticoagulant for blood collection if the result is to be read by fluorescence. The use of heparin as anticoagulant is recommended.

MATERIALS REQUIRED BUT NOT PROVIDED

- Loopamp™ PURE DNA Extraction Kit (REF LMC802) (optional)

For Visual fluorescence detection

(For LF-160 incubator)

- LF-160 (REF MVKM17)

(For other incubator using UV lamp)

- Incubator (temperature accuracy: ±0.5°C: with hot bonnet)
- Heating block
- UV lamp (wavelength: 240 to 260 nm, and 350 to 370 nm)
- Goggles/glasses or other UV-blocking eye mask

For real-time turbidity detection

- Real-time turbidimeter LA-500 (REF MVL300) (only for use in the LAMP method; wavelength: 600 to 700 nm; amplification temperature: 65.0°C)

For reagent and sample mixing

- Micropipettes (10 to 100μL, and 20 to 200μL) and pipette tips with filter
- Centrifuge for micro-tubes (optional)
- Centrifuge for eight connected tubes (optional)

PREPARATION OF SAMPLE DNA SOLUTION

The following DNA extraction methods are recommended.

(For boil and spin)

To extract the DNA from blood sample, follow the standard operating procedures published at http://www.finddiagnostics.org/programs/malaria-afs/lamp/standard_procedures/index.html.

(For PURE)

To extract the DNA from blood sample, follow the instruction for the Loopamp™ PURE DNA Extraction Kit[6] (sold separately). Use the DNA solution obtained for LAMP amplification.

PREPARATION OF REAGENTS

(1) Malaria Pan detection reagent

Remove the required number of tubes from the aluminium pouch and put them in the rack provided. (number of samples + number of controls).

Note: After removing the required tubes, seal the aluminium pouch with any unused tubes immediately.

(2) Negative control Mal (NC Mal)

Flick (or spin) down the tube before using, in order to collect the content at the bottom of the tube.

Note: NC Mal should be used with every run.

(3) Positive control Mal (PC Mal)

Flick (or spin) down the tube before using, in order to collect the content at the bottom of the tube.

Note: PC Mal should be used with every run.

MEASUREMENT PROCEDURE

Reagent and sample mixing

(1) Turn on the incubator or the real-time turbidimeter.
(2) Dispense 30μL of extracted DNA solution into a reaction tube using the pipette, and close the cap.
(3) Dispense 30 μL of NC Mal into a reaction tube using the pipette, and close the cap.
(4) Dispense 30μL of PC Mal into a reaction tube using the pipette, and close the cap.
(5) Flick (or spin) down all tubes to collect the solution at the bottom of the tubes.

Note: When using the PURE device make sure the liquid level is closer to the upper line of the two lines on a reaction tube to ensure that the correct volume has been dispensed.

(6) Reconstitute the dried reagent in the cap by inverting the reaction tubes and collecting the DNA solution in the cap. Leave the tubes standing upside down for 2 minutes to reconstitute the dried reagent.
(7) Invert the reaction tubes five times to mix the contents. Make sure that the dried reagent in the cap is fully dissolved (the solution should have a slight orange colour).
(8) Flick (or spin) down all tubes to collect the solution at the bottom of the tubes.

Amplification

For visual fluorescence detection

(for LF-160)

(1) Check that the temperature showing on the incubator is 65.0°C.
(2) Load the reaction tubes into the LF-160 incubator and press the green button to start the LAMP reaction (40 minutes at 65.0°C). See the LF-160 instruction manual for details on how to operate the incubator.
(3) Confirm the completion of polymerase inactivation (automatically completed by the LF-160). Take all reaction tubes from the LF-160.

(for other incubator using UV lamp)

(1) Set the temperature of the incubator at 65.0°C (with hot bonnet temperature set to 10 degrees above the reaction temperature or as near to this figure as possible – temperature accuracy: ±0.5°C). Wait until the temperature displayed reaches the set value.
(2) Load the reaction tubes, and then start amplification reaction (for 40 minutes at 65.0°C).
(3) Forty minutes later, inactivate the polymerase using the heating block (for 5 minutes at 80°C, or for 2 minutes at 95°C) to terminate the reaction.

