ADDITIVE STABILIZATION OF *PLASMODIUM* PROTEINS PRESENT IN DRIED INFECTED BLOOD SPECIMENS

BY

COLLINS MISITA MORANG'A

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CELL AND MOLECULAR BIOLOGY

SCHOOL OF PHYSICAL AND BIOLOGICAL SCIENCES

MASENO UNIVERSITY

©2016

DECLARATION

I declare that this is my original work and has never been presented in any University or Institution for any award.

Signature________________________ Date______________________

Collins Misita Morang'a
MSC/SC/00057/013

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

Supervisors

Dr. Cyrus Ayieko
Department of Zoology,
Maseno University,
Maseno, Kenya.

Signature _________________________ Date _____________________

Dr. Elizabeth Wanja
Malaria Diagnostics Centre,
USAMRD-Kenya, Walter Reed Project
Kisumu, Kenya.

Signature __________________________ Date ____________________

ACKNOWLEDGEMENT

First and foremost, I wish to acknowledge all the lecturers in the Department of Zoology, Maseno University for their professional support in making this research undertaking a success.

Tremendous and special appreciation goes to my supervisors; Dr. Cyrus Ayieko and Dr. Elizabeth Wanja for their immense support, direction and guidance during the entire period of this undertaking. This thesis would not have been accomplished without their mentorship.

In a special way, I thank Dr. John Waitumbi for allowing me to work in his laboratory and all the invaluable assistance granted to me. I also wish to extend my appreciation to Basic Science Laboratory staff for teaching me the skills to carry out this work.

I would also like to acknowledge MAJ. Derek Monthei for his massive support throughout this work, and his guidance in writing this thesis. I wish to extend my gratitude to Carol Moseti, Rachel Achilla, Nickline Kuya and the rest of Malaria Diagnostic Centre staff for their enormous support throughout the duration of this work.

I wish to acknowledge the Almighty God for His favors and my family members for their patience and understanding during my study period.

DEDICATION

To my loving family: James Morang'a and Jenyfer Kwamboka, Brothers; Cyprian and Benjamin, Sisters; Brigid, Christine, Sophy and Sylvia. In appreciation for their encouragement, inspiration and overwhelming support.

ABSTRACT

Malaria rapid diagnostic tests (RDTs) are a great achievement to the implementation of parasite based diagnosis before treatment. They use a nitrocellulose strip coated with antibodies that target the *Plasmodium* antigens; histidine rich protein II (HRP2), lactose dehydrogenase (LDH), and aldolase. A major drawback of the method is that the majority of RDTs do not have positive control materials to test different batches of RDTs at the point of care. Dried *Plasmodium*-infected samples with the RDT target antigens has been suggested as a possible positive control, but their utility is hampered by rapid loss of activity over time and variability of performance over different ranges of temperatures. This study hypothesized that the temporal and thermal stability HRP2, LDH and Aldolase can be improved by addition of chemical additives. The objectives of the study were to determine the effect of chemical additives on temporal stability of HRP2, LDH and aldolase proteins as well as to investigate the effect of chemical additives on thermal stability of HRP2, LDH and aldolase proteins, and finally, to compare the effect of chemical additives on stability of HRP2, LDH and aldolase obtained from wild versus cultured parasites present in dried *Plasmodium* infected samples. This was a retrospective cross-sectional study utilizing forty archived whole blood patient samples from Kombewa Sub-County Hospital. *In vitro* cultivated *Plasmodium falciparum* parasites were utilized; prepared at Walter Reed Project. Three RDTs (SD Bioline, First Response and Binax NOW) were selected based on round two of the World Health Organization's performance evaluations and their ability to detect at least two of the target antigens. Blood samples were tested for baseline activity of the antigens then treated with chemical additives. Repetitive RDT testing of the stabilized specimens was done for a period of eight months. To determine temporal and thermal stability of the proteins, the Z-test of proportions was used to compare the proportionate stability time difference between control and stabilized samples. Logistic regression model for repeated measures was used to determine the differences in responses between cultured samples and patient samples. HRP2 was shown to loses stability in <21 weeks (63% stability) while aldolase and LDH lost stability in <12 week (33% stability) during the eight months of storage. Temporal stability of HRP2, LDH, and aldolase increased significantly (>18%, $P<0.001$) in the presence sucrose, trehalose, sucrose/trehalose, biostab/trehalose, and LDH stabilizer/trehalose. Thermal stability of HRP2 was shown to be lost in <21weeks at temperatures of 37°C and 45°C while LDH and aldolase lost stability in <12 weeks at similar conditions. There was significant increase in stability (>24%, $P<0.05$) of HRP2, LDH, and Aldolase in the presence of trehalose, sucrose/trehalose, and LDH stabilizer/trehalose at both 37°C and 45°C across all three parasite densities. Cultured samples at 2000p/µL can be used to develop the positive controls in the same capacity as patient samples ($P>0.05$). In conclusion, trehalose and its combination with biostab enzyme stabilizer, sucrose and LDH stabilizer improved the temporal stability of HRP2, LDH and Aldolase for eight months of storage. Thermal stability of HRP2, LDH and Aldolase was increased at 25°C, 37°C and 45°C in the presence of trehalose, sucrose/trehalose, and LDH stabilizer/trehalose. We recommend that trehalose or in combination with sucrose, biostab or LDH stabilizers can be used in the development of stable field deployable positive controls for validation of malaria rapid tests. Routine validation of malaria RDTs would improve reliability and confidence of the results thereby ensuring accurate treatment of malaria.

TABLE OF CONTENTS

DECLARATION ii
ACKNOWLEDGEMENT iii
DEDICATION iv
ABSTRACT v
TABLE OF CONTENTS vi
ACRONYMS AND ABBREVIATIONS ix
DEFINITION OF TERMS x
LIST OF TABLES xi
LIST OF FIGURES xii
LIST OF APPENDICES xiii
CHAPTER ONE 1
INTRODUCTION 1
1.1 Background 1
1.2 Problem Statement 6
1.3 Justification 7
1.4 Significance of the study 8
1.5 Research Questions 9
1.6 General Objective 9
1.6.1 Specific Objectives 9
CHAPTER TWO 10
LITERATURE REVIEW 10
2.1 Malaria 10
2.2 Rapid Diagnostic Tests 10
2.3 *Plasmodium* proteins in dried blood specimens 12

2.4 Temporal Stability of Proteins ... 14

2.5 Thermal Stability of Proteins ... 18

2.6 Mechanisms of additive thermal and temporal stabilization of proteins ... 19

2.7 Effectiveness of using malaria cultures ... 20

2.8 Summary of literature review ... 22

CHAPTER THREE ... 23

METHODS ... 23

3.1 Study Area ... 23

3.2 Study Population ... 23

3.3 Study Design ... 23

3.4 Sample Size ... 24

3.5 Collection of Patient Samples ... 24

3.5.1 Inclusion and Exclusion Criteria ... 25

3.5.2 Ethical Consideration ... 25

3.6 Selection of Malaria Rapid Diagnostic Tests ... 26

3.7 Selection of Chemical Additives ... 26

3.8 Sample Procedures ... 27

3.8.1 Determining temporal Stability ... 27

3.8.2 Determining the Thermal Stability ... 28

3.8.3 Effect of Using Culture Sample In Comparison with Patient Samples ... 28

3.9 Data Collection ... 30

3.10 Data analysis ... 31

CHAPTER FOUR ... 32

RESULTS ... 32

4.1 Determination of Temporal Stability of *Plasmodium* Proteins ... 32

4.1.1 Temporal Stability of HRP2 and *Plasmodium* LDH by SD Bioline 32
4.1.2 Temporal stability of HRP2 and *Plasmodium* LDH by First Response 35
4.1.3 Temporal stability of HRP2 and *Plasmodium* Aldolase by Binax NOW 37
4.2 Thermal Stability 39
4.2.1 Thermal Stability of HRP2 and LDH by SD Bioline Malaria kit 39
4.2.2 Thermal Stability of HRP2 and LDH by First Response malaria kit 41
4.2.3 Thermal Stability of HRP2 and Aldolase by BinaxNOW Malaria Kit 43
4.3 Effectiveness of Using Cultured Samples. 45
CHAPTER FIVE 47
DISCUSSION 47
5.1 Temporal Stability 47
5.2 Thermal Stability 52
5.3 Effectiveness of Using Cultured Samples 54
CHAPTER SIX 56
CONCLUSIONS AND RECOMMENDATIONS 56
6.1 Conclusions 56
6.2 Recommendations 57
REFERENCES 58
APPENDIX 63

ACRONYMS AND ABBREVIATIONS

DBS	-	Dried Blood Spots
DBT	-	Dried Blood Tubes
HRP2	-	Histidine Rich Protein 2
kDa	-	Kilo Daltons
LDH	-	Lactose Dehydrogenase
mRNA	-	messenger Ribonucleic Acid
PBS	-	Phosphate Buffered Saline
PEG	-	Polyethylene Glycol
PEI	-	Polyethyleneimine
RBC	-	Red Blood Cell
RDT	-	Rapid Diagnostic Tests
WHO	-	World Health Organization

DEFINITION OF TERMS

Temporal Stability-Ability of the *Plasmodium* HRP2, LDH, and aldolase to remain stable for eight months of storage at 37°C

Thermal Stability- Ability of the *Plasmodium* proteins to remain stable at high temperatures of 25°C, 37°C, and 45°C

Reactivity- *Plasmodium* proteins (antigens) are captured by the malaria RDT antibodies

Chemical Additives- Substances that increase or decrease the thermal and temporal stability of the *Plasmodium* proteins

Preferential Interactions: Is the interaction that determines the effect of additives on the stability of a protein.

LIST OF TABLES

Table 1: Additives and exploratory tests 27
Table 2: Percentage increase in thermal stability of HRP2 and LDH in patient samples measured for 33 weeks by SD Bioline malaria kit 40
Table 3: Percentage increase in thermal stability of HRP2 and LDH in patient samples measured for 33 weeks by First Response malaria kit 42
Table 4: Percentage increase in thermal stability of HRP2 and LDH in patient samples measured for 33 weeks by BinaxNOW malaria kit.. 44
Table 5: Logistic Regression model for repeated measures showing the relationship between sample type and antigen reactivity at different parasite concentrations 46

LIST OF FIGURES

Figure 1: Temporal percentage stability of chemically stabilized *Plasmodium* HRP2 and LDH in patient samples measured for eight months by SD Bioline malaria kit at different parasite densities: 34

Figure 2: Temporal percentage stability of chemically stabilized *Plasmodium* HRP2 and LDH in patient samples measured for eight months by First Response malaria kit at different parasite densities: 36

Figure 3: Temporal stability of chemically stabilized HRP2 and *Plasmodium* aldolase in patient samples measured for 33 weeks by BinaxNOW malaria kit at different parasite densities: 38

LIST OF APPENDICES

Appendix 1: Map of the study area 63
Appendix 2: Consent Forms 64
Appendix 3: Research Approvals 71

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria is still a leading cause of morbidity and mortality, with 214 million cases and 438,000 deaths reported in 2015 (WHO, 2015a). Parasite based malaria diagnosis is recommended by World Health Organization (WHO) and various *Plasmodium* proteins have been used extensively as biomarkers in developing malaria diagnostic kits (Wongsrichanalai *et al.*, 2007). Malaria rapid diagnostic tests (RDTs) have been shown to have false positives, false negatives, low specificities (<70%) and sensitivities (<80%), and varying temperature tolerances when used in the field (Mouatcho & Goldring, 2013). There are measures in place to ensure good manufacturing quality and providing guidelines for selection of RDTs for purchase (WHO/FIND/CDC, 2015), but lack of proper quality control and quality assurance mechanisms of RDT during transportation and storage in peripheral hospitals remains a major problem. Dried infected blood specimens containing the RDT target proteins have been proposed to be used as possible positive control specimens for quality checks of malaria RDTs (Aidoo *et al.*, 2012; Tamiru *et al.*, 2015; Versteeg & Mens, 2009).

The target proteins in the dried infected blood specimens are; *Plasmodium falciparum* histidine rich protein II (HRP2), enzymes of the *Plasmodium* glycolytic cycle lactate dehydrogenase (LDH) and aldolase (Mouatcho & Goldring, 2013). HRP2 is found in the parasite cytoplasm where it facilitates the polymerization of toxic heme to nontoxic malaria pigment hemozoin. It contains repetitive B-cell epitopes that enable the protein to be targeted by different antibodies developed on different kits (Howard *et al.*, 1986). On the other hand, lactate dehydrogenase is the last enzyme on the parasite glycolytic pathway and is produced by both sexual and asexual stages of the parasite. The water soluble enzyme shares 26% amino acid

sequence identity with that of the human LDH, and up to 90% of the sequence identity with other *Plasmodium* species (Brown *et al.*, 2004). Aldolase is also a glycolytic enzyme produced during the asexual stages of malaria parasite and contains strong sequence conservation across all the *Plasmodium* species (Lee. *et al.*, 2006). Since majority of the malaria kits use monoclonal antibodies that target a common epitope of LDH and aldolase so as to detect all malaria species in blood, the performance of RDT pivot on the long term stability of these proteins under different environmental and storage conditions. Consequently, there is need to stabilize these target proteins to ensure their applicability in low resource health facilities. This will ensure cost-effectiveness of the supply and resupply chains enabling continuous and universal availability of the controls.

A study by Aidoo *et al* (2012) showed that the stability of HRP2 was highly variable and reactivity of *Plasmodium* LDH reduced drastically during the 12 week storage duration at 4°C, room temperature, and 35°C. There is therefore need to improve their stability for long term storage. A number of methods have been explored to stabilize proteins for long term storage. These methods include; storage at a frozen state (-80°C), use of specific ligands such as amino acids that bind to the native state, high ionic strength of the protein system, salted out precipitates, freeze dried solids, and chemical additives (Balcão & Vila, 2015). Chemical additives commonly used in protein stabilization are salts containing ions such as lithium, sodium and potassium as well as sugars such as trehalose, mannitol, sucrose and sorbitol and finally, organic solvents such as polyethylene glycol (PEG), polyethyleneimine (PEI) and glycerol (Balcão & Vila, 2015). Chemical additives provide conformational stability to proteins thereby preventing the loss of enzymatic activity, inhibiting irreversible aggregation, and protecting them against chemical instabilities (Ohtake *et al.*, 2011). In a previous study trehalose

and sucrose were shown to maintain the relative activity of freeze-dried LDH for three months of storage (Kawai & Suzuki, 2007). Glycerol has been shown in previous studies to stabilize proteins by interacting preferentially with the hydrophobic parts of the protein structures (Vagenende *et al.*, 2009). Other blood and protein stabilizers such as Alserver's solutions and LDH stabilizer have been developed and demonstrated to stabilize proteins during long term storage (GwentGroup, 2010; Lowe *et al.*, 1973). Biostab enzyme stabilizer has been shown previously to stabilize lysozyme and prevent the loss of its mass over storage time through a mechanism of preferential hydration on the surface of the protein (Subbaraman *et al.*, 2005). Chemical additives can improve the temporal stability of RDT target proteins thereby developing stable positive controls, which in turn can ensure proper cost effectiveness, reduced resupply chains, and overall quality of malaria RDT tests in the peripheral health centers.

Among the challenges hampering deployment of LDH and HRP2 based RDTs is their limited stability over wide ranges of temperatures suggesting that the capture antibodies lose stability when temperature condition increase above the recommended conditions (>37°C) (Chiodini *et al.*, 2007). The implication is that positive controls samples for monitoring performance of these kits could lose reactivity with increase in temperature. Aidoo *et al* (2012) showed that reactivity of the HRP2 on dried infected blood specimens was stable at 4°C but highly variable between room temperature (RT) and 35°C. Similarly, reactivity of LDH was fairly stable at 4°C, but reduced drastically at room temperature and 35°C. This shows that the utility of dried infected blood specimens as controls will be inapplicable in many regions given that the temperature conditions in the world have registered marked increase over recent years (WMO, 2016). Many malaria endemic areas such as; Senegal, Burkina Faso, Ethiopia and Kenya register diurnal temperature of up to 40°C (Albertini *et al.*, 2012; KMD, 2012)**.** At high

temperatures proteins undergo destabilization through a thermal irreversible process of denaturation where by a protein is transformed from the native functional state to a disorderly state (Bischof & He, 2006). There is need to explore ways to stabilize aldolase, LDH and HRP2 present in dried specimens over a range of temperatures to provide a wide array for their applicability in different temperature conditions. Several methods have been explored for improving the thermal stability of the proteins including the use of chemical additives (Arakawa *et al.*, 2001). Previous studies have shown that trehalose, sucrose, maltose, and lactose can improve stability of proteins against thermal denaturation (Jain & Roy, 2009; Kawai & Suzuki, 2007). The thermal stabilizing abilities of Glycerol (Vagenende *et al*., 2009), Alsever's (Lowe *et al.*, 1973), and LDH stabilizer (GwentGroup, 2010) have also been highlighted. However, whether these additives may be useful in stabilizing proteins in dried blood specimens remains an open question.

Use of patient biological samples has been preferred as it mimics the matrix of a patient disease condition. Several ethical, social and legal issues emerge, whereby research ethics committees are mandated to protect the research participants interests such as, harm to the individual, consent, ownership and confidentiality (Moodley *et al.*, 2014). The proposed use of whole blood from patient in the development of dried infected patient blood may be successful but it will involve the risk of drawing large quantities of blood from the patient for worldwide utilization of the controls amidst other ethical concerns. The alternative is to use in-vitro cultivated parasites which use laboratory based chemicals, small amounts of serum and small amount of human negative blood to culture large amounts of parasites which can be used in the proposed development (Aidoo *et al.*, 2012; Tamiru *et al.*, 2015). All four malaria species have been cultured or maintained *in vitro*; however, *P. falciparum*, is the only species for which all

life cycle stages have been established in culture (Schuster, 2002). In the human host, *P. falciparum* exhibits a synchrony of about 48-h duration and this synchrony can be artificially imposed *in vitro* (Schuster, 2002). This alternative provides a simpler method that has minimum ethical concerns and poses minimum risk to the malaria positive patients. Determination of whether cultured samples will mimic the patient parasite matrix and whether detection by RDTs is similar to patient samples is the only way to justify the use of cultured samples in development of dried blood specimens.