For real-time turbidity detection (see Flow chart of the procedure)

(1) If not already correctly configured, configure the real-time turbidimeter for detection with this product.
(2) Check if the temperature displayed reaches 65.0°C (allow the turbidimeter to warm up for 20 minutes before use).
(3) Load the reaction tubes, and start measurement.
(4) Watch the display of the turbidimeter to check the positive and negative controls for any increase in turbidity. If the turbidity increases in the positive but not in the negative control solution, amplification reaction is proceeding properly (Fig1). If this is not the case, amplification reaction may be proceeding in a wrong way. In such a case, restart testing from reagent preparation.
(5) Confirm the completion of polymerase inactivation (automatically completed by the turbidimeter). Take all reaction tubes from the turbidimeter and discard them without opening.

Amplification plots by Malaria Pan detection reagent

(Analyzer: real-time turbidimeter LA-500 (two different reaction blocks are shown here))

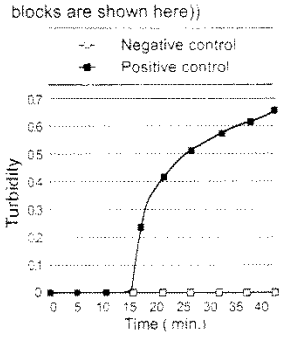

Fig1 : Amplification plots for controls

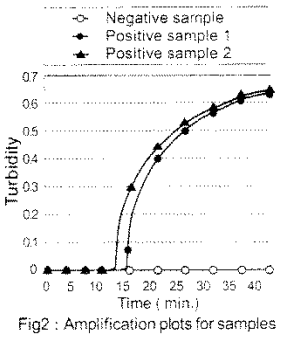

Fig2 : Amplification plots for samples

PROCEDURAL NOTES

(1) The LAMP reaction is very sensitive, and contamination with small amounts of amplified product might lead to false positive results.
(2) Separate the sample preparation and the amplification areas.
(3) Clean benches with over 0.5% sodium hypochlorite before and after performing the test.

(4) Change gloves after transferring the blood or if the gloves come into contact with the DNA solution.
(5) When handling this product, avoid microbial contamination and nuclease contamination. Even a small amount of contamination of the reaction tube from sweat or saliva may decompose DNA and cause a false result.
(6) Furthermore, read the instruction manual of DNA extraction kit when performing DNA extraction.
(7) The DNA solution should ideally be used immediately after preparation.
(8) **(For LF-160 or other incubator using UV lamp)**
If bubbles are present, flick the tubes to release them.
(For real-time turbidimeter)
Since bubbles in reaction solution may interfere with turbidity measurement and cause a false result, avoid forming any bubble when mixing reagent and sample solution. If bubbles occur, spin or flick the tube to release them.
(9) dMAL Pan should be fully dissolved. Any undissolved portion may influence performance, such as causing a decrease in sensitivity.
(10) The PC Mal contains a high copy number of control DNA. Avoid any contamination of other samples with the PC Mal. Dispense the samples and the NC Mal and close all reaction tubes before dispensing the PC Mal.
(11) Flick (or spin) down the PC Mal tube before opening it, in order to collect the content at the bottom of the tube. Close the tube immediately after dispensing the PC Mal.
(12) Never open the reaction tubes once the LAMP reaction has started or after completion. Be particularly careful when unloading the reaction tubes from the incubator to avoid opening the tubes accidentally.
(13) When the LF-160 incubator or the real-time turbidimeter is used, polymerase inactivation is automatically performed.
(14) For other incubators, when visual fluorescence judgment is chosen, inactivate the polymerase (for 5 minutes at 80°C, or for 2 minutes at 95°C) before reading, or false results will be caused.
(15) Do not reuse any amplified product in the tubes for electrophoresis or other applications.

INTERPRETATION OF RESULTS

For visual fluorescence detection

(For LF-160)

Set each reaction tube into the Fluorescence Visual Check Unit, irradiate and observe the tube from the side.

(For other incubator using UV lamp)

Irradiate the bottom of each reaction tube and observe from the side through goggles or other UV-protective eye shielding.

For a valid run, the following results must be obtained when read at the specified time:

- Positive Control: Green fluorescent light is emitted.
- Negative Control: No fluorescent light is emitted.

If any control is invalid, all samples in the run should be reported as invalid and the test should be repeated.

After confirming that the run is valid, evaluate samples as follows:

- Positive Sample: Green fluorescent light is emitted.
- Negative Sample: No fluorescent light is emitted.

For real-time turbidity detection

After confirming that the turbidity increases in the positive control but doesn't in the negative control solution, evaluate samples in accordance with the following criteria (Fig 1 and 2).

- Positive: Some increase is observed in turbidity.
- Negative: No increase is observed in turbidity.

Notes:

(1) The minimum detectable sensitivity of the MALARIA Pan detection kit is 5.0 copies per test.
(2) Although the primers have been designed to target a region containing a relatively small number of variations, it is conceivable that *Plasmodium* infections may occur with variations in this region that are not well detected by this product.
(3) Test results may be affected by specimen collection, transport, specimen preparation, inhibitors and other laboratory procedural errors. A negative test does not exclude the presence of *Plasmodium* DNA from the specimen.
(4) This product is a kit for qualitative detection; it is not designed for quantitative measurement. The intensity of fluorescent light observed or the rise time of turbidity measured by the real-time turbidimeter does not correlate with the concentration of template DNA.

INTERFERING SUBSTANCES

Our in-house studies have revealed that turbidimetry measurement was not affected by the presence of EDTA (6.0 nmol/test), free bilirubin (1.2 µg/test), conjugated bilirubin (1.2 µg/test), chyle (formazine turbidity: 14,400), and hemolytic hemoglobin (3.4 µg/test), and human genomic DNA (1.2 µg/test). EDTA may cause false positive results when the result is read by fluorescence.

With regard to drugs, our in-house studies have revealed that measurement was not affected by the presence of isoniazid (840 ng/test), ethambutol (204 ng/test), rifampicin (958 ng/test), pyrazinamide (4.2 µg/test), acetaminophen (322 ng/test), loxoprofen sodium (637 ng/test), cefotaxime sodium (12 µg/test), levofloxacin (270 ng/test), atovaquone (1.59 µg/test), proguanil (20 ng/test), chloroquine (40 ng/test), quinine (960 ng/test), doxycycline hydrochloride (360 ng/test), mefloquine (168 ng/test), primaquine (18 ng /test), and artemisinin (93 ng/test).

PERFORMANCE CHARACTERISTICS

(1) Sensitivity and accuracy

In testing the following samples:

- negative sample (concentration: 0 copy/test)
- positive sample 1 (100 copies/test)
- positive sample 2 (1000 copies/test);

The negative sample shall test negative, and the positive samples 1 and 2 shall test positive.

(2) Within-run reproducibility

In testing five negative and five positive samples simultaneously, the negative samples shall test negative throughout, and the positive samples shall test positive throughout.

(3) Limit of detection

5.0 copies/test

(4) Cross-reactivity

The measurement system tested positive for *Plasmodium* species and negative for other pathogens, as detailed in the table below:

Plasmodium genus	
Plasmodium falciparum	Positive
Plasmodium vivax	Positive
Plasmodium ovale	Positive
Plasmodium malariae	Positive
Other pathogens	
Trypanosoma brucei	Negative
Trypanosoma cruzi	Negative
Leishmania donovani	Negative
Schistosoma mansoni	Negative
Theileria parva	Negative
Mycobacterium tuberculosis	Negative
Influenza A virus	Negative
Human immunodeficiency virus	Negative

(5) Information about a Calibrator

The performance test for this product used plasmid DNA containing the mitochondrial DNA of *Plasmodium falciparum* as a calibrator.