1.2 Problem Statement

Currently there is lack of proper quality control and quality assurance mechanisms for validation of malaria RDTs after transportation and during routine testing. While use of dried infected blood specimens as positive controls has been suggested, the target antigens; HRP2, *Plasmodium* LDH and aldolase have short-half life in terms of storage duration(<12 weeks), limited temperature tolerance (at RT and 35°C) and require refrigeration requirements (4°C). A number of additives have been shown to increase stability of various proteins and can potentially increase stability of LDH and HRP2 but their potential has not been studied on dried infected blood specimens. These additives include sucrose, trehalose, alserver's solution, glycerol, Biostab enzyme stabilizer, and LDH stabilizer. Lastly, patient samples are viewed as the only source of developing the dried infected blood specimens because they mimic patient parasite matrix, but use of patient blood for large production of the controls creates serious ethical concerns and pose a risk to the subjects. There is a necessity to develop stable field dried infected blood specimens with long term storage stability, thermal stability at higher temperature conditions, and are made from in-vitro cultured samples.

1.3 Justification

The RDTs have several advantages such as ease of use, cost, real-time diagnosis, but they lack reliable quality control systems for performance assessment in the field which greatly impacts diagnostic performance (Mouatcho & Goldring, 2013). The only methods employed for performance assessment include; comparison of the RDT test results with microscopy, directly observing on how the test is being performed and asking health workers to interpret pictorial RDT procedures. All these methods are faced with numerous challenges that make them imperfect methods for quality assurance (Tamiru *et al.*, 2015). In the absence of recombinant positive control antigens (Lon *et al.*, 2005), well-characterized dried infected blood specimens have been suggested in monitoring performance of different brands of RDTs (Aidoo *et al.*, 2012). The challenge facing the dried infected blood specimens is loss of reactivity of the target proteins (HRP2, LDH and aldolase) due to the loss of thermal and temporal stability during prolonged storage and high temperature conditions (Aidoo *et al.*, 2012; Versteeg & Mens, 2009). The utilization of the dried infected blood specimens will depend on the long term stability of HRP2, LDH and aldolase at different climatic conditions especially poor resource areas where refrigeration equipments are unavailable. Addition of commercially available additives may potentially improve the temporal and thermal stability of target biomarkers and enhance operational utility of dried blood specimens as control materials for malaria RDTs in resource limited settings. The additives can improve the stability of HRP2, LDH and aldolase through dampening the molecular motions at the molecular, physical, chemical, and thermodynamic levels. The stabilization will prevent the irreversible loss of the unique chemical structure of the proteins, hence maintaining the native structure which eventually determines specific epitopes of the protein that acts as antibody binding sites (Bischof & He, 2006; Carpenter *et al.*, 2002).

1.4 Significance of the study

The WHO recommends the 'Test', 'Treat' and 'Track' of all suspected malaria cases. The development and utilization of malaria RDTs is a great realization towards malaria free world. In 2015 alone, 521.4 million RDTs were utilized in the world, with 149 million in Africa and 8.3 million in Kenya. All these malaria RDTs are utilized without proper quality control materials to monitor the quality of the test in routine use. Additive stabilization of HRP2, LDH, and aldolase in dried *Plasmodium* infected blood specimens provides with thermally stable ($<45^{\circ}C$) positive controls that can be used in routine checks. The additive stabilization mechanisms provide for stable positive controls that can be stored under ambient conditions for eight months of storage. The improvement of stability of the *Plasmodium* RDT target proteins ensures efficient utilization in peripheral health facilities located in high temperatures conditions and reduces the costs of resupply of the positive controls. The stable positive controls can be used to scale up RDT quality monitoring and enhance personnel proficiency testing programs.

1.5 Research Questions

1. What is the effect of storage duration on chemically stabilized HRP2, LDH and aldolase present in dried *Plasmodium* infected samples over eight months of storage?
2. What is the effect of storage temperature on chemically stabilized HRP2, LDH and aldolase present in dried *Plasmodium* infected samples?
3. What is the effectiveness of using cultured samples in comparison with wild samples in developing chemically stabilized positive controls?

1.6 General Objective

To investigate the effect of storage duration, storage temperature, and source of parasitized sample on chemically stabilized HRP2, LDH and aldolase present in dried *Plasmodium* infected samples.

1.6.1 Specific Objectives

1. To determine the temporal stability of chemically stabilized HRP2, LDH and aldolase present in dried *Plasmodium* infected samples over eight months of storage.
2. To determine the thermal stability of chemically stabilized HRP2, LDH and aldolase present in dried *Plasmodium* infected samples.
3. To determine the effectiveness of using cultured samples in comparison with patient samples in developing chemically stabilized positive controls.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria

Malaria is caused by *Plasmodium* parasites such as *P. falciparum*, *P. ovale, P. malariae, and P. vivax*, which are transmitted by mosquito vectors to humans. Globally, 3.2 billion people remain at risk of malaria and millions do not access services to be tested and treated for malaria (WHO, 2015a). To address the burden of malaria towards elimination, the World Health Organization (WHO) has developed the Global Technical Strategy for Malaria 2016-2030 which includes increasing percentage of diagnostic testing for malaria before patient treatment. Diagnosis of malaria will ensure reduction of the over-use of artemisinin-based combination therapies which are the first line of treatment which in turn reduces the occurrence of resistance that has been reported in five countries in the world (WHO, 2015b). Microscopy has been the mainstay for malaria diagnosis but the upsurge in developing malaria rapid diagnostic tests (RDT) has improved the diagnosis of malaria in areas where microscopy was poor or unavailable (Wongsrichanalai *et al.*, 2007).

2.2 Rapid Diagnostic Tests

Rapid Diagnostic tests are malaria testing platforms that detect *Plasmodium* protein (HRP2, LDH, and aldolase) in patient blood. There are over 120 types of malaria kits available from different manufacturers, developed on the type of antigen tested or combination of antigens (WHO/FIND/CDC, 2015). RDTs use an immune-chromatographic technique, whereby a colored detecting antibody binds to a parasite antigen in the blood sample and is moved by capillary action on a wicking strip and arrested by a capture antibody, resulting in a colored band. The RDTs have several advantages but studies show differences in diagnostic performance among

kits and between the test antigens such as HRP-2 and LDH (Mouatcho & Goldring, 2013). A study by Houze *et al* (2009), showed that HRP-2 tests gave 5% false negative results for *P. falciparum* cases and 5% false positive results in people without *P. falciparum*. On the other hand, *Plasmodium* LDH tests give about 8% to 9% false negative results for *P. falciparum* cases and about 1% false positive results in people without *P. falciparum* tests (Houze *et al.*, 2009). Generally, HRP-2 antibody-based tests are more sensitive while LDH antibody-based tests and pan aldolase are more specific. The lower specificity of HRP-2 tests is due to continual antigenemia after a person is treated for *P. falciparum* malaria (Murray *et al.*, 2008).

Rapid diagnostic tests can be performed by both laboratory staff and community health workers because it has simple procedure. (Mouatcho & Goldring, 2013). Despite the many advantages, the number of false positives, false negatives, low specificities (<70%) and sensitivities (<80%), varying temperature tolerances, manufacturer product variation, inter-lot disparity, and lack of proper quality assurance during use, are some of the difficulties facing malaria RDTs (Chiodini *et al.*, 2007; Mouatcho & Goldring, 2013). The World Health Organization (WHO), Foundation for Innovative New diagnostic devices and Centre for Disease Control and Prevention have taken measures to ensure good manufacturing quality and providing guidelines for selection of RDTs for purchase based on the intended endemic area (WHO/FIND/CDC, 2015). The guidelines include the panel detection score against *P. falciparum* and *P. vivax* samples (at least 75% at 200 parasites/µL in all transmission areas), the false positive rate (less than 10%), the invalid rate (less than 5%), meet stability requirements at temperatures of intended storage, transport and use, the RDT’s ease of use, and training requirements for the health workers. Majority of the RDTs being used currently meet the criteria for a good RDT, but they lack of quality control and quality assurance mechanisms during

transportation and after delivery in the field. Continuous monitoring of the kits is imperative because the performance of RDTs have been shown to be compromised when stored for long periods at different temperatures (Chiodini *et al.*, 2007).

One of the main methods being employed for quality checks is to compare the RDT results with light microscopy blood smears, but the method of parasite detection is quite different between the two tests. Microscopy detects whole parasites present in RBC while RDTs detect parasite antigens, furthermore microscopy requires an expert microscopist who is normally not available in peripheral health centers. Another method is training and observing how well health workers perform the rapid test, but this only assures the quality of the process and not the accuracy of the test. Frozen parasite sent to the health centers can be used but maintaining the frozen specimens at freezing temperatures during transportation and storage at -70°C makes them impractical in areas where the equipment is unavailable. Recombinant antibodies for quality checks on malaria RDT are being developed (Ravaoarisoa *et al.*, 2010) but it's quite unclear when such antibodies will be available for use. In the absence of recombinant positive controls to validate malaria RDTs, *Plasmodium* infected dried blood specimens have been developed for validation of malaria RDTs (Aidoo *et al.*, 2012).

2.3 *Plasmodium* proteins in dried blood specimens

The dried infected specimens contain *Plasmodium* proteins which are the target for majority of malaria RDTs. Protein expression for *Plasmodium* is a complex mechanism producing several proteins across all parasite stages (Florens *et al.*, 2002) but majority of the RDTS target three proteins; that is *P. falciparum* specific biomarker Histidine Rich Protein 2 (HRP-2) and pan specific parasite glycolytic enzymes namely *Plasmodium* Lactate dehydrogenase (LDH) and aldolase.

The enzyme LDH is important for the anaerobic lifestyle of *Plasmodium* because the parasite lacks the functional citric acid cycle for mitochondrial ATP production during their erythrocyte stages (Mouatcho & Goldring, 2013). *Plasmodium* LDH is coded by a gene located on chromosome 13 and expressed as a 1.6 kb m-RNA, which translates to a 140 kDa protein (Brown *et al.*, 2004). *Plasmodium falciparum* LDH has almost a 26% amino acid sequence identity with human LDH and is 90% among all Plasmodia species with highly conserved catalytic residues. The gene sequences are overly conserved but it has almost 18 residue insertions that are different and with various residue insertions and deletions. (Hewitt *et al.*, 1997). This information forms the model of *Plasmodium* LDH which can be used in structural explanation like stability of the protein.

Aldolase is also a target for malaria RDTs and is a glycolytic protein found in the host tissues and malaria parasite where it catalyzes the breakdown of fructose-1,6-bis-phosphate to dihydroxyacetone-phosphate and glyceralaldehyde-3-phophate (Wongsrichanalai *et al.*, 2007). In humans/vertebrates there are three different kinds of tissue specific to aldolase but in *P. falciparum and P. vivax* one kind exists which shares a 68% sequence similarity with the vertebrate aldolase. The *P. falciparum and P. vivax* aldolase sequences are 368 amino acids in length and relatively conserved (Lee. *et al.*, 2006). The aldolase gene is represented only once in the *Plasmodium* genome and transcribes at a high rate as a 2.4-kb mRNA in the *P. falciparum* blood stage. The mRNA encodes a protein of 41 kDa, which is 68% homologous to known eukaryotic aldolases. There is a strong sequence conservation of this protein among the *Plasmodium* species (Lee. *et al.*, 2006).

There are three types of histidine rich proteins synthesized by the parasite during the blood stage HRP I, II and III. *Plasmodium* HRP II is expressed by both the knob positive and

knob negative infected red blood cells. HRP2 contains 34% histidine and 37% alanine and 12% aspartate shown by sequence data. The protein is synthesized and present throughout all the erythrocyte growth stages. It is then exported through the erythrocyte cytoplasm and the surface membrane to accumulate outside the cell, whereby it can be found in the culture supernatant. The extracellular protein can also arise from the artefactual lysis of immature RBCs or mature RBCs at the time of rupture to release merozoite. The protein can be recovered in 2-8 hours of ring stage culture supernatants if the mature RBCs can be ruptured (Howard *et al.*, 1986; Parra *et al.*, 1991).

2.4 Temporal Stability of Proteins

The main challenge facing the development of dried infected blood specimens as positive controls is loss of antigen reactivity during prolonged storage durations. HRP2 was shown to lose stability after 12 weeks of storage, while LDH was shown to lose stability after one week of storage (Aidoo *et al.*, 2012). It is critical that the functional structure of the proteins remain unaltered during storage to ensure long term storability of the *Plasmodium* proteins in dried specimens. During storage, proteins undergo various mechanisms that cause instabilities including chemical degradation, oxidation, chemical modifications, hydrolysis, disulfide exchange, beta elimination and aggregation (Manning *et al.*, 2010). The implication of this is that the protein in the dried blood specimens can lose stability causing loss of reactivity during prolonged storage.

Several methods have been explored that ensure proteins retain their native structure during prolonged storage. Freezing proteins at -80°C for long term storage or as unfrozen aqueous solutions in refrigerators at 4°C-8°C for short term storage have been explored (Carpenter *et al.*, 2002), but in low resource areas effective storage requirements would be a

challenge. Storage of protein in salted out precipitates is another commercially used method for storage of enzymes. Storage as freeze dried solids enables proteins to remain stable for years but the method requires the use of appropriate additives to stabilize the proteins against denaturation during the terminal stages of freezing and drying (Carpenter *et al.*, 2002). Other methods of protein storage are; in a non-frozen aqueous solutions containing a narrower pH range, specific ligands that bind to the native state, reduced concentration of the protein, and a high ionic strength that favors the formation of non-native aggregates (Balcão & Vila, 2015). The alternative to these methods is the use of cheaper additives with simpler application methods to maintain temporal stability of proteins.

Additives stabilize the long term storability of proteins by two mechanisms; one is the water replacement hypothesis which predicts that the water molecules are replaced from the hydration shell of the protein entity by the additives, and the second is the preferential exclusion hypothesis which states that the additives are excluded from the surface of the protein forming protective and stabilizing shields around the protein (Arakawa *et al.*, 2001). Additives that have been used in stabilizing proteins include salts such as lithium, sodium and potassium, sugars such as trehalose, mannitol, sucrose and sorbitol, and organic solvents such as polyethylene glycol (PEG), polyethyleneimine (PEI) and glycerol.

Previous studies have shown that trehalose stabilizes proteins through the vetrification theory, preferential exclusion theory, and water replacement theory. Trehalose is a white, odorless, non-reducing disaccharide with several properties that are useful in industries including cosmetics, pharmaceuticals, food processing, starch retro gradation, and stabilization of lipids and proteins (Jain & Roy, 2009). Trehalose was shown to stabilize monoclonal antibodies (Yazdani *et al.*, 2015), and pyro-phosphatase (Zancan & Sola-Penna, 2005). Hedoux *et al*, (2009)

indicated that trehalose stabilizes Bovine serum albumin by reducing the dynamic fluctuations of the polar side chains at the protein solvent interface (Hedoux *et al.*, 2009). Another study indicated the stability of ribosomal protein S6 was increased in the presence of trehalose in a non-specific thermodynamic stabilization (Chen *et al.*, 2005). The effect of trehalose was evaluated on lysozyme by means of molecular simulations under different conditions and it was shown that trehalose provides relative stabilization to native state of the protein due to an interplay of many interactions (Katyal & Deep, 2014).

Glycerol has also been used for many years to stabilize proteins. The mechanism of this stabilization involves nonspecific interactions between protein and solvent components in the presence of glycerol. It exerts its effects by being preferentially excluded from the immediate surface of the protein (Gekko & Timasheff, 1981). Addition of 10-100% glycerol to gluco-amylase increased its thermal stability and the thermal stability of the protease was increased in 50-70% glycerol. Glycerol has also been shown previously to increase the stability of lysozyme, ribonuclease A alpha amylase from bacillus bacteria (when trehalose is present), and native catalase enzyme (Iyer & Ananthanarayan, 2008).

The BioStab Enzyme Stabilizer contains a mixture of protectants including the ectoines (osmo-protectants) and similar compounds in an optimized combination to protect enzymes and other proteins in the best possible way. The product stabilizes the proteins molecules through preferential interactions such as preferential exclusion and preferential hydration. This product has been shown to protect the mass loss of lysozyme over time (Subbaraman *et al.*, 2005). Stabilization of lactate dehydrogenase by the LDH stabilizer was developed by the Gwent group with the stabilizer containing several additives combined together. The LDH stabilizer protects the protein against loss of activity and confers extended shelf life. In their studies, the Gwent

group has shown that LDH can be stabilized for 80 days. The stabilizer molecules bind to multiple protein molecules in the solution forming soluble complexes by electrostatic interactions including hydrogen bonds which stabilize the protein (GwentGroup, 2010).

Sucrose is also disaccharide which has been used widely in different industries due to its inherent properties like solubility, high melting point, relative density, and low chemical reactivity. Sucrose stabilizes proteins by preferential exclusion thereby increasing the chemical potential of the protein which adopts a more stable conformation (Lee & Timasheff, 1981). Previous studies have shown that it can stabilize recombinant interleukin 1 receptor antagonist (Kendrick *et al.*, 1997), monoclonal antibodies (mAbs) against recombinant HBsAg (rHBsAg) epitopes (Yazdani *et al.*, 2015), alpha-lactalbumin and ribonuclease-A, lysozyme and ribonuclease (Balcão & Vila, 2015). Sucrose was also shown to stabilize Recombinant Factor XIII stored at $40^{\circ}C$ (Kreilgaard *et al.*, 1998).

Alsever's solution is a balanced isotonic salt solution routinely used as an anticoagulant blood preservative. The solution permits storage of whole blood in a refrigerator for close to 10 weeks. A study investigated the stabilities of erythrocytic glucose-6-phosphate dehydrogenase, protein kinase, triose phosphate isomerase, gluthathione reductase, and NADH dehydrogenase when blood was collected in heparin tubes, EDTA-glucose, or Alsever's solution. Alsever's solution maintained the longest stability of 25 days for all the five proteins and the solution can be dried before sample addition and achieve the same results (Lowe *et al.*, 1973).

2.5 Thermal Stability of Proteins

Temperature tolerance of both HRP2 and LDH malaria tests is one of the difficulties and challenges facing the utilization of these kits. The lack of tolerance of the capture antibodies at high temperature conditions is considered a possible cause for loss of reactivity of malaria kits under different storage temperatures (Chiodini *et al.*, 2007; WHO/FIND/CDC, 2015). Different temperature condition in various geographical locations could be a challenge to the deployment of positive controls samples which could lose reactivity with increase in temperature. The HRP2 in dried infected samples was shown to loose stability at 35°C within two weeks while LDH lost stability within a few days (Aidoo *et al.*, 2012). In recent times world temperature conditions have markedly increased up to 40°C as shown in European countries such as Spain, France, the United Kingdom, Germany, Netherlands and Italy as well as African countries such as Morocco and Algeria (WMO, 2016). Countries like Senegal, Burkina Faso, Ethiopia, and Philippines where malaria is endemic have shown higher temperatures than ever before. (Albertini *et al.*, 2012). In Kenya, temperatures have registered higher than they have historically although the temperature varies across counties (KMD, 2012)**.**

The applicability of the positive controls in high temperature conditions will be hampered by the poor thermal stability of the RDT target proteins. In normal cellular environments proteins are protected against denaturation by heat shock proteins. Likewise, mechanical and chemical modification of HRP2 and LDH can inhibit the denaturation process. Chemical additives such as sucrose, trehalose, glycerol, Biostab enzyme stabilizer, and LDH stabilizer have been shown in previous studies to protect proteins against thermal denaturation (Chen *et al.*, 2005; GwentGroup, 2010; Katyal & Deep, 2014; Kendrick *et al.*, 1997; Subbaraman *et al.*, 2005; Vagenende *et al.*, 2009; Yazdani *et al.*, 2015). Sucrose and Trehalose have also been shown to stabilize dried formulation of LDH stored at 60°C for over 90 days (Kawai & Suzuki, 2007).

Trehalose was also shown to delay the thermal unfolding of cutinese by delaying the rate constant of the protein intermediate (Baptista *et al.*, 2008)

2.6 Mechanisms of additive thermal and temporal stabilization of proteins

Protein stabilization preserves their structure and functionality during storage at different temperature conditions through establishment of thermodynamic equilibrium of the protein microenvironment. The native structural state of a protein molecule depends mostly on intermolecular interactions between amino acids and intra-molecular interactions with the solvent or solute surrounding the protein in the microenvironment(Wang, 1999). Additives thermally stabilize proteins by dampening the molecular motions and eliminating the conformational transitions while the molecule is still in its native structural state (Castronuovo, 1991). It is easy to generalize that the additives have nonspecific indirect effects on the molecular motions of the proteins at four levels; molecular motions, chemical properties, thermodynamic properties (Wang, 1999). The stability of a protein is gained as a result of enhancing the translational, vibrational, and rotational viscosity leading to a more rigid and three dimensional structure with decreased entropy and increased enthalpy leading to protein stabilization (Khechinashvili, 1990).

Water forms a hydration shell around the protein thereby dictating both stability and flexibility of the protein by fluctuating among different equilibrium structures and maintaining the plasticity of the protein (Arakawa *et al.*, 2001). Therefore, exposing the water to specific additives thereby reduces the chemical activity of water around the protein and completely removes the water by drying, achieving protein stabilization. A protein oscillates between many slightly different 3D conformations and between each two conformations the oscillation is ruled by the second law of thermodynamics which involves Gibbs free energy of the two 3D

conformations as a function of transition enthalpy subtracted by transition entropy and absolute temperature (Murphy *et al.*, 1992). The conformational enthalpy of a protein depends on the intra-molecular interactions hence any physical or chemical instability that breaks the molecular bond of a protein will result in the reduction of the protein enthalpy resulting in a negative Gibbs free energy meaning the protein is denatured.

The entropy of a protein increases as the protein unfolds and vice versa, but presence of additives increase stability of a protein entropic-ally by decreasing entropic gain during unfolding. Another mechanism involves the hydrophobic interaction between the protein non polar amino acids and the additive leading to protein rigidfication and attaining thermos-stability (Balcão & Vila, 2015). The two mechanisms lead to various hypothesis such as water replacement hypothesis which predicts that the water molecules are replaced from the hydration shell of the protein entity by the additives and the preferential exclusion hypothesis which states that the additives are excluded from the surface of the protein forming protective and stabilizing shields around the protein, and the limited water molecules can interact preferentially with the protein thus stabilizing the native protein (Arakawa *et al.*, 2001)

2.7 Effectiveness of using malaria cultures

Quality assurance for malaria RDTs at peripheral health centers requires the use of fresh sample with known parasite levels or recombinant antigen that can be used to mimic the human blood antigens (Versteeg & Mens, 2009). In the absence of these recombinant antigens, the use of well characterized *Plasmodium* infected dried blood specimens as a positive control is imperative. Human blood can be used to develop dried blood samples that are infected with *Plasmodium falciparum* since they present an exact matrix of the patient disease. However, limitations of current ethical and regulatory frameworks and the associated risk to the blood

donor, pose a number of concerns in the use of patient blood in developing these controls (Moodley *et al.*, 2014). Specific issues such as benefit sharing, consent, privacy, validity of the broad consent, exporting, and future use of human blood have been highlighted as concerns around the use of human samples for research. The potential of drawing more volume could cause harm and pose a huge risk especially to sick children leading to anemia whereby the child does not have enough blood (Tindana *et al.*, 2014). Determining how much blood is too much to draw from sick child with malaria will become a challenge in developing these controls. This is in line with the fact that developing enough controls to serve all endemic areas with these positive controls will require drawing enough blood to prepare the specimens.

The alternative to using human blood is the use of in-vitro cultivated malaria parasites which mimics the normal life cycle of the parasite in the human host. The life cycle of *Plasmodium* is complex, with the parasite alternating between sexual reproduction in an invertebrate (mosquito) host and asexual reproduction in a vertebrate host. All four species have been cultured or maintained *in vitro*; however, *P. falciparum*, is the only species for which all life cycle stages have been established in culture. Synchrony can be artificially imposed *in vitro* upon developing malaria parasites by the use of sorbitol. Infected cells are treated with 5% sorbitol, which causes lysis of erythrocytes containing late stages and preferentially selects for red blood cells with early ring stages(Schuster, 2002). This indicates that in-vitro cultivation of parasites mimic the true life cycle of the parasites in the erythrocytic stages. This can be exploited to develop the dried infected blood specimens which will have minimum ethical concerns.

2.8 Summary of literature review

There is a need to develop stable field deployable positive controls that are thermally stable and can be stored for long durations in the peripheral health facilities. Using additives the temporal stability of the *Plasmodium* proteins can be achieved ensuring the proteins are protected against physical and chemical instabilities experienced during storage of proteins. Various additives have been shown to stabilize proteins through various mechanisms in achieving the necessary stability for storage of proteins. These additives have also been shown in this section that they can stabilize the proteins against thermal denaturation which can be experienced in different climatic conditions where malaria is endemic. Stabilizing the proteins against thermal denaturation will ensure that they do not undergo aggregation, coagulation or gelation. Cultured samples as an alternative to human blood in the development of these controls will reduce the ethical issue and concern that are likely to be faced when doing large scale production of the dried infected blood specimens. This study investigated the effect of storage duration, storage temperature, and source of parasitized sample on chemically stabilized HRP2, LDH and aldolase present in dried *Plasmodium* infected samples.

CHAPTER THREE

METHODS

3.1 Study Area

The study was conducted at the Walter Reed Project, Kisumu. The patient samples with parasitized blood were collected from the Kombewa Clinical Research Center, situated 40km west of Kisumu city, as part of the Kombewa Health and Demographic Surveillance System (HDSS). The site lies between longitudes 34°24’00”E and 34° 41’30”E, and latitudes 0° 11’30” to N-0°11’30”S, at an average altitude of 1400 meters above sea level. Malaria is holoendemic in this area, and transmission occurs throughout the year (Sifuna *et al.*, 2014). The long rainy season from late March to May produces intense transmission from April to August. The short rainy season from October to December produces another, somewhat less intense, transmission season from November to January (Sifuna *et al.*, 2014).

3.2 Study Population

The study populations were people in the Kombewa clinical research centre; health and demographic surveillance system (HDSS). The study population comprised approximately 141,956 individuals. The population has a mean age of 23 years with 48% of the population below the age of 15 years. Malaria accounts for approximately (35%), followed by cold/flu (20%), cough (18%), diarrhoea and vomiting (9%) (Sifuna *et al.*, 2014).

3.3 Study Design

This was a retrospective cross-sectional study utilizing archived whole blood samples from Kombewa Sub-County Hospital.

3.4 Sample Size

The WHO recommends that malaria diagnostic tests should detect 200 parasites /µL with at least 95% sensitivity. Parasitized samples were used therefore a sensitivity of 99% for the RDT was used in this study to measure the reactivity status of the samples. Estimating required sample size to achieve 80% power to detect 20% reduction in a hazard of not detecting reactivity by using a two-sided 0.05-level log-rank test under four different storage conditions (4°C, 25°C, 37°C, and 45°C below). All of these study conditions assume:- Alpha (a) = 0.05, Power (1−β) = 0.8, Effect size = (0.20) we used the formula below to calculate n (Buderer, 1996).

$$\boldsymbol{n = Z^2{}_{a/2} \text{ x } \frac{S_N(1 - S_N)}{L^2 \text{ x Prevalence}}}$$

$$\boldsymbol{n = 1.96^2 \text{ x } \frac{0.99 \times (1 - 0.99)}{0.05^2 \text{ x } 0.38}}$$

$$\boldsymbol{n = 40\ samples}$$

$\boldsymbol{Z^2}_{\alpha/2}$ (*standard Normal Quartile*) = 1.96, $\boldsymbol{S_N}$ (*Sensitivity*) =*0.99*, $\boldsymbol{L}$ *(significance level) = 0.05,* Prevalence = 0.38

3.5 Collection of Patient Samples

The patient samples were obtained from archived blood samples that were collected from volunteers using a WRAIR/KEMRI approved protocol #1720. Volunteers were mostly symptomatic (fever, chills., headache, sweats, fatigue, nausea and vomiting, dry cough, muscle or back pain, enlarged spleen) individuals with no history of anti-malaria treatment in the last two weeks before blood collection. The subjects were briefed and consented before a finger prick was conducted. Subjects were screened by RDT and the presence of *Plasmodium* parasites was confirmed by microscopic examination of a malaria blood film. Patients were selected for venous puncture if malaria RDT results were positive and malaria microscopy indicates a parasite density of≥2000 parasites/µL (Parasite density = Number of parasites counted/200 Leukocytes

multiplied *8000). Three milliliters of venous blood was collected in EDTA tubes from individuals with parasites as stated in the consent form and then the samples were anonymized before use in this study.

3.5.1 Inclusion and Exclusion Criteria

Individuals of any age were included in this study. Individuals were excluded from this study if they were unwilling to donate blood or give informed consent, if the person was less than 18 years old, or if the parent or guardian was unwilling to give consent for those ages 1-17. Individuals were also excluded if they had any contraindication to donating 3-20 mL of blood (depending on age, clinical status, etc.), as determined by the study clinician or the attending physician, if they had any acute or chronic illnesses that may compromise their health because of blood donation, if children less than18years old were without an available parent or guardian, and finally, those who had already started taking malaria medication for treatment.

3.5.2 Ethical Consideration

The study participation was voluntary. Each participant was provided with oral and written information describing the nature and duration of the study before signing the consent form. If the participant is a child, the consent document was signed by the parent or legal guardian. If the participant was able to give only oral consent, a witness to the consent process signed the consent form on his /her behalf. The subject received a copy of the signed consent document prior to initiation of any activities related to this study. Scientific and ethical approval for the study was obtained from the Ethical Review Committee and Scientific Steering Committee of the Kenya Medical Research Institute, Nairobi (SSC#2008) and the Walter Reed

Army Research Institute of Human Use Research Committee, Silver Spring, Maryland, USA (WRAIR #1720.002).

3.6 Selection of Malaria Rapid Diagnostic Tests

The RDTs were selected based on performance in Round 2 of WHO/FIND/CDC (2010) RDT performance evaluations and national guidelines on required performance and test characteristics for different levels of use. Three different types of RDTs were selected; SD Bioline Malaria-Ag 05FK60 (Standard Diagnostics, Inc. Korea), First Response -I16FRC (Premier Medical Co. Ltd, India), and BinaxNOW Malaria (Binax, Inc., Inverness Medical, ME, USA). These RDTs detect pf-LDH, pan-LDH, Pan-Aldolase and *P. falciparum*-specific pfHRP-2. The selection was based on panel detection score (PDS) at 200 parasite/µL of ≥90.

3.7 Selection of Chemical Additives

Additives chosen for this project were polyethylene glycol (Sigma-Aldrich, MO, USA), sucrose (Sigma-Aldrich, MO, USA), Biostab enzyme stabilizer, Alsever's Solution (Fisher Scientific, USA), trehalose (Fisher-Scientific, USA), lactate dehydrogenase stabilizer solution (The Gwent Group. UK), polyethyleneimine (Fisher-Scientific, USA), and glycerol (Sigma-Aldrich, MO, USA). Stepwise exploratory tests were conducted to determine which additives, combinations and concentrations gave optimal RDT test outcomes. Additives were added to the start-up culture samples individually (mono) or in combination (mixed) with other additives at concentrations of 200 p/µL and 2000 p/µL. The ratio of additives used in combination was 1:1 as done in previous studies (Kreilgaard *et al.*, 1998). The ratio of additives or combinations to blood culture was 1:2, so as to achieve appropriate concentration of the additive in the blood (Hamada *et al.*, 2009). A control start-up sample (no additive/s added) was also included.

Table 1: Additives and exploratory tests

Additive	Code	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7	Test 8
Biostab Enzyme Stabilizer	1	Neat	1+2	1+3	1+4	1+5	1+6	1+7	1+2+3+4+5+6+7+8
Alsever's	2	Neat	2+3	2+4	2+5	2+6	2+7	2+8	
Trehalose	3	Neat	3+4	3+5	3+6	3+7	3+8		
Polyethyleneimine	4	Neat	4+5	4+6	4+7	4+8			
Glycerol	5	Neat	5+6	5+7	5+8				
Polyethylene Glycol	6	Neat	6+7	6+8					
Sucrose	7	Neat	7+8						
LDH Stabilizer	8	Neat							

In the event that an additive, combination or concentration was found to interfere with RDT test results, that particular additive or combination was withdrawn. Additives that showed no or little effect on the stability of the proteins were also withdrawn. Ten additives/ combinations were selected from the 36 combinations; 0.5M sucrose, 10% glycerol/0.5M sucrose, Alsever's solution/0.5M sucrose, 0.5M trehalose, 0.5M sucrose/0.5M trehalose, 10% glycerol/0.5M trehalose, 0.5M trehalose/biostab stabilizer, biostab stabilizer/0.5M sucrose, LDH Stabilizer, and LDH Stabilizer/0.5M trehalose. The concentration formulations were selected according to extensive previous studies on the effect of different additives on keeping the structure of proteins (Jain & Roy, 2009; Kendrick *et al.*, 1997; Vagenende *et al.*, 2009).

3.8 Sample Procedures

3.8.1 Determining temporal Stability

Plasmodium positive malaria samples obtained had a parasite concentration ranging from 1053p/µL-453,605p/µL and were diluted to the prioritized parasitemia using malaria negative group O+ blood. Ninety mL of Group O+ blood was obtained through a USAMRD-K SSC approved study (SSC#1330). It was used to standardize the samples at 2,000p/µL, 500p/µL, and 200p/µL using the formula for making dilutions ($C_1 \times V_1 = C_2 \times V_2$). The ten selected additives

and their combinations were added to the samples in a ratio of 1:2, and a control sample (without any additive) was prepared. Dried blood in tubes were prepared by depositing 40μL aliquots at the bottom of 2mL vials, the tubes were left uncapped to air dry overnight in a bio-safety cabinet, then sealed tightly by closing the vial cap then put to storage at 37°C. The major challenge in most health centers is lack of resources to maintain health commodities at the required temperature conditions. It is possible to minimize the transport and storage time of commodities in the health facility, but it's necessary to ensure facilities have adequate stocks and reduce the durations of resupply. Temperature conditions in the world normally vary across different climatic conditions, with high temperatures in tropical regions and lower temperatures in other regions.

3.8.2 Determining the Thermal Stability

Patient sample were standardized to the required concentration of 2,000p/μL, 500p/μL, and 200p/μL. The dried infected blood tubes were prepared by depositing 40μL aliquots at the bottom of 2mL vials; the tubes were left uncapped to air dry overnight in a bio-safety cabinet, and then sealed tightly by closing the vial cap. The dried blood tubes were stored at four different temperatures; refrigerator at 4°C, room temperature (~25°C), dry incubator set at 37°C, and dry incubator 45°C. Thermal stability of proteins can be determined by subjecting them to a range of temperatures for a fixed duration and then measuring their activity at these temperatures in comparison to a given favorable temperature (Bischof & He, 2006)

3.8.3 Effect of Using Culture Sample In Comparison with Patient Samples

Cryopreserved stocks of *Plasmodium falciparum* strains (3D7) maintained at the KEMRI/ Walter Reed Project - Kisumu were used to initiate cultures according to the WRAIR/KEMRI

approved protocol. The culture medium was prepared by dissolving one sachet (1x) of commercial RPMI1640 media (Sigma-Aldrich, MO, USA) into one liter of distilled water, then adding 2 grams of sodium bicarbonate and 1 mL of 50 mg/mL gentamicin.