ORDERING INFORMATION

Product Code	Product Name	Contents
LMP561	***Loopamp™ MALARIA Pan Detection Kit***	***480 tests***
MVKM17	LF-160	1 Main unit 1 Fluorescence Visual Check Unit
LMC802	Loopamp™ PURE DNA Extraction Kit	90 tests
MVL300	LA-500	1 control unit 1 amplification unit

Note: For detection of DNA of *P. falciparum* parasites in blood, use Malaria Pf detection reagent (dMAL Pf) in the Loopamp™ MALARIA Pan/Pf Detection Kit (REF LMC562) (sold separately).

REFERENCES

1) Notomi T., et al.: Nucleic Acids Research 28, No. 12, e63 (2000)
2) Nagamine K., et al.: Clin. Chem. 47, No. 9, 1742–1743 (2001)
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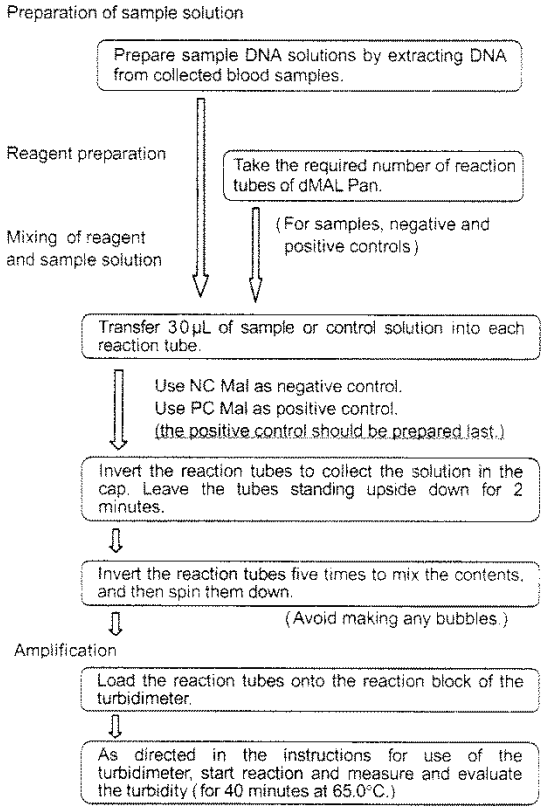
4) Tomita N., et al.: Nat. Protoc. 3, No. 5, 877–882 (2008)
5) The guideline for the bio-safety and bio-hazard (by the Japanese Society for Bacteriology): Japanese Journal of Bacteriology 54, No. 3, 667–715 (1999)
6) The package insert of Loopamp™ PURE DNA Extraction Kit.

Flow chart

Operation procedure for real-time turbidity detection



Confirm the completion of polymerase inactivation (for 5 minutes at 80°C, or for 2 minutes at 95°C). Take all reaction tubes from the turbidimeter and discard them without opening. Be careful not to damage the tubes.

EIKEN CHEMICAL CO.,LTD.
4-19-9, Taito, Taito-ku, Tokyo 110-8408, Japan

Date of Issue: April 1, 2013

CareStart™ Malaria HRP2/pLDH (Pf/PAN) Combo Test

Rapid One Step Malaria HRP2/pLDH Combo Test

A rapid test for the detection of HRP2 and parasite LDH in human blood

Intended Use

For the rapid qualitative determination of Malaria Histidine-rich Protein 2 (HRP2) and parasite lactate dehydrogenase (pLDH) in human blood as an aid in the diagnosis of Malaria infection.