The media was filtered using a 0.45nm Millipore filter then stored at $4^{\circ}C$-$8^{\circ}C$. A 10% complete media with serum (CMS) was prepared by adding 25 mL of human serum to 225 mL of incomplete media. Malaria negative O+ blood (5mL) was washed in 10mL RPMI through centrifugation at 3000 rpm for 10 minutes three times to give 100% red blood cell hematocrit. The washed cells were diluted to 5% hematocrit with CMS in 25 cm^2 tissue culture flasks (0.3 mL of blood cells +5.7 mL of CMS to make a final volume of 6 mL). *Plasmodium* strains were added then the flasks were gassed using a mixture of (95% N, 5% CO_2, and 5% O_2) before being placed in the incubator at $37^{\circ}C$. The CMS was replaced daily and parasite cultures were sub-cultured after every two weeks. Giemsa-stained thin smears were prepared during every media change and parasitemia estimated by microscopy.

The *P. falciparum* cultures were grown to higher parasitemia (>5000 parasites/µL) and synchronized to >95% ring stages using 5% sorbitol. The culture samples were standardized to the required concentration of 2,000p/µL, 500p/µL, and 200p/µL. Dried blood tubes were prepared by depositing 40µL aliquots at the bottom of 2mL vials, the tubes were left uncapped to air dry overnight in a bio-safety cabinet, then sealed tightly by closing the vial cap. The dried blood tubes were stored at four different temperatures; refrigerator at $4^{\circ}C$, room temperature (~$25^{\circ}C$), dry incubator set at $35^{\circ}C$, and another set at $45^{\circ}C$.

3.9 Data Collection

Baseline data was collected by testing of all the aliquots to confirm the reactivity of the antigens using SD Bioline, First Response and Binax Now. The test was conducted according to manufacturer instructions whereby; a sample is deposited on the sample spot, two-four drops of the buffer are added to the buffer spot, and reactivity of the antigens is observed between 15-30 minutes. Reactivity or presence of the HRP2, LDH, and aldolase is demonstrated by color changes at the test lines. A successful test is confirmed by the presence of a control line. Results were captured as either positive or negative through visual examination of the kit. It was recorded positive if both the control band and the tests bands were visible and it was recorded negative if the control line was visible but the test lines were absent. The test was recorded invalid if the control line was absent.

Temporal stability and thermal stability data was collected by retesting both the control and the stabilized samples (both culture and patient samples). The samples were retested consecutively after 1, 2, 4, 6, 8, 10, 12, 15, 18, 21, 24 and 33 weeks of storage. Retesting was completed by rehydrating the dried blood pellets for the control and each additive. One sachet of PBS-Tween 20 was dissolved in one liter of distilled water at a pH of 7.4, then 40µL was used to rehydrate each sample so that to obtain the exact original volume. The tubes were placed on a shaker for one hour to ensure complete rehydration. Each sample was tested on SD Bioline, First Response and Binax Now and results captured and recorded on forms as either positive or negative.

3.10 Data analysis

To determine temporal and thermal stability of chemically stabilized HRP2, LDH, and aldolase, the time taken (in weeks) to lose reactivity was compared between the control sample and stabilized samples. Analysis was done for each of the three different RDT kits (SD Bioline, First Response, and Binax Now). Percentage stability was calculated against the endpoint (week 33) and the Z-test was used to compare the proportions between control and the stabilized specimens (Hansen, 2004). The test of significant difference was set at $p<0.05$ for the Z-test and if the p-value was less than 0.05, then there was sufficient evidence to conclude that the proportions between the control and the stabilized sample was different. Logistic regression model for repeated measures was used to determine the effectiveness of using cultured samples as compared to patient samples. The test of significant difference was also set at $p<0.05$. Analysis was performed using Stata version 12 (StataCorp, College Station, TX).

CHAPTER FOUR

RESULTS

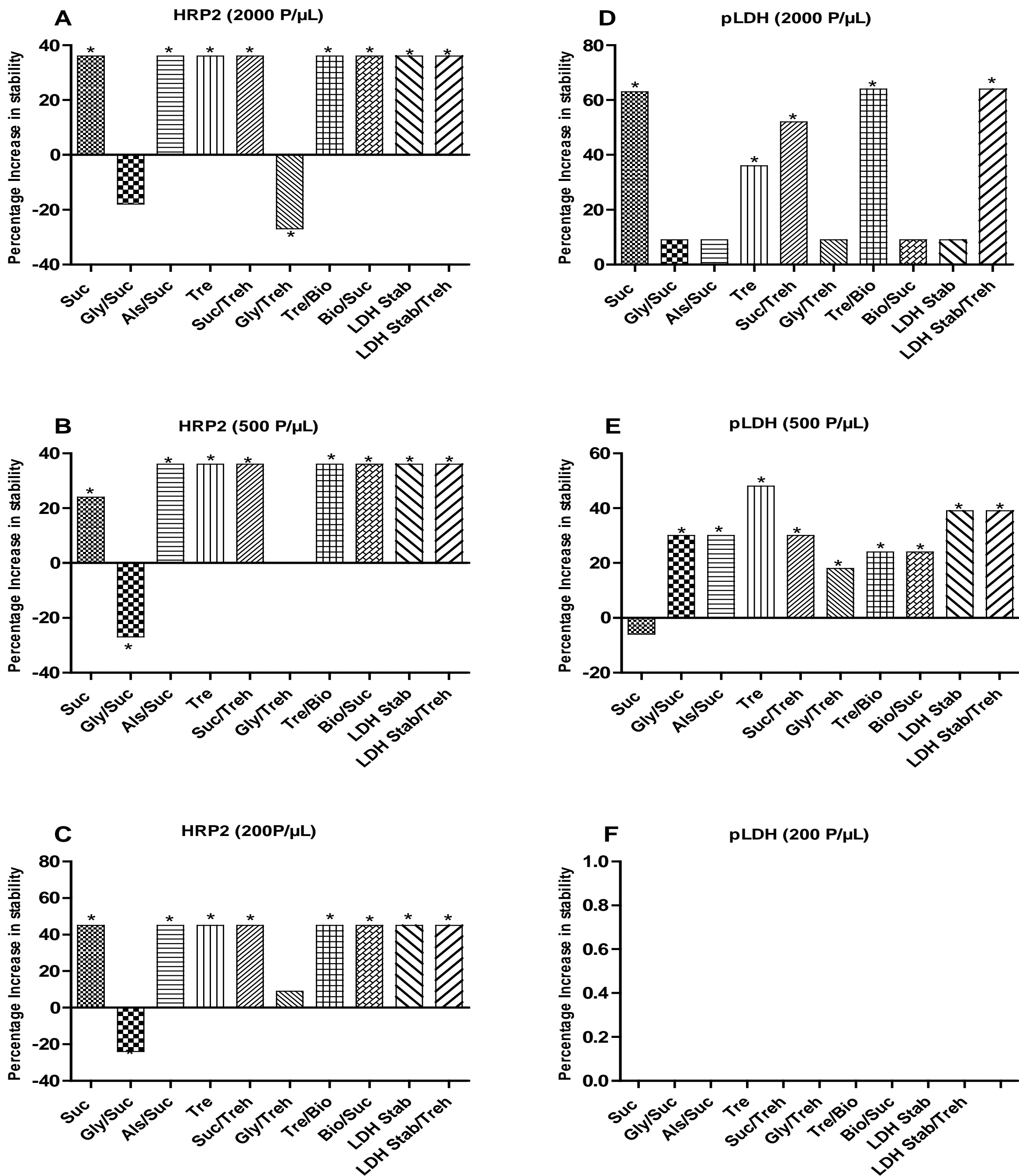
4.1 Determination of Temporal Stability of *Plasmodium* Proteins

4.1.1 Temporal Stability of HRP2 and *Plasmodium* LDH by SD Bioline

Figure 1 shows the ability of additives to improve the stability of the HRP2 and LDH in dried blood specimens when measured by SD Bioline. The reactivity of the HRP2 in the positive control sample was lost after 21 weeks at 2000p/µL, 21 weeks at 500p/µL and 18 weeks at 200p/µL during the 33 weeks of analysis. Thus, the baseline percentage stability of HRP2 was 64% at 2000p/µL and 500p/µL, but was lower at 200p/µL (54%). Addition of sucrose, alservers/sucrose, trehalose, sucrose/trehalose, biostab/trehalose, biostab/sucrose, and LDH stabilizer/trehalose increased the percentage stability of the HRP2 by 36% (Z= -3.87; P=0.0001) at 2000p/µL and 500p/µL and by 45% (Z=-4.4062, p=0.0001) at 200p/µL. However the results show that addition of glycerol/sucrose decreases the stability of HRP2 at all parasite concentrations while glycerol/trehalose decreases stability at 2000p/µL, does not improve stability at 500p/µL, and increases stability by 9% at 200p/µL (Z=-0.7517, P= 0.4522).

The reactivity of LDH in the positive control sample was lost after twelve weeks at 2000p/µL and two weeks at 500p/µL during the 33weeks of analysis. Therefore, the baseline percentage stability of HRP2 was 36% at 2000p/µL and 6% at 500p/µL. Addition of sucrose, trehalose/sucrose, trehalose/biostab, and trehalose/LDH stabilizer improved the percentage stability of *Plasmodium* LDH by 64% (z= -5.55, P=0.0001), and in trehalose 36% (z=-2.96, P=0.0030) at 2000p/µL. The rest of the additives improved stability of LDH by 9% (z=-0.76, P=0.447) at the same parasite concentration.

At 500p/µL, addition of trehalose improves stability of LDH by 48% (z=-4.29, P=0.0000), LDH stabilizer and LDH stabilizer/trehalose by 39% (z=-3.66, P=0.0002), glycerol/sucrose, Alsever's/sucrose, glycerol/trehalose and sucrose/trehalose improved stability by 30% (z=-3.00, P=0.003), trehalose/biostab and sucrose/biostab by 24% (z=-2.50, P=0.01) , and addition of glycerol/trehalose by 18% (z=-3.02, P=0.003). The antigen was not reactive from baseline on SD Bioline malaria kit at 200p/µL so there was no stability results recorded for this concentration. (Figure 1)



*indicates statistical significance (P<0.05), HRP2-Histidine Rich Protein 2, LDH-*Plasmodium* Lactate Dehydrogenase

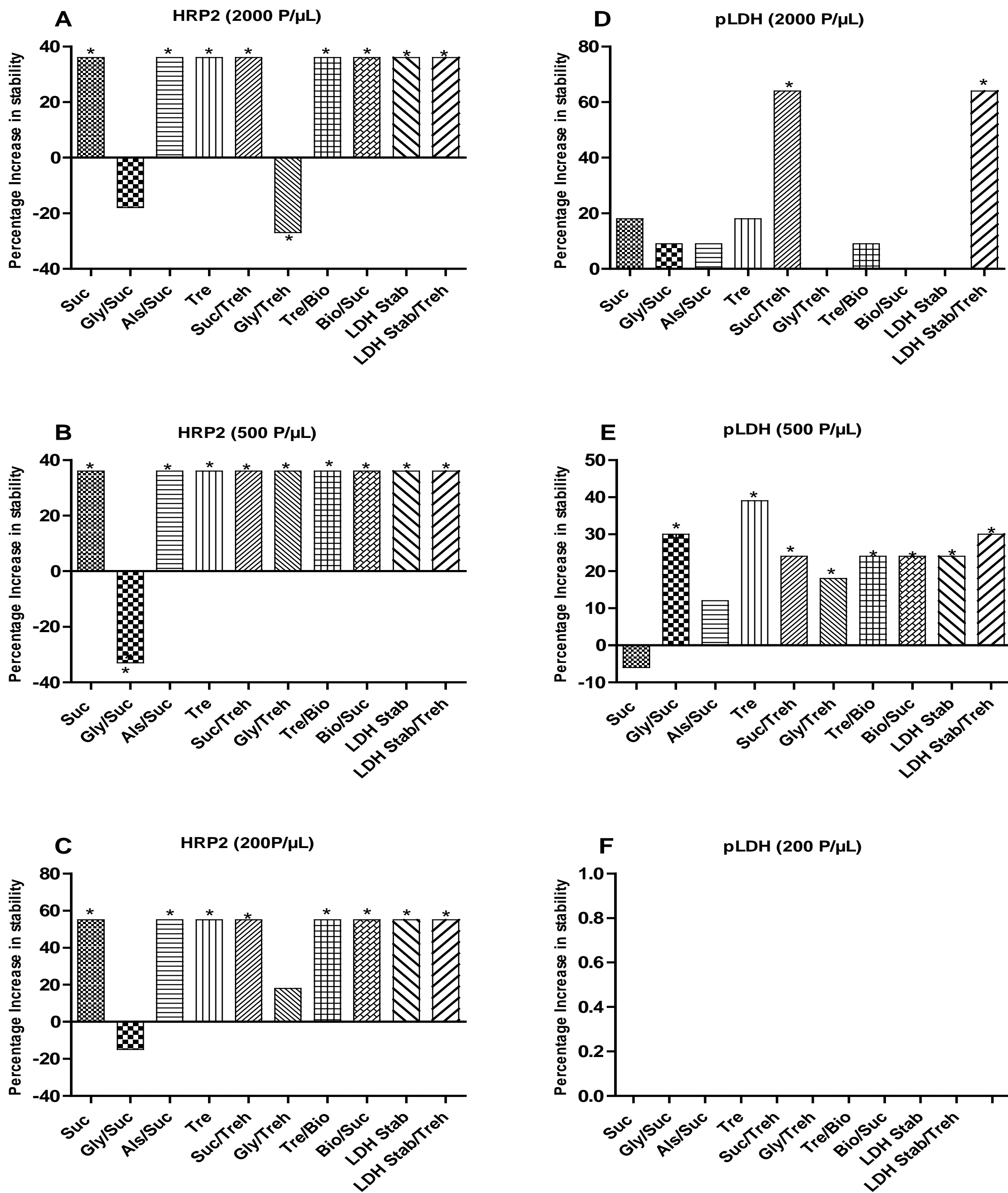
Figure 1: Temporal percentage stability of chemically stabilized *Plasmodium* HRP2 and LDH in patient samples measured for eight months by SD Bioline malaria kit at different parasite densities:

1A: Percentage stability of HRP2 in 2000p/µL, 1B: Percentage stability of HRP2 in 500p/µL, 1C: Percentage stability of HRP2 in 200p/µL, 1D: Percentage stability of LDH in 2000p/µL, 1E: Percentage stability of LDH in 500p/µL, 1F: Percentage stability of LDH in 200p/µL

4.1.2 Temporal stability of HRP2 and *Plasmodium* LDH by First Response

The First Response malaria kit indicated that the temporal stability of HRP2 was retained for 21 weeks at 2000p/µL, 21 weeks at 500p/µL and 15 weeks at 200p/µL during 33 weeks of analysis. Thus, the baseline percentage stability of HRP2 was 64% at 2000p/µL and 500p/µL, but was lower at 200p/µL (45%). The presence of sucrose, alservers/sucrose, trehalose, sucrose/trehalose, biostab/trehalose, biostab/sucrose, and LDH stabilizer/trehalose increased the percentage stability of the HRP2 by 36% (z= -3.81; P=0.0001) at 2000p/µL and 500p/µL and by 55% (z= -4.97, p=0.0000) at 200p/µL. The results indicate that addition of glycerol/sucrose decreases the stability of HRP2 at all parasite concentrations while glycerol/trehalose decreases stability at 2000p/µL, but improved stability at 500p/µL by 36% (z= -3.83, P= 0.0001), and 18% at 200p/µL (z=-1.48, P= 0.139) (Figure 2).

The reactivity of LDH in the positive control sample was also shown to be lost after 12weeks at 2000p/µL and two weeks at 500p/µL during the 33weeks of analysis. As a result, the baseline percentage stability of LDH was 36% at 2000p/µL and 6% at 500p/µL. Addition of trehalose/sucrose and trehalose/LDH stabilizer improved the percentage stability of *Plasmodium* LDH by 64% (z= -5.55, P=0.0001) at 2000p/µL. In the presence of sucrose and trehalose stability was improved by 18% (z=-1.48, P=0.138), while addition of glycerol/sucrose, Alsever's/sucrose, and trehalose/biostab improved stability by 9% (z=-0.75, P=0.0.454) at the same concentration. At 500p/µL, addition of trehalose improved stability of LDH by 39% (z=-3.65, P=0.0003), glycerol/sucrose and LDH stab/trehalose by 30% (z=-3.01, P=0.0026). Addition of sucrose/trehalose, trehalose/biostab, LDH stabilizer, and sucrose/biostab improved stability by 24% (z=-2.50, P=0.01). At parasite density of 200p/µL, the antigen was not reactive from baseline as tested by First Response malaria kit. (Figure 2)



*indicates statistical significance (P<0.05), HRP2-Histidine Rich Protein 2, LDH-*Plasmodium* Lactate Dehydrogenase

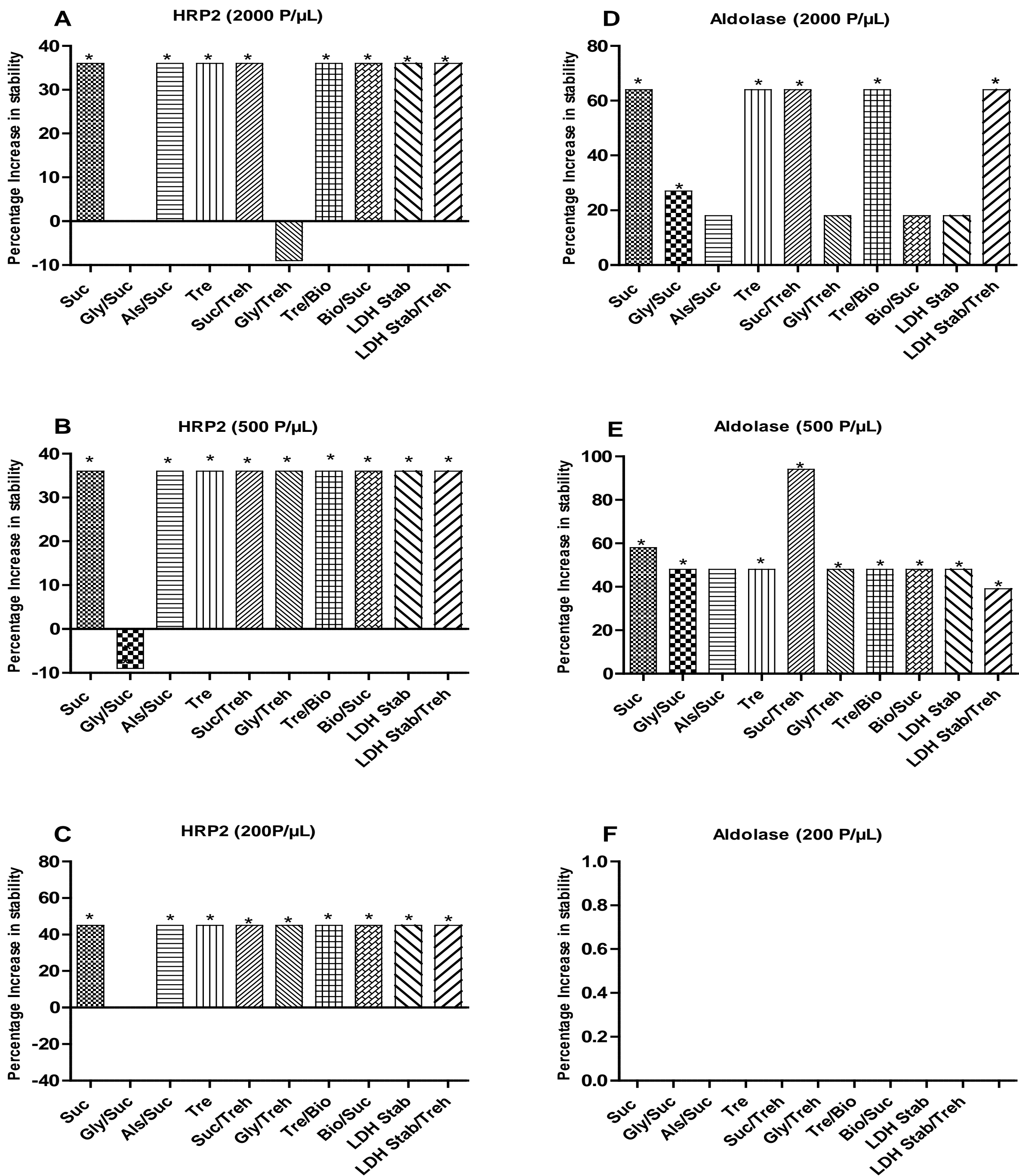
Figure 2: Temporal percentage stability of chemically stabilized *Plasmodium* HRP2 and LDH in patient samples measured for eight months by First Response malaria kit at different parasite densities:

1A: Percentage stability of HRP2 in 2000p/µL, 1B: Percentage stability of HRP2 in 500p/µL, 1C: Percentage stability of HRP2 in 200p/µL, 1D: Percentage stability of LDH in 2000p/µL, 1E: Percentage stability of LDH in 500p/µL, 1F: Percentage stability of LDH in 200p/µL

4.1.3 Temporal stability of HRP2 and *Plasmodium* Aldolase by Binax NOW

Figure 3 shows the reactivity of the HRP2 on Binax NOW malaria kit was lost after 21 weeks at 2000p/µL, 21 weeks at 500p/µL and 18 weeks at 200p/µL during 33 weeks of analysis. Therefore, the baseline percentage stability of HRP2 was 64% at 2000p/µL and 500p/µL, but was lower at 200p/µL (54%). Addition of sucrose, alservers/sucrose, trehalose, sucrose/trehalose, biostab/trehalose, biostab/sucrose, and LDH stabilizer/trehalose increased the percentage stability of the HRP2 by 36% (z= -3.81; P=0.0001) at 2000p/µL and 500p/µL; by 45% (z= -4.41, p=0.0000) at 200p/µL. The results show that the addition of glycerol/trehalose decreases stability at 2000p/µL only, but improves stability at 500p/µL by 36% (z= -3.83, P= 0.0001), and 45% at 200p/µL (z=-4.41, P= 0.0000) (Figure 3).

The reactivity of Aldolase in the positive control sample was also shown here to be lost after 12weeks at 2000p/µL and two weeks at 500p/µL during the 33weeks of analysis. Hence, the baseline percentage stability of aldolase was 36% at 2000p/µL and 6% at 500p/µL. Addition of sucrose, trehalose, sucrose/trehalose, trehalose/biostab and LDH stabilizer/trehalose improved the percentage stability of *Plasmodium* aldolase by 64% (z= -5.55, P=0.0001) at 2000p/µL. In the presence of glycerol/sucrose stability improved by 27% (z=-2.21, P=0.0271), while addition of Alsever's/sucrose, glycerol/trehalose, biostab/sucrose, and LDH stabilizer stability was improved only by 18% (z=-1.48, P=0.1398) at the same concentration. At 500p/µL, addition of sucrose/trehalose improved stability of aldolase by 94% (z=-7.64, P=0.0000). In the presence of sucrose stability of aldolase was improved by 58% (z=-4.90, P=0.0000) while the rest of the additives improved stability by 48% (z=-4.28, P=0.0000). There was no stability results recorded for aldolase because the antigen was not reactive from baseline on Binax NOW malaria kit at 200p/µL (Figure 3).



*indicates statistical significance (P<0.05), HRP2-Histidine Rich Protein 2

Figure 3: Temporal percentage stability of chemically stabilized HRP2 and *Plasmodium* aldolase in patient samples measured for 33 weeks by BinaxNOW malaria kit at different parasite densities:

1A: Percentage stability of HRP2 in 2000p/µL, 1B: Percentage stability of HRP2 in 500p/µL,

1C: Percentage stability of HRP2 in 200p/µL, 1D: Percentage stability of aldolase in 2000p/µL,

1E: Percentage stability of aldolase in 500p/µL, 1F: Percentage stability of aldolase in 200p/µL

4.2 Thermal Stability

4.2.1 Thermal Stability of HRP2 and LDH by SD Bioline Malaria kit

The thermal stability of HRP2 in the control sample was determined to be 33 weeks at 4°C and 25°C across all three parasite densities. At higher thermal conditions it was shown to reduce to 21weeks at 37°C for both 2000p/µL and 500p/µL and 18weeks for 200p/µL. Stability was reduced further at extreme temperature conditions (45°C), to 18 weeks for both 2000p/µL and 500p/µL and 10 weeks for 200p/µL. Addition of sucrose, Alservers/sucrose, trehalose, sucrose/trehalose, biostab/trehalose, biostab/sucrose, LDH stabilizer and LDH stabilizer/trehalose significantly increased the percentage thermal stability of HRP2 at both 37°C and 45°C ($P<0.05$) across all three parasite densities. The presence of glycerol/sucrose and glycerol/trehalose showed a tendency to reduce the thermal stability of HRP2 at both temperature conditions including 25°C across all parasite densities (Table 2).

Plasmodium LDH (2000p/µL) was shown to retain thermal stability for 18weeks, 15weeks, 12weeks and 8 weeks when stored at 4°C, 25°C, 37°C and 45°C respectively. At parasite density of 500p/µL the thermal stability was lost after twelve weeks at 4°C, four weeks at 25°C, and two weeks at both 37°C and 45°C. Three additives (trehalose, sucrose/trehalose, and LDH stabilizer/trehalose) significantly increased the percentage thermal stability of *Plasmodium* LDH across all four temperature conditions and all three parasite densities ($P<0.05$). Addition of sucrose, biostab/trehalose, sucrose/glycerol, trehalose/glycerol, biostab/sucrose, and LDH stabilizer significantly increased the percentage thermal stability across all temperatures at 500p/µL ($P<0.05$), but was quite variable at 2000p/µL (Table 2). The percentage increase in thermal stability of LDH was not recorded because the antigen was not reactive at baseline on SD Bioline malaria kit.

Table 2: Percentage increase in thermal stability of HRP2 and LDH in patient samples measured for 33 weeks by SD Bioline malaria kit

	Percentage Increase in Stability by SD Bioline Malaria Kit																			
Protein	HRP2 antigen												LDH antigen							
Parasite Density	2000 P/µL				500 P/µL				200 P/µL				2000 P/µL				500 P/µL			
Temperature	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**
Sucrose	0	0	36*	45*	0	0	24*	9	0	0	45*	33*	45*	54*	63*	30*				
Glycerol/Sucrose	0	0	-18	-18	0	0	-27	-24	0	-27	-24	-6	45*	27*	9	12	64*	76*	30*	24*
Alsever's/Sucrose	0	0	36*	45*	0	0	36*	45*	0	0	45*	33*	45*	18*	9	21	64*	42*	30*	18*
Trehalose	0	0	36*	45*	0	0	36*	45*	0	0	45*	70*	45*	55*	36*	30*	64*	61*	48*	39*
Sucrose/Trehalose	0	0	36*	45*	0	0	36*	45*	0	0	45*	70*	45*	55*	52*	48*	64*	42*	30*	24*
Glycerol/Trehalose	0	0	-27	-24	0	-12	0	-18	0	0	9	24*	45*	42*	9	6	64*	52*	18*	18*
Trehalose/Biostab	0	0	36*	45*	0	0	36*	45*	0	0	45*	70*	45*	55*	64*	21	27*	24*	24*	24*
Biostab/Sucrose	0	0	36*	45*	0	0	36*	45*	0	0	45*	70*	45*	9	9	12	64*	42*	24*	18*
LDH stabilizer	0	0	36*	45*	0	0	36*	45*	0	0	45*	70*	45*	55*	9	12	64*	52*	39*	24*
LDH stabilizer/Trehalose	0	0	36*	45*	0	0	36*	45*	0	0	45*	70*	45*	55*	64*	76*	64*	42*	39*	39*

*indicates statistical significance at P<0.05

The target antigen *Plasmodium* LDH was not reactive from baseline at 200p/µL, so there was no stability results recorded

4.2.2 Thermal Stability of HRP2 and LDH by First Response malaria kit

The thermal stability of HRP2 in the control sample was determined to be 33 weeks at 4°C and 24weeks at 25°C across all three parasite densities. At higher thermal conditions it was shown to reduce to 21weeks at 37°C for both 2000p/µL and 500p/µL and 15weeks for 200p/µL. At extreme temperature conditions (45°C), stability reduced further to 18weeks and 21 weeks for both 2000p/µL and 500p/µL respectively and eight weeks for 200p/µL. Addition of sucrose, Alservers/sucrose, trehalose, sucrose/trehalose, biostab/trehalose, biostab/sucrose, LDH stabilizer and LDH stabilizer/trehalose significantly increased the percentage thermal stability of HRP2 at 25°C, 37°C and 45°C ($P<0.05$) across all three parasite densities. The presence of glycerol/sucrose and glycerol/trehalose showed a tendency to reduce the thermal stability of HRP2 at both temperature conditions except 25°C across all parasite densities (Table 2).

Plasmodium LDH (2000p/µL) was shown to retain thermal stability for 18 weeks, 15 weeks, 12 weeks and eight weeks when stored at 4°C, 25°C, 37°C and 45°C respectively. At parasite density of 500p/µL the thermal stability was lost after six weeks at 4°C, four weeks at 25°C, and two weeks at both 37°C and 45°C. Two additives (sucrose/trehalose and LDH stabilizer/trehalose) significantly increased the percentage thermal stability of *Plasmodium* LDH across all four temperature conditions and all three parasite densities ($P<0.05$). Addition of trehalose, sucrose, Alservers/sucrose, biostab/trehalose, trehalose/glycerol, sucrose/glycerol, biostab/sucrose, and LDH stabilizer increased/maintained the percentage thermal stability across all temperatures at 500p/µL, but at 2000p/µL reactivity was quite variable because the control sample remained reactive for twice the durations at 2000p/µL as compared to 500p/µL (Table 3). The antigen was not reactive from baseline on First Response malaria kit at 200p/µL so there was no thermal stability results recorded.

Table 3: Percentage increase in thermal stability of HRP2 and LDH in patient samples measured for 33 weeks by First Response malaria kit

	Percentage Increase in Stability by First response Malaria Kit (%)																			
Protein	HRP2 antigen												LDH antigen							
Parasite Density	2000 P/µL				500 P/µL				200 P/µL				2000 P/µL				500 P/µL			
Temperature	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**
Sucrose	0	27*	36*	45*	0	27*	36*	0*	0	45*	55*	39*	45*	27*	18	30*				
Glycerol/Sucrose	0	27*	-18*	-18	0	27*	-33*	-33*	0	45	-15	0	45	27*	9	12	45*	52*	30*	24*
Alsever's/Sucrose	0	27*	36*	45*	0	27*	36*	36*	0	45*	55*	76*	45*	0*	9	21	18	18	12	24*
Trehalose	0	27*	36*	45*	0	27*	36*	36*	0	45*	55*	76*	45*	55*	18	30*	55*	33*	39*	39*
Sucrose/Trehalose	0	27*	36*	45*	0	27*	36*	36*	0	45*	55*	76*	45*	55*	64*	30*	82*	52*	24*	24*
Glycerol/Trehalose	0	27*	-27*	-24*	0	27*	36*	-33*	0	45*	18	30*	45*	0	0	6	45*	24*	18*	18*
Trehalose/Biostab	0	27*	36*	45*	0	27*	36*	36*	0	45*	55*	76*	45*	55*	9	6	18	18	24*	24*
Biostab/Sucrose	0	27*	36*	45*	0	27*	36*	36*	0	45*	55*	76*	45*	0	0	12	36*	42*	24*	18*
LDH stabilizer	0	27*	36*	45*	0	27*	36*	36*	0	45*	55*	76*	45*	0	0	12	27*	24*	24*	18*
LDH stabilizer/Trehalose	0	27*	36*	45*	0	27*	36*	36*	0	45*	55*	76*	45*	55*	64*	21*	27*	24*	30*	30*

*indicates statistical significance at $P<0.05$

The target antigen LDH was not reactive from baseline at 200p/µL, so there was no stability results recorded

4.2.3 Thermal Stability of HRP2 and Aldolase by BinaxNOW Malaria Kit

The thermal stability of HRP2 in the control sample was determined to be 33 weeks at 4°C and 25°C across all three parasite densities. At higher thermal conditions it was shown to reduce to 21 weeks at 37°C for both 2000p/µL and 500p/µL and 18weeks for 200p/µL. At extreme temperature conditions (45°C), stability was reduced further to 18 weeks for both 2000p/µL and 500p/µL and ten weeks for 200p/µL. Addition of sucrose, Alservers/sucrose, trehalose, sucrose/trehalose, biostab/trehalose, biostab/sucrose, LDH stabilizer and LDH stabilizer/trehalose significantly increased the percentage thermal stability of HRP2 at both 37°C and 45°C ($P<0.05$) across a ll three parasite densities. The presence of glycerol/sucrose and glycerol/trehalose showed a tendency to reduce the thermal stability of HRP2 at both temperature conditions including 25°C across all parasite densities (Table 2).

Plasmodium aldolase (2000p/µL) was shown to retain thermal stability for 18weeks, 15weeks, 12weeks and eight weeks when stored at 4°C, 25°C, 37°C and 45°C respectively. At a parasite density of 500p/µL the thermal stability was lost after 12weeks at 4°C, four weeks at 25°C, and two weeks at both 37°C and 45°C. Five additives (trehalose, sucrose/glycerol, trehalose/sucrose, trehalose/biostab, and LDH stabilizer/trehalose) significantly increased the percentage thermal stability of *Plasmodium* aldolase across all four temperature conditions and all three parasite densities ($P<0.05$). Addition of sucrose, Alservers/sucrose, trehalose/glycerol, sucrose/glycerol, biostab/sucrose, and LDH stabilizer significantly increased the percentage thermal stability across all temperatures at 500p/µL ($P<0.05$) (Table 4). The increase in thermal stability of aldolase was not recorded at 200p/µL because the sample was not reactive from baseline analysis.

.

Table 4: Percentage increase in thermal stability of HRP2 and aldolase in patient samples measured for 33 weeks by BinaxNOW malaria kit

Percentage Increase in Stability by Binax NOW Malaria Kit

Protein	HRP2 antigen												Aldolase							
Parasite Density	2000 P/µL				500 P/µL				200 P/µL				2000 P/µL				500 P/µL			
Temperature	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**	**4°C**	**25°C**	**37°C**	**45°C**
Sucrose	0	0	36*	45*	0	0	36*	9	0	0	45*	33*	45*	55*	64*	39*				
Glycerol/Sucrose	0	0	0	9	0	0	-9	0	0	-27*	0	24*	45*	55*	27*	39*	36*	61*	48*	48*
Alsever's/Sucrose	0	0	36*	45*	0	0	36*	45*	0	0	45*	33*	45*	55*	18	30*	18	42*	48*	48*
Trehalose	0	0	36*	45*	0	0	36*	45*	0	0	45*	70*	45*	55*	64*	30*	64*	61*	48*	48*
Sucrose/Trehalose	0	0	36*	45*	0	0	36*	45*	0	0	45*	70*	45*	55*	64*	76*	64*	88*	94*	48*
Glycerol/Trehalose	0	0	-9	0	0	0	36*	0	0	0	45*	24*	45*	55*	18	30*	27*	52*	48*	48*
Trehalose/Biostab	0	0	36*	45*	0	0	36*	45*	0	0	45*	70*	45*	55*	64*	39*	18	42*	48*	48*
Biostab/Sucrose	0	0	36*	45*	0	0	36*	45*	0	0	45*	42*	45*	9	18	30*	36*	61*	48*	48*
LDH stabilizer	0	0	36*	45*	0	0	36*	45*	0	0	45*	70*	45*	55*	18	30*	18	42*	48*	48*
LDH stabilizer/Trehalose	0	0	36*	45*	0	0	36*	45*	0	0	45*	70*	45*	55*	64*	76*	64*	42*	39*	39*

*indicates statistical significance at P<0.05

The target antigen *Plasmodium* aldolase was not reactive from the baseline at 200p/µL, so there was no stability results recorded

4.3 Effectiveness of Using Cultured Samples.

Table 5 indicates the regression coefficients for the antigen reactivity being predicted by the culture samples when patient samples are used as the reference term. Reactivity of the antigens varied with concentration. At 2000p/µL there was no significant difference ($P>0.05$) in using cultured samples as compared to patient samples in preparing dried blood specimens. Reactivity of all the proteins (HRP2, LDH, and aldolase) that were stabilized or not stabilized at this concentration did not depend on the source of the sample.