Summary

Malaria is a serious parasitic disease characterized by fever, chills, and anemia and is caused by a parasite that is transmitted from one human to another by the bite of infected Anopheles mosquitoes. There are four kinds of malaria that can infect humans: *Plasmodium falciparum, P. vivax, P. ovale,* and *P. malariae*. In humans, the parasites (called sporozoites) migrate to the liver where they mature and release another form, the merozoites. The disease is now occurs in more than 90 countries worldwide, and it is estimated that there are over 500 million clinical cases and 2.7 million malaria-caused deaths per year. At the present, malaria is diagnosed by looking for the parasites in a drop of blood. Blood will be put onto a microscope slide and stained so that the parasites will be visible under a microscope.

The *CareStart*™ Malaria pLDH/HRP2 combo Test contains a membrane strip, which is pre-coated with two monoclonal antibodies as two separate lines across a test strip. One monoclonal antibody (test line 2) is pan specific to lactate dehydrogenase (pLDH) of the *Plasmodium* species (*P. falciparum, vivax, malariae, ovale)* and the other line (test line 1) consists of a monoclonal antibody specific to Histidine-Rich Protein 2 (HRP2) of the *Plasmodium falciparum* species. The conjugate pad is dispensed with monoclonal antibodies, which are pan specific to pLDH and *P. falciparum* specific to HRP2.

So, the ***CareStart***™ Malaria pLDH/HRP2 Antigen Test is designed for the differential diagnosis between *Plasmodium falciparum* and the other *Plasmodium* species.

Materials Provided

CareStart™ Malaria Antigen Test Kit contains following items to perform the assay:

- Test Device
- Package Insert
- Assay Buffer
- Sample Pipette (Optional)
- Lancet (Optional)
- Alcohol Swab (Optional)

Precautions

In order to obtain reproducible results, the following rules must be observed:

1) For *in vitro* diagnostic use only.
2) Use disposable gloves while handling potentially infectious material and performing the assay. Wash hands thoroughly afterwards.
3) Do not use it beyond the expiration date.
4) Do not eat or smoke while handling specimens.
5) Clean up spills thoroughly using an appropriate disinfectant.

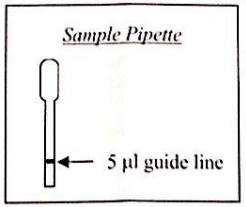
Specimen Collection and Storage

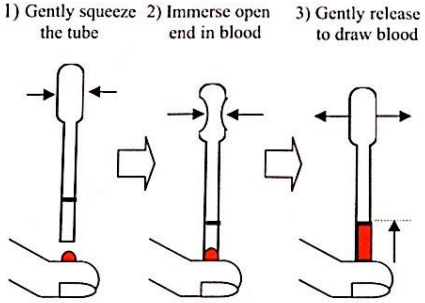
[Collection by venipuncture]

1) Collect the whole blood into the collection tube (containing EDTA, citrate, or heparin) by venipuncture.
2) If specimens are not immediately tested, they should be refrigerated at 2 ~ 8°C. For storage periods greater than three days, freezing is recommended. They should be brought to room temperature prior to use. Using the specimen in the long-term keeping more than three days can cause non-specific reaction.
3) When storage at 2 ~ 8°C, the whole blood sample should be used within three days.

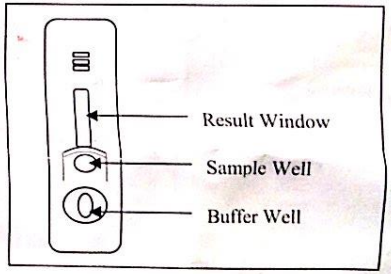
[Collection using a lancet]

1) Clean the area to be lanced with an alcohol swab.
2) Squeeze the end of the fingertip and pierce with a sterile lancet provided.
3) Wipe away the first drop of blood with sterile gauze or cotton.
4) Take a sample pipette provided, and while gently squeezing the tube, immerse the open end in the blood drop and then gently release the pressure to draw blood into the sample pipette up to the black line.

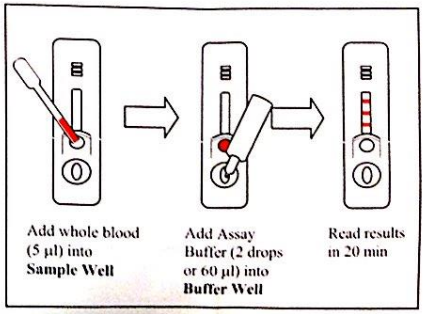




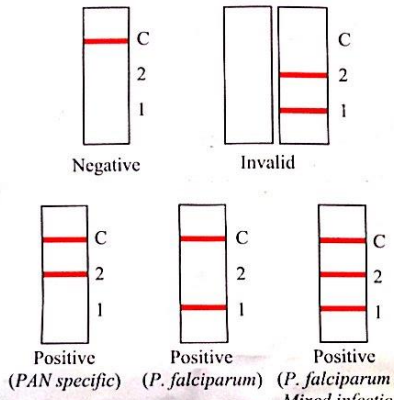
Test Procedure



1) Add 5 µl of whole blood into the Sample Well (small well).
2) Add two drops (60 µl) of assay buffer into the buffer well.
3) Read the test result in 20 min.



Interpretation of the test



1) Negative reaction
The presence of only one band in the Control Area within the result window indicates a negative result.

2) Invalid
The test is invalid if the line in the Control Area does not appear. If this occurs, the test should be repeated using a new strip.

3) *P. vivax, P. Malariae, or P. Ovale* Positive reaction
The presence of two color bands (one band in the Control Area and another band in the "2" area) indicates a positive result for *P. vivax, P. malariae,* or *P. ovale.* The pLDH present in the sample reacts with the pan anti-pLDH conjugate and move through the test strip where the pLDH is captured by pan specific anti-pLDH.

4) *P. falciparum* Positive reaction

The presence of three color bands (three bands in the Control, "2" and "1" areas) or two bands (one band in the Control Area and another band in the "1" area) indicates a positive result for *P. falciparum.*

5) Mixed infection of *P. falciparum* and other species Positive reaction

The presence of three color bands (three bands in the Control, "2" and "1" areas) indicates a positive result for *P. falciparum* or Mixed infection of ***P. falciparum*** and other species.

Limitations and Interferences

1) The test procedure, precautions and interpretation of results for this test must be followed when testing.
2) Anti-coagulants such as heparin, EDTA, and citrate do not affect the test result.
3) Do not mix reagent of different lots.
4) The test is limited to the detection of antigen to Malaria *Plasmodium* sp. Although the test is very accurate in detecting HRP2 and pLDH, a low incidence of false results can occur. Other clinically available tests are required if questionable results are obtained. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the results of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.

Performance Characteristics

The ***CareStart*™ Malaria HRP2/pLDH combo kit** has tested with positive and negative clinical samples tested by microscopic examination of whole blood.

1) Malaria *P. vivax* evaluation results

	Pv-positive confirmed specimen		Sensitivity
	Positive	Negative	
***CareStart*™** Malaria pLDH/HRP2	96	4	96/100 x 100% = 96%

2) Malaria *P. falciparum* evaluation results

	Pf-positive confirmed specimen		Sensitivity
	Positive	Negative	
***CareStart*™** Malaria pLDH/HRP2	98	2	98/100 x 100% = 98%

3) Malaria-negative normal human specimen evaluation results

	Random normal human specimen		Specificity
	Positive	Negative	
***CareStart*™** Malaria pLDH/HRP2	5	195	195/200 x 100%=97.5%

Precision

Within-run and between-run precisions have been determined by the testing 10 replicates of three specimens: a negative, a low positive and a strong positive. The agreement between the test results and the expected results were 100%.

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***Access Bio*, Inc.**
2033 Route 130, Unit-H
Monmouth Jct.
New Jersey 08852, USA.

Tel: 1-732-297-2222
Fax: 1-732-297-3001
E-mail: info@accessbio.net
Web Site: www.accessbio.net

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