The model shows that a culture sample significantly predicts the reactivity of HRP2 at 500p/µL, measured by SD Bioline and BinaxNOW ($P<0.05$). Although, the First Response malaria kit indicates that there is no significant difference ($P>0.05$). Aldolase and LDH reactivity on the malaria kits whether stabilized or not was shown to be predicted by the source of the sample ($P<0.0001$).

At parasite density of 200p/µL, the reactivity of aldolase and LDH was not included in the model since the sample tested negative from baseline. Reactivity of HRP2 at 200p/µL shows a significant difference if the source of the sample was a culture as tested by SD Bioline and BinaxNOW, but and First response did not show a statistically significant difference.

Table 5: Logistic regression model for repeated measures showing the relationship between sample type and antigen reactivity at different parasite concentrations

	Coeff	95% CI		Std. Err.	z	P-value
2000 p/µL						
HRP2 (SD Bioline)	0.124	-0.0919	0.3394	0.110	1.12	**0.261**
Plasmodium LDH (SD Bioline)	-0.088	-0.3077	0.1311	0.112	-0.79	**0.430**
HRP2 (First Response)	0.083	-0.1291	0.2948	0.108	0.77	**0.444**
Plasmodium LDH (First Response)	-0.178	-0.3970	0.0411	0.112	-1.59	**0.111**
HRP2 (BinaxNOW)	0.298	-0.0111	0.6077	0.158	1.89	**0.059**
Aldolase (BinaxNOW)	-0.136	-0.4431	0.1708	0.157	-0.87	**0.385**
500 p/µL						
HRP2 (SD Bioline)	0.264	0.0259	0.5031	0.122	2.17	0.030
Plasmodium LDH (SD Bioline)	-0.579	-0.7878	-0.3708	0.106	-5.45	0.000
HRP2 (First Response)	0.229	-0.0038	0.4627	0.119	1.93	**0.054**
Plasmodium LDH (First Response)	-0.568	-0.7842	-0.3513	0.110	-5.14	0.000
HRP2 (BinaxNOW)	0.408	0.2130	0.6022	0.099	4.11	0.000
Aldolase (BinaxNOW)	-0.54	-0.8111	-0.2684	0.138	-3.90	0.000
200 p/µL						
HRP2 (SD Bioline)	0.291	0.0536	0.5289	0.121	2.40	0.016
HRP2 (First Response)	0.216	-0.0122	0.4446	0.117	1.86	**0.064**
HRP2 (BinaxNOW)	0.321	0.0085	0.6337	0.159	2.01	0.044

HRP2-Histidine Rich Protein 2, LDH-Lactose Dehydrogenase

The table indicated detection of the antigens present in culture sample by the specific kits. Both sample types (Patient and Culture) can be used to prepare the dried blood tubes as positive controls for validation of HRP2 detecting malaria RDTs and LDH detecting kits at 2000 p/µL.

CHAPTER FIVE

DISCUSSION

5.1 Temporal Stability

Good quality assurance of malaria RDTs is vital to proper patient management (McMorrow *et al.*, 2008). Microscopy is the main method for ensuring diagnostic performance of RDTs, but it is scarce and poor microscopy has been recognized in practice (Wongsrichanalai *et al.*, 2007). Many countries are implementing the use of malaria RDTs without a good QA system as a result there is need for simple and reliable method is essential for validation of malaria kits (Abeku *et al.*, 2008). Dried blood specimens have been proposed for monitoring malaria RDT performance but they have to be stored for shorter durations at peripheral hospitals (Aidoo *et al.*, 2012; Tamiru *et al.*, 2015). Long term storage stability of the dried blood specimens depends on the stability of the RDT target *Plasmodium* proteins (HRP2, LDH, and aldolase).

The percentage stability of HRP2, LDH and aldolase was shown to be >63% after eight months of storage. The chemical additives significantly improved the long term storage stability of HRP2, LDH, and aldolase as determined by reactivity on the three malaria RDTs (SD Bioline, First Response, and BinaxNOW). Specifically, addition of sucrose, trehalose, biostab/trehalose, LDH stabilizer/trehalose, and trehalose/sucrose improved the stability of all the three proteins during the eight months of storage. During the stabilization process, the disaccharides, biostab and LDH stabilizer are preferentially excluded from the surface of the HRP2, LDH, and aldolase thereby increasing the chemical potential of the protein. Thus by the Le Chateliers principle, the sugars favor the native state of the protein (N-state) over the more disordered state (D-state). Therefore, the Gibbs free energy changes that is normally associated with the transition of the protein to a disordered state ($N \leftrightarrow D$) is increased in the presence of the trehalose and sucrose ($\Delta GD = -RT\ln K$) (Arakawa *et al.*, 2001). During drying, trehalose and sucrose substitute the

water forming hydrogen bonds with the proteins thereby increasing the thermal unfolding temperature of the stabilized protein and inhibiting the irreversible chemical instabilities. In the dried state, the HRP2, LDH and aldolase were entrapped in shells by the surrounding complex molecular structures of sucrose and trehalose. The un-stabilized protein lost reactivity in less than 21 weeks of storage because the proteins lost inter and intra-molecular interactions because of unfolding and various destabilizing effects of protein-protein interactions. The entrapment localized HRP2, LDH and aldolase in a defined portion of space, which limits the movement of the proteins that decreases protein-protein interaction and unfolding.

Glycerol/trehalose and glycerol/sucrose are the only additives shown to destabilize or decrease stability of HRP2, LDH, or aldolase. This study indicates that the sugars increase the stability of the protein individually, but the presence of glycerol as a combination decreases stability. This is so because during addition of the additives into blood, glycerol preferentially hydrates HRP2, LDH, or aldolase thereby enhancing the ordering of water around the hydrophobic amino acid chains. This ordering decreases the interaction of the disaccharides with the protein structures preventing the formation of hydrogen bonds and encapsulation of the protein in the sugar matrix core. This trend was shown to be reversed at reduced concentration of the proteins (500 p/µL and 200 p/µL), which can infer that reduction in the levels of accessible proteins increases the chance of interaction between the sugar molecules and proteins. Another explanation is that glycerol is also preferentially excluded from the surface of the protein but the exclusion by glycerol is thermodynamically unfavorable which favors the unstructured form of HRP2, LDH, or aldolase. This was shown in previous studies on the mechanisms by which sucrose stabilizes proteins (Gekko & Timasheff, 1981).

Previous studies have shown that through preferential interactions; sucrose, trehalose, biostab enzyme stabilizer, and LDH stabilizer improve the long term stability of various proteins including; ribonuclease A, lysozyme, chymo-trypsinogen recombinant interleukin 1 receptor antagonist and monoclonal, antibodies pyro-phosphatase, Bovine serum albumin, Ribosomal protein S6, cutinese, LDH and lysozyme (Balcão & Vila, 2015; Baptista *et al.*, 2008; Chen *et al.*, 2005; Hedoux *et al.*, 2009; Katyal & Deep, 2014; Kawai & Suzuki, 2007; Kendrick *et al.*, 1997; Subbaraman *et al.*, 2005; Yazdani *et al.*, 2015; Zancan & Sola-Penna, 2005). The findings of the present study imply that the use of these additives in preparation of dried blood specimens will ensure prolonged storage in the low resource facilities, thereby reducing the costs of resupply and increased reliability on them as positive controls.

It should also be noted, that stabilization of HRP2, LDH and aldolase increases as concentration decreases for these additives. This can be due to the dilution effect and surface area to volume ratio (Chang *et al.*, 2005). In additive protein mixtures there is a distribution of the additives around the proteins at a particular concentration and size of the protein. If the protein concentration is reduced and the same amount of the additive is maintained the proteins achieve more stability because all the binding sites of the protein will be saturated depending on the molecular weight. A previous study on effect of sucrose concentration on protein stability showed that; a 1:1 weight ratio provided a six fold stabilization toward aggregation for hGH(22kD), a 4-fold stabilization for rHSA (66kD), and a 20-fold stabilization for IgG1 antibody(150kD) protein (Chang *et al.*, 2005).

In stabilizing these proteins there were differences in the stabilization of HRP2 antigen versus the LDH and aldolase antigen. This can be explained by the inherent differences in the levels of these proteins. The levels of HRP2 have been shown to be higher in individuals

(approx. 7 fold), and can persist in the bloodstream for more than two weeks as compared to the LDH which cannot be detected in smaller volumes of blood and the clearance time rate is less than five days (Martin *et al.*, 2009). The differences between these proteins in terms of quantity can be seen as a factor in the level of stabilization achieved for this protein since HRP2 tends to achieve better stabilization across all the additives than aldolase and LDH. The differences in the secondary, tertiary and quaternary structures of HRP2, LDH, or aldolase can be at a confounding in the differences observed in stabilization especially the exposure levels of the hydrophobic cores. The increased or decreased interaction of sucrose and trehalose with the hydrophobic fragments of HRP2, LDH, or aldolase can lead to increase or decrease in the Gibbs free energy of the system and the concomitant stabilization (Arakawa, 2001). Future work on development of stable proteins will have to involve exact quantization of the protein (not concentration of the parasites) so as to achieve uniform protein levels.

One of the limitations of the study is that there was noticeable dissimilarity between SD Bioline, First Response and Binax NOW in the detection of antigen reactivity. The results indicate (Figure 1-3) that the detection of LDH in sucrose or trehalose/biostab showed reactivity up to 33weeks by SD Bioline and Binax Now at 2000p/µL and 500p/µL, but was reactive for 18 and 15 weeks respectively on First response. The three RDTs are made by three different manufacturers and the differences the capture of the antigens can be attributed in the manufacturer differences especially because they use different capture antibodies. Previous studies on patient samples and WHO panel detection scores, have shown that the antigen capture of malaria RDTs varies between different manufacturer brands and between different lots from the same manufacturer (Aidoo *et al.*, 2012; Mouatcho & Goldring, 2013; WHO/FIND/CDC, 2015; Wongsrichanalai *et al.*, 2007). Manufacturer factors such as the type of immunoglobulin

used for antigen capture, the type of strip used, type of buffer used, the quality of fluorescence particles, and the overall kit development, play a critical role in the variability of results among kits (Mouatcho & Goldring, 2013). The results of this study also indicate the need to implement these stabilized positive controls for monitoring the test performance of malaria kits at the point of care.

The parasite density of the specimens played a significant role on the outcome of the results. The detection of HRP2 antigen by the three kits increased as the parasite density increased, and it has to be noted that despite the addition of additives, at 200p/µL the loss of antigen reactivity was detected earlier as compared to the specimens standardized at 2000p/µL. Aidoo et al (2012) also indicated that reactivity of the samples standardized at 200p/µL lost reactivity faster on all the ten malaria kits used as compared to samples at 2000p/µL. In this study, *Plasmodium* LDH and aldolase were detectable from baseline by all the three malaria kits but their reactivity was detectable as parasite density increased. The loss of reactivity of these antigens both in the positive control and the stabilized samples was faster at 500p/µL as compared to the specimens standardized at 2000p/µL. The degree of stability depends on the quantity of proteins that can be exposed to the additives for temporal stabilization, and the decrease in the levels of proteins can be directly proportional to the decreased amount of proteins stabilized at lower concentrations. A study by Versteg and Mens (2009) indicated that none of the samples containing a parasite density of 300p/µL gave a signal throughout their study while the samples containing 3000p/µL were positive for four weeks and samples at 30,000p/µL yielded a signal that remained visible for some time.

5.2 Thermal Stability

The applicability of these positive controls depends on their usefulness in diverse climatic conditions. Reports have indicated that temperatures can rise up to >40°C in many parts of the world especially in malaria endemic areas (Albertini *et al.*, 2012; KMD, 2012; WMO, 2016). Proteins undergo denaturation at high temperature conditions whereby they transform from a native ordered state to a disordered state due to rearrangement of the hydrogen bonds. To show the effect of chemical additives on thermal stability of HRP2, LDH and aldolase, the dried infected samples were exposed to four different temperature conditions 4°C, 25°C, 37°C, and 45°C, then reactivity was determined on the three malaria RDTs.

This study indicated that the addition of sucrose, trehalose, biostab/trehalose, and LDH stabilizer/trehalose increased the percentage thermal stability of HRP2, LDH and aldolase at 25°C, 37°C, and 45°C when compared to the control. Disrupting the thermodynamic stability of the protein molecule is the least work needed to disrupt the tertiary native structure of a protein. At temperatures of 25°C, 37°C, and 45°C this involved disrupting the intra-molecular bonds (hydrogen and hydrophobic bonds) and other types of interactions such as non-covalent bonds hence effecting the highly co-operative process of protein transition from the folded to unfolded state (Balcão & Vila, 2015). Thermodynamically the thermal protein stability is attained by the balance between the enthalpic (stabilizing) and entropic forces (destabilizing). The control sample lost reactivity due to changes in the conformational structure of the HRP2, LDH and aldolase at the different temperature conditions (4°C, 25°C, 37°C, and 45°C). Since it’s impossible for the proteins to retain their native structures at high temperature condition, the presence of sucrose, trehalose and biostab tailored the structure through hydrogen bonding and reduced the molecular motions of the proteins so as to achieve thermal stability.

More so, in the dried state the proteins (HRP2, LDH and aldolase) are encapsulated in the matrix core surrounded by sucrose and trehalose, and this eliminates the rotational, translational, and vibrational molecular motions of the proteins. Permanent inactivation of the protein function is achieved when high temperatures initiate the unfolding process of the protein (denaturation) and the breakdown of both hydrophobic and electrostatic interactions of the protein structure. Thus, the presence of the sugars improved the thermal stability of HRP2, LDH and aldolase by decreasing the entropic gain on unfolding at high temperatures, thereby preserving the native functional structure of the proteins at the high temperature conditions.

This study is in line with previous studies that have shown the effect of these additives on the thermal stability of other proteins. A US Research Patent by Roser (1990) showed that antigens and antibodies coupled with red blood cells then dried in the presence of trehalose, could completely retain their function as antibody or antigen at 40°C. They also showed that trehalose can be used to preserve other protein such as R-phycocerthrin, rat IgG, lymphocytes, serum complement, and alkaline phosphatase at a wide range of temperature conditions (Roser, 1990). Another study also showed that three enzymes ribonuclease, chymotrypsin, and chymotrypsinogen in varying amounts of sucrose could be recovered after storage at 90°C (Balcão & Vila, 2015). During storage at 60°C for 90 days fully dried formulations of LDH in sucrose or trehalose remained in glassy state and only demonstrated 35% loss in LDH activity (Kawai & Suzuki, 2007). The ability of Alsever's solution to provide thermal stability has also been shown previously by enhancing the preservation glucose-6-phosphate dehydrogenase, pyruvate kinase, triose phosphate isomerase, glutathione reductase, and NADH diaphorase activities at 30°C for three and half weeks (Lowe *et al.*, 1973).

5.3 Effectiveness of Using Cultured Samples

Well characterized patient samples are ideal in the production of positive controls for validation of the malaria RDTs. However challenges such as; regulatory structures governing the use of human blood and ethical issues associated with risk to the blood donor limit the use of patient blood for production of controls (Moodley *et al.*, 2014). This study determined the effectiveness of using cultured samples as compared to patient samples in the preparation of dried infected blood specimens. This was done by preparation of cultures samples and subjecting them to the same condition as patient samples.

There was no significant difference in the effectiveness of using cultured samples over the use of patient samples at 2000p/µL, but there were significant difference at parasite densities of 500p/µL and 200p/µL. Overall, this indicates that well characterized cultured samples treated the same way as patient samples at higher concentration would mimic patient matrix of the proteins, and can be used successfully in developing the positive controls. Similarly, previous studies by Aidoo *et al* (2012) and Tamiru *et al* (2015) used in-vitro cultured *Plasmodium* samples to develop dried blood specimens indicating that they can be adopted for the development of stable positive controls.

The only challenge is the low concentration effect whereby the cultured samples produced more significant reactivity of LDH and aldolase as compared to patient samples. The possible cause of this can be due to the expression profiles of these protein genes when it is in its natural host environment. The expression profiles of the parasite genes depend on the interaction signals between the parasite and the human body. The expression is also related to the distinct physiological states of the parasite; that is the glycolytic growth, starvation response and general stress response (Daily *et al.*, 2007).

These makes the two proteins LDH and aldolase involved in the glycolytic growth to be highly regulated in the host but they are not regulated as much *in vitro* cultures, hence the levels are higher in cultures than patient samples (Daily *et al.*, 2007). Also, LDH or aldolase levels are normally very low in patient sample as compared to HRP2 (Martin *et al.*, 2009); which leads to challenges in standardizing the antigen matrix during dilutions and hence unequal distribution of the protein. So the cultured samples cannot compare to patient samples at low concentration and the inference of this is that, it proves to be a major limitation in the development of dried infected blood specimens as positive controls for RDT validation.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The studies demonstrated that the presence of sucrose, trehalose, Biostab/trehalose, and LDH stabilizer/trehalose in dried infected *Plasmodium* samples significantly improves the temporal stability of HRP2, LDH, and aldolase for eight months of storage.

2. The presence of trehalose, Biostab/trehalose, sucrose/trehalose, sucrose, and LDH stabilizer/trehalose maintained the thermal stability of HRP2, LDH, and aldolase present in dried *Plasmodium* infected blood specimens at 25°C, 37°C, and 45°C.

3. We conclude that cultured samples at 2000p/µL can be used to develop the positive controls in the same capacity as patient samples, except at lower concentrations (500p/µL and 200p/µL. The use of cultured samples will reduce the ethical issues and concerns over drawing large amounts of blood to prepare the dried infected blood specimens.

6.2 Recommendations

1. The recommends the use of sucrose/trehalose or biostab/trehalose in improving the thermal and temporal stability (8 months) of HRP2, LDH, and aldolase in dried *Plasmodium* infected blood specimens. Stabilized cultured specimens at concentration above 2000p/µL can be used to develop stable field deployable positive controls for validation of malaria RDTs.

2. This study also recommends that future exploration in the field be carried out. Dried *Plasmodium* infected specimens as RDT positive controls should be sent to off-site facilities in different climatic zones both in Kenya and other countries for testing under ambient temperature conditions in order to determine the operational viability of the stabilized specimens. If the field evaluations prove successful, attempts should be made to produce these specimens under regulatory approvals for quality control of malaria RDTs.

REFERENCES

Abeku, T. A., Kristan, M., Jones, C., Beard, J., Mueller, D. H., Okia, M., Rapuoda, B., Greenwood, B., & Cox, J. (2008). Determinants of the accuracy of rapid diagnostic tests in malaria case management: evidence from low and moderate transmission settings in the East African highlands. *Malar J, 7*, 202. doi: 10.1186/1475-2875-7-202

Aidoo, M., Patel, J. C., & Barnwell, J. W. (2012). Dried *Plasmodium* falciparum-infected samples as positive controls for malaria rapid diagnostic tests. *Malar J, 11*, 239. doi: 10.1186/1475-2875-11-239

Albertini, A., Lee, E., Coulibaly, S. O., Sleshi, M., Faye, B., Mationg, M. L., Ouedraogo, K., Tsadik, A. G., Feleke, S. M., Diallo, I., Gaye, O., Luchavez, J., Bennett, J., & Bell, D. (2012). Malaria rapid diagnostic test transport and storage conditions in Burkina Faso, Senegal, Ethiopia and the Philippines. *Malar J, 11*, 406. doi: 10.1186/1475-2875-11-406

Arakawa, T., Prestrelski, S. J., Kenney, W. C., & Carpenter, J. F. (2001). Factors affecting short-term and long-term stabilities of proteins. *Advanced Drug Delivery Reviews, 46*(1–3), 307-326. doi: http://dx.doi.org/10.1016/S0169-409X(00)00144-7

Balcão, V. M., & Vila, M. M. D. C. (2015). Structural and functional stabilization of protein entities: state-of-the-art. *Advanced Drug Delivery Reviews, 93*, 25-41. doi: http://dx.doi.org/10.1016/j.addr.2014.10.005

Baptista, R. P., Pedersen, S., Cabrita, G. J., Otzen, D. E., Cabral, J. M., & Melo, E. P. (2008). Thermodynamics and mechanism of cutinase stabilization by trehalose. *Biopolymers, 89*(6), 538-547. doi: 10.1002/bip.20926

Bischof, J. C., & He, X. (2006). Thermal Stability of Proteins. *Annals of the New York Academy of Sciences, 1066*(1), 12-33. doi: 10.1196/annals.1363.003

Brown, W. M., Yowell, C. A., Hoard, A., Vander Jagt, T. A., Hunsaker, L. A., Deck, L. M., Royer, R. E., Piper, R. C., Dame, J. B., Makler, M. T., & Vander Jagt, D. L. (2004). Comparative structural analysis and kinetic properties of lactate dehydrogenases from the four species of human malarial parasites. *Biochemistry, 43*(20), 6219-6229. doi: 10.1021/bi049892w

Buderer, N. M. (1996). Statistical methodology: I. Incorporating the prevalence of disease into the sample size calculation for sensitivity and specificity. *Acad Emerg Med, 3*(9), 895-900.

Carpenter, J. F., Manning, M. C., & Randolph, T. W. (2002). Long-term storage of proteins. *Curr Protoc Protein Sci, Chapter 4*, Unit 4.6. doi: 10.1002/0471140864.ps0406s27

Castronuovo, G. (1991). Proteins in aqueous solutions. calorimetric studies and thermodynamic characterization. *Thermochimica Acta, 193*, 363-390. doi: http://dx.doi.org/10.1016/0040-6031(91)80193-M

Chang, L., Shepherd, D., Sun, J., Ouellette, D., Grant, K. L., Tang, X. C., & Pikal, M. J. (2005). Mechanism of protein stabilization by sugars during freeze-drying and storage: native structure preservation, specific interaction, and/or immobilization in a glassy matrix? *J Pharm Sci, 94*(7), 1427-1444. doi: 10.1002/jps.20364

Chen, L., Cabrita, G. J., Otzen, D. E., & Melo, E. P. (2005). Stabilization of the ribosomal protein S6 by trehalose is counterbalanced by the formation of a putative off-pathway species. *J Mol Biol, 351*(2), 402-416. doi: 10.1016/j.jmb.2005.05.056

Chiodini, P. L., Bowers, K., Jorgensen, P., Barnwell, J. W., Grady, K. K., Luchavez, J., Moody, A. H., Cenizal, A., & Bell, D. (2007). The heat stability of *Plasmodium* lactate dehydrogenase-based and histidine-rich protein 2-based malaria rapid diagnostic tests. *Trans R Soc Trop Med Hyg, 101*(4), 331-337. doi: http://dx.doi.org/10.1016/j.trstmh.2006.09.007

Daily, J. P., Scanfeld, D., Pochet, N., Le Roch, K., Plouffe, D., Kamal, M., Sarr, O., Mboup, S., Ndir, O., Wypij, D., Levasseur, K., Thomas, E., Tamayo, P., Dong, C., Zhou, Y., Lander, E. S., Ndiaye, D., Wirth, D., Winzeler, E. A., Mesirov, J. P., & Regev, A. (2007). Distinct physiological states of *Plasmodium falciparum* in malaria-infected patients. *Nature, 450*(7172), 1091-1095. doi: http://www.nature.com/nature/journal/v450/n7172/suppinfo/nature06311_S1.html

Florens, L., Washburn, M. P., Raine, J. D., Anthony, R. M., Grainger, M., Haynes, J. D., Moch, J. K., Muster, N., Sacci, J. B., Tabb, D. L., Witney, A. A., Wolters, D., Wu, Y., Gardner, M. J., Holder, A. A., Sinden, R. E., Yates, J. R., & Carucci, D. J. (2002). A proteomic view of the *Plasmodium falciparum* life cycle. *Nature, 419*(6906), 520-526. doi: 10.1038/nature01107

Gekko, K., & Timasheff, S. N. (1981). Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry, 20*(16), 4667-4676.

GwentGroup. (2010). Stabiliser Solution for Lactate dehydrogenase; Product Information Sheet. *Gwent Group Website*.

Hamada, H., Arakawa, T., & Shiraki, K. (2009). Effect of additives on protein aggregation. *Curr Pharm Biotechnol, 10*(4), 400-407.

Hansen, J. P. (2004). CAN'T MISS: Conquer any number task by making important statistics simple. Part 6. Tests of Significance (Z-test statistic, Rejecting Null Hypothesis, p-value), t Test, z Test for proportions, Statistical significance Versus Meaningful difference. *Journal of Healthcare Quality, 26*(4).

Hedoux, A., Willart, J. F., Paccou, L., Guinet, Y., Affouard, F., Lerbret, A., & Descamps, M. (2009). Thermostabilization mechanism of bovine serum albumin by trehalose. *J Phys Chem B, 113*(17), 6119-6126. doi: 10.1021/jp900330r

Hewitt, C. O., Sessions, R. B., Dafforn, T. R., & Holbrook, J. J. (1997). Protein engineering tests of a homology model of *Plasmodium falciparum* lactate dehydrogenase. *Protein Eng, 10*(1), 39-44.

Houze, S., Boly, M. D., Le Bras, J., Deloron, P., & Faucher, J. F. (2009). PfHRP2 and PfLDH antigen detection for monitoring the efficacy of artemisinin-based combination therapy (ACT) in the treatment of uncomplicated falciparum malaria. *Malar J, 8*, 211. doi: 10.1186/1475-2875-8-211

Howard, R. J., Uni, S., Aikawa, M., Aley, S. B., Leech, J. H., Lew, A. M., Wellems, T. E., Rener, J., & Taylor, D. W. (1986). Secretion of a malarial histidine-rich protein (Pf HRP II) from *Plasmodium* falciparum-infected erythrocytes. *J Cell Biol, 103*(4), 1269-1277.

Iyer, P. V., & Ananthanarayan, L. (2008). Enzyme stability and stabilization—Aqueous and non-aqueous environment. *Process Biochemistry, 43*(10), 1019-1032. doi: http://dx.doi.org/10.1016/j.procbio.2008.06.004

Jain, N. K., & Roy, I. (2009). Effect of trehalose on protein structure. *Protein Sci, 18*(1), 24-36. doi: 10.1002/pro.3

Katyal, N., & Deep, S. (2014). Revisiting the conundrum of trehalose stabilization. *Phys Chem Chem Phys, 16*(48), 26746-26761. doi: 10.1039/c4cp02914c

Kawai, K., & Suzuki, T. (2007). Stabilizing effect of four types of disaccharide on the enzymatic activity of freeze-dried lactate dehydrogenase: step by step evaluation from freezing to storage. *Pharm Res, 24*(10), 1883-1890. doi: 10.1007/s11095-007-9312-6

Kendrick, B. S., Chang, B. S., Arakawa, T., Peterson, B., Randolph, T. W., Manning, M. C., & Carpenter, J. F. (1997). Preferential exclusion of sucrose from recombinant interleukin-1 receptor antagonist: role in restricted conformational mobility and compaction of native state. *Proc Natl Acad Sci U S A, 94*(22), 11917-11922.

Khechinashvili, N. N. (1990). Thermodynamic properties of globular proteins and the principle of stabilization of their native structure. *Biochim Biophys Acta, 1040*(3), 346-354.

KMD. (2012). Weather And Crop Review For Dekad 03, 2012 *DEKADAL AGROMETEOROLOGICAL BULLETIN*: Kenya Meteorological Department.

Kreilgaard, L., Frokjaer, S., Flink, J. M., Randolph, T. W., & Carpenter, J. F. (1998). Effects of additives on the stability of recombinant human factor XIII during freeze-drying and storage in the dried solid. *Arch Biochem Biophys, 360*(1), 121-134. doi: 10.1006/abbi.1998.0948

Lee, J. C., & Timasheff, S. N. (1981). The stabilization of proteins by sucrose. *J Biol Chem, 256*(14), 7193-7201.

Lee., Baker, J., Bell, D., McCarthy, J., & Cheng, Q. (2006). Assessing the genetic diversity of the aldolase genes of *Plasmodium falciparum* and *Plasmodium* vivax and its potential effect on performance of aldolase-detecting rapid diagnostic tests. *J Clin Microbiol, 44*(12), 4547-4549. doi: 10.1128/jcm.01611-06

Lon, C. T., Alcantara, S., Luchavez, J., Tsuyuoka, R., & Bell, D. (2005). Positive control wells: a potential answer to remote-area quality assurance of malaria rapid diagnostic tests. *Trans R Soc Trop Med Hyg, 99*(7), 493-498. doi: 10.1016/j.trstmh.2005.01.001

Lowe, M. L., Gin, J. B., & Demetriou, J. A. (1973). Stability of erythrocytic enzymes for screening tests. *Clin Chem, 19*(5), 529-530.

Manning, M. C., Chou, D. K., Murphy, B. M., Payne, R. W., & Katayama, D. S. (2010). Stability of Protein Pharmaceuticals: An Update. *Pharmaceutical Research, 27*(4), 544-575. doi: 10.1007/s11095-009-0045-6

Martin, S. K., Rajasekariah, G. H., Awinda, G., Waitumbi, J., & Kifude, C. (2009). Unified parasite lactate dehydrogenase and histidine-rich protein ELISA for quantification of *Plasmodium* falciparum. *Am J Trop Med Hyg, 80*(4), 516-522.

McMorrow, M. L., Masanja, M. I., Abdulla, S. M., Kahigwa, E., & Kachur, S. P. (2008). Challenges in routine implementation and quality control of rapid diagnostic tests for malaria--Rufiji District, Tanzania. *Am J Trop Med Hyg, 79*(3), 385-390.

Moodley, K., Sibanda, N., February, K., & Rossouw, T. (2014). "It's my blood": ethical complexities in the use, storage and export of biological samples: perspectives from South African research participants. *BMC Med Ethics, 15*, 4. doi: 10.1186/1472-6939-15

Mouatcho, J. C., & Goldring, J. P. (2013). Malaria rapid diagnostic tests: challenges and prospects. *J Med Microbiol, 62*(Pt 10), 1491-1505. doi: 10.1099/jmm.0.052506-0

Murphy, K. P., Bhakuni, V., Xie, D., & Freire, E. (1992). Molecular basis of co-operativity in protein folding. III. Structural identification of cooperative folding units and folding intermediates. *J Mol Biol, 227*(1), 293-306.

Murray, C. K., Gasser, R. A., Magill, A. J., & Miller, R. S. (2008). Update on rapid diagnostic testing for malaria. *Clin Microbiol Rev, 21*. doi: 10.1128/cmr.00035-07

Ohtake, S., Kita, Y., & Arakawa, T. (2011). Interactions of formulation excipients with proteins in solution and in the dried state. *Adv Drug Deliv Rev, 63*(13), 1053-1073. doi: 10.1016/j.addr.2011.06.011

Parra, M. E., Evans, C. B., & Taylor, D. W. (1991). Identification of *Plasmodium falciparum* histidine-rich protein 2 in the plasma of humans with malaria. *J Clin Microbiol, 29*(8), 1629-1634.

Ravaoarisoa, E., Zamanka, H., Fusai, T., Bellalou, J., Bedouelle, H., Mercereau-Puijalon, O., & Fandeur, T. (2010). Recombinant antibodies specific for the *Plasmodium falciparum* histidine-rich protein 2. *MAbs, 2*(4), 416-427.

Roser, B. J. (1990). United States Patent: U. S. Patent.

Schuster, F. L. (2002). Cultivation of *Plasmodium* spp. *Clin Microbiol Rev, 15*(3), 355-364. doi: 10.1128/cmr.15.3.355-364.2002

Sifuna, P., Oyugi, M., Ogutu, B., Andagalu, B., Otieno, A., Owira, V., Otsyula, N., Oyieko, J., Cowden, J., Otieno, L., & Otieno, W. (2014). Health & Demographic Surveillance System Profile: The Kombewa Health and Demographic Surveillance System (Kombewa HDSS). *Int J Epidemiol, 43*(4), 1097-1104. doi: 10.1093/ije/dyu139

Subbaraman, L. N., Glasier, M. A., Senchyna, M., & Jones, L. (2005). Stabilization of lysozyme mass extracted from lotrafilcon silicone hydrogel contact lenses. *Optom Vis Sci, 82*(3), 209-214.

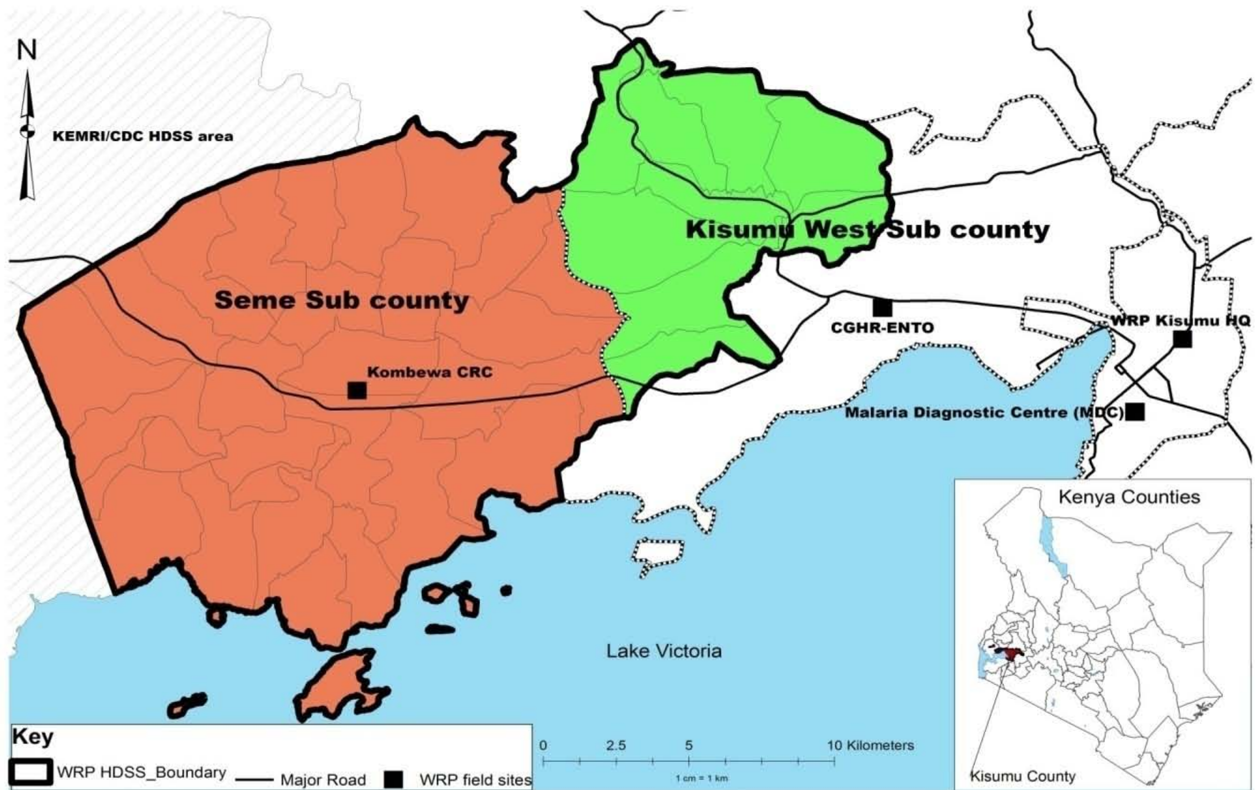
Tamiru, A., Boulanger, L., Chang, M. A., Malone, J. L., & Aidoo, M. (2015). Field assessment of dried *Plasmodium falciparum* samples for malaria rapid diagnostic test quality control and proficiency testing in Ethiopia. *Malar J, 14*, 11. doi: 10.1186/s12936-014-0524-z

Tindana, P., Molyneux, C. S., Bull, S., & Parker, M. (2014). Ethical issues in the export, storage and reuse of human biological samples in biomedical research: perspectives of key

stakeholders in Ghana and Kenya. *BMC Med Ethics, 15*, 76. doi: 10.1186/1472-6939-1576
Vagenende, V., Yap, M. G., & Trout, B. L. (2009). Mechanisms of protein stabilization and prevention of protein aggregation by glycerol. *Biochemistry, 48*(46), 11084-11096. doi: 10.1021/bi900649t
Versteeg, I., & Mens, P. F. (2009). Development of a stable positive control to be used for quality assurance of rapid diagnostic tests for malaria. *Diagn Microbiol Infect Dis, 64*(3), 256-260. doi: 10.1016/j.diagmicrobio.2009.03.012
Wang, W. (1999). Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int J Pharm, 185*(2), 129-188.
WHO. (2015a). WHO Global Technical Strategy for Malaria 2016-2030. *World Health Organization*.
WHO. (2015b). The World Malaria Report 2015. *World Health Organization*.
WHO/FIND/CDC. (2015). Malaria Rapid Diagnostic Test Performance Results of WHO product testing of malaria RDTs: round 6 (2014–2015). *World Health Organization*.
WMO. (2016). World Meteorological Organization Bulletin. *The Journal of WMO, 65*(1), 29.
Wongsrichanalai, C., Barcus, M. J., Muth, S., Sutamihardja, A., & Wernsdorfer, W. H. (2007). A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). *Am J Trop Med Hyg, 77*(6 Suppl), 119-127.
Yazdani, Y., Mohammadi, S., Yousefi, M., & Shokri, F. (2015). Preliminary Assessment of Various Additives on the Specific Reactivity of Anti- rHBsAg Monoclonal Antibodies. *Avicenna J Med Biotechnol, 7*(4), 145-150.
Zancan, P., & Sola-Penna, M. (2005). Trehalose and glycerol stabilize and renature yeast inorganic pyrophosphatase inactivated by very high temperatures. *Arch Biochem Biophys, 444*(1), 52-60. doi: 10.1016/j.abb.2005.09.014

APPENDIX

Appendix 1: Map of the study area



Appendix 2: Consent Forms

Voluntary Consent to Participate (Blood Collection)

Study Title:

Human blood collection for the maintenance of quality diagnostics in clinical and research settings

What Organizations Are Involved?

1. Centre for Clinical Research, Kenya Medical Research Institute
2. U.S. Army Medical Research Unit-Kenya (USAMRU-K)
3. Walter Reed Army Institute of Research (WRAIR)
4. Ministry of Health facilities:

a. Kondele (Obama) Children's Hospital, Kisumu, Nyanza Province
b. Kisumu West District Hospital, Kombewa, Nyanza Province
c. Kisumu East District Hospital, Kisumu, Nyanza Province
d. Kisii District Hospital, Kisii, Nyanza Province
e. Kericho District Hospital, Rift Valley Province
f. Isiolo District Hospital, Eastern Province
g. Malindi District Hospital, Coast Province
h. Alupe District Hospital, Busia, Western Province

Who is the doctor in charge of the study?

Dr. Bernhards Ogutu R., Kenya Medical Research Institute/U.S. Army Medical Research Unit-Kenya, Kisumu, Kenya P.O. Box 54-40100. Telephone 0733-812-613, 0733-333-530, 057-2022699 or 057-2022645.

Purpose of the Study

This is a research project and you/your child are one of the people from Kenya, or visiting Kenya, being asked to donate blood for the purpose of creating standardized malaria diagnostic samples (for example, malaria blood film slides) to be used to improve the quality of malaria diagnostics by teaching and testing laboratory technologists on malaria microscopy, to implement laboratory quality assurance programs, and conduct research on ways to diagnose malaria more easily, rapidly, and accurately. The Kenya Medical Research Institute - Centre for Clinical Research and the U.S. Army Medical Research Unit-Kenya (together known as The Walter Reed Project) are managing this project.

A similar study has been conducted previously by the Walter Reed Project, during which almost 60,000 slides have been made from a total of 220 blood samples from participants. These slides have already been heavily used for the training of microscopists both in Kenya and internationally. However, the majority of the samples obtained were of falciparum malaria, which is the most common form of malaria found here in Kenya. There is still need to get patients with less common forms of malaria, and it is anticipated that this current study will be able to achieve this objective

Malaria is a disease caused by a parasite which can cause fever, muscle aches, cough, headache, nausea, vomiting, abdominal cramping, diarrhea, seizures, weakness, unconsciousness, and death. Malaria is transmitted through bites from certain infected female mosquitoes. The primary way to diagnose this disease is by seeing the parasites that cause malaria in your/your child's blood under a microscope. Being able to know which malaria parasite is in /your child's blood helps doctors decide the best way to treat your/your child's illness. In many clinics and laboratories, however, this diagnostic capability either does not exist or is supported by staff with inadequate skills. This project is designed to prepare standardized malaria diagnostic samples (for example, malaria blood film slides) to be used to improve the quality of malaria diagnostics by teaching and testing laboratory technologists on malaria microscopy, to implement laboratory quality assurance programs, and conduct research on ways to diagnose malaria more easily, rapidly, and accurately.

Procedures

As a volunteer blood donor for this project, you/your child may or may not have malaria. Blood without malaria is just as important as blood with malaria because slide readers must be able to determine when blood does not contain malaria parasites just as well as determining when parasites are present. If you/your child do have malaria as determined by our tests, you/your child will be referred to the healthcare provider at the local clinic for free treatment.

You/your child will have a finger stick (or heel stick for infants) to collect the first blood sample. For this, we will wipe your/your child's finger (or heel for infants) with alcohol, and stick it with a lancet. A few drops of blood will be used to make a malaria blood smear and use a rapid diagnostic test. If you/your child is found to have the malaria parasites that meets the standards for training, we will collect a second blood sample from a vein to create the malaria blood smear slides. For this, the individual taking your/your child's blood will clean part of your arm with iodine or alcohol, apply a rubber tourniquet to the arm, and insert a clean unused needle into a vein of your arm to collect blood into a syringe. The amount of blood withdrawn from your/your child's arm will be based on your/your child's weight, or the child's weight and clinical status, with a maximum of 20 milliliters (4 teaspoons). If the vein is missed on initial attempt, the procedure may have to be repeated, but you can stop the procedure during the process without penalty or loss of any benefits to which you are otherwise entitled.

For Malaria Positive Cases

If your/your child's blood is confirmed to be malaria positive by malaria rapid diagnostic test and/or microscopy and has parasites with the desired characteristics, we will request you/your child donate a second blood sample by venous draw. Approximately 3ml to 20ml (1/2 to 4 teaspoons) of blood will be obtained during the second blood draw. The second blood sample will be used to produce malaria blood smears that can be used for our training and testing program. A very small portion of your/your child's second blood sample will be used for laboratory techniques such as Polymerase Chain Reaction (PCR) which will help confirm the diagnosis of malaria, characterize the types of malaria parasites present in your/your child's blood, and allow us to see how well these tests work.

For Malaria Negative Cases

If you are participating as a potential malaria negative case and satisfying the inclusion criteria, we will request you to donate a blood sample by venous draw. Approximately 3ml to 20ml (1/2 to 4 teaspoons) of blood will be obtained during the blood draw. The blood sample will be used to produce blood smears that can be used for this training and testing program. A very small portion of your blood sample will also be used for laboratory techniques such as Polymerase Chain Reaction (PCR) to confirm the absence of malaria parasites.

You/your child will be informed of the results of the rapid diagnostic tests and microscopy tests; however you/your child will not be informed of the results of the PCR tests.

Storage, Exportation and Future Studies

Some blood samples may be stored for future use and the stored blood samples may be used for testing and development/evaluation of emerging malaria diagnostic technologies in future, however before samples are used for testing and development/evaluation of emerging malaria diagnostic technologies or for any other purpose not explained in this form, permission will be sought from KEMRI SSC, KEMRI ERC and WRAIR IRB.

Inclusion and exclusion criteria.

Malaria negative individuals

A. You will be allowed to participate if;

1. You are a healthy adults (male or female)
2. You have a Negative blood smear for presence of malaria parasites.

B. You will not be allowed to participate if;

1. You are Unwilling to donate blood or sign an informed consent.
2. You are not capable of signing an informed consent.
3. You have a history of malaria in the past 10 years.
4. You are from a/you have been in a malaria-endemic area of Kenya or another endemic country for more than 5 days currently.
5. You are an individual who, at discretion of an investigator, would be adversely affected by donation of 3-20 mls of blood, such as anemia, history of bleeding tendency, etc.

Malaria Positive Individuals

A. You will be allowed to participate if;

1. You/your child are an individual of any age.

B. You will not be allowed to participate if;

1. You/your child are unwilling to donate blood or give informed consent, or, if you are under 18 years old, unwillingness to indicate assent (ages 12-17) and/or the unwillingness of a parent or guardian >18 years old to provide minor consent.

2. There is any contraindication to donation of 3-20 mls of blood (depending on age, clinical status, etc.), as determined by either the study clinician, or, in the case of hospital cases, their attending physician.
3. You/your child have any acute or chronic illnesses that may make blood donation compromise your health.
4. Children <18 years old without available parent or guardian >18 years old.
5. You/your child already taking medication for treatment of malaria.

Your/your child's participation and rights

Your/your child's participation as a donor is completely voluntary. You/your child can withdraw from participation for any reason and at anytime. If you/your child agree to participate, you may withdraw at any point without penalty or loss of benefits to which you are otherwise entitled. Your consent to this procedure applies to a finger stick (or heel stick for infants) and a blood sample collection from your/your child's vein. You/your child may have a finger stick (or heel stick for infants), a vein stick, or both depending on if we already know you have malaria or not and if your malaria is needed in our training sets. This document does not consent to future collection of your blood.

If you have any questions or concern about complications that arise after your blood is taken, you can call or visit our clinician at the location where your/your child's blood will be drawn and evaluated in a timely manner. The physicians who can be contacted include Dr. Bernhards Ogutu and Mr. Nickline Kuya. If you do not have phone access, the head of the clinic where your blood was drawn can help you contact them.

What your/your child's blood will be used for

Your/your child's blood (sample from your/your child's vein) will be used for the purpose stated above: creation of teaching and testing malaria blood smear slides to improve the quality of malaria diagnosis, and blood samples will be preserved by freezing or drying to confirm the microscopy and rapid diagnostic test results and evaluate diagnostic assays such as PCR. As part of this process, your/your child's blood may be used for a complete blood count; you will not be given the results of the blood count in case it is done. Your/your child's blood will not be kept for future use except for the malaria blood smear slides and the small amount of blood for confirmatory testing by PCR. Your/your child's blood will not be tested for the infectious agents of AIDS virus or hepatitis. The malaria slides and blood specimens made from the blood you donate will be maintained at KEMRI/USAMRU-K and may be given or loaned to institutions that are participating in research and malaria diagnosis training programs. Your/your child's privacy will be protected by not linking your name to the samples collected.

Duration of participation

If you/your child is seen from our clinic, the study duration will last not more than five hours from the time your consent is obtained. Sometimes we shall have to go out to seek some rare types of malaria infections. If you/your child are a participant during these surveys, we shall only take the screening samples. If you/your child are found to have the infection that meet the standards for training, we shall contact you again to inform you the test results and request

you/your child to donate the blood for making the training blood smears. This may take upto 24 to 48 hours. In either case, you are free to withdraw your participation during the process without penalty or loss of any benefits that you are otherwise entitled to.

Risks and discomforts

The risks of complications from this procedure are minimal. However, you may suffer pain at the place where the lancet or needle was inserted that may persist for a few days after blood collection. Potential complications of taking a sample of your blood from a vein include local infection, bleeding, bruising, or blood clot formation. Donation of blood is usually well tolerated, but some healthy individuals may experience transient dizziness, nausea, or mild headache. All precautions to minimize the risk of infection will be applied. In the event of infection which is rare, potential treatments might include antibiotics (by mouth or through a vein) or drainage of localized infection. The risk of bleeding significant enough to require blood transfusion is rare. The study personnel who will perform the blood draws are well trained and experienced, and will take every precaution to minimize the risks of the above mentioned complications.

Direct benefits

There is no direct benefit to you for participating in this project as a blood donor. However all study participants will be provided with their laboratory results and all participants that are diagnosed as having malaria will be referred to the healthcare provider at the local clinic for free treatment.

Indirect benefits

You/your child will potentially benefit your community and people worldwide by donating blood sample to be used in the improvement of malaria diagnosis.

Termination of participation

Your/your child's participation in the study may be stopped for reasons such as failure on your part (your child's) to follow study requirements, or if the investigator decides that it is in your (your child's) best interest to discontinue, or occurrence of circumstances that might make your (your child) participation dangerous or detrimental to your (your child's) health, or other reasons not currently known.

What Should I Do If I/My Child Has An Illness Or Injury?

If you think you/your child has a medical problem, illness, or injury related to donation of blood in this study, please report to the nearest health facility. If for some reason this is not possible, contact Dr. Bernhards R. Ogutu, Walter Reed Project; P.O Box 54, Kisumu, Kenya, or by telephone: 0733-812-613,0733-333-530,057-2022699 or 057-2022645.

Who Should I Contact For Information Or Answers To Questions Concerning The Rights Of A Research Participant?

You may contact the Chairman of the Kenya National Ethical Review Committee, c/o Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya tel.020-2722541.

Safety and Confidentiality – (who will have access to my/child's information and blood sample).

The study personnel will ensure your/your child's safety and confidentiality during and after blood donation. Your/your child's name will neither appear on the blood sample, your /your child's history sheet, nor on any of the blood smears that will be made from it. A unique identifier (site code and study number) will be assigned to each sample to avoid traceability to the individual patient or subject. The consent form and information obtained from you or your child will be stored securely at a KEMRI/USAMRU-K facility for a period of not less than 5 years after the completion of all study activities. The consent forms will be stored in locked cabinets. These will only be made available to study personnel or the KEMRI Ethical Review Committee and WRAIR Institutional Review Board, or their representatives who will be eligible to review research records as part of their responsibility to protect human subjects in research.

Compensation

You/your child will receive no money or other direct compensation for your blood donation.You/your child may be provided a small amount of food and drink at the time your/your child's blood is taken.

Medical Care for Protocol-Related Injury

Should you or your child be injured as a direct result of participating in this research project, you will be provided medical care, at no cost to you, for that injury. You will not receive any injury compensation, only medical care. You should understand that this is not a waiver or release from your legal rights. You should discuss this issue thoroughly with the principal investigator before you or your child enrolls in this study. In the event of a research related injury or if you or your child experience an untoward event, please immediately contact Dr. Bernhards Ogutu (Walter Reed Project; P.O Box 54, Kisumu, Kenya, or by telephone: 0733-812-613, 0733-333-530,057-2022699 or 057-2022645).

Number of participants.

You/your child will be one of up to 10,000 potential volunteers who will be screened to reach the target of up to 500 volunteer required for this study.

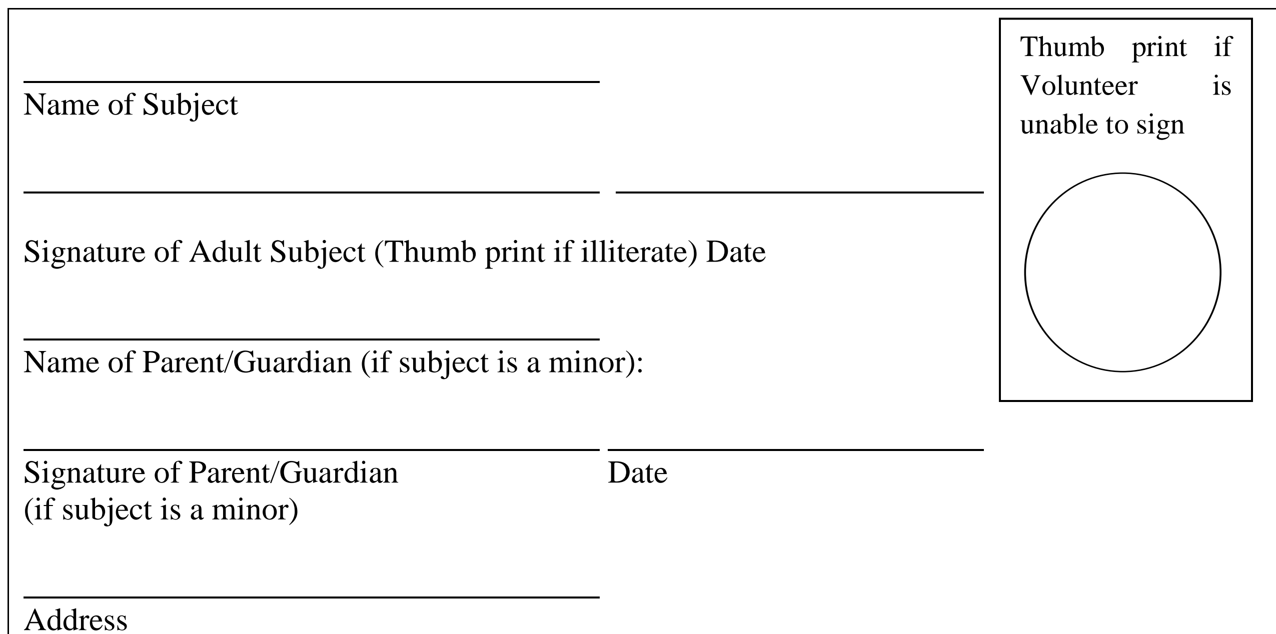
Consent for Optional Second Blood Donation

In the event that there is a problem with the blood sample collected (e.g. accidental clotting, haemolysis or spillage) we may request you (your child) to provide an additional blood sample. This is not a conditional requirement but a voluntary option and does not in any way result in penalty or loss of any benefits to which you are otherwise entitled.

Informed Consent

I hereby confirm that I have read or have had the consent information explained to me and questions related to this study have been answered satisfactorily. A signed copy of this consent document has been given to me for my personal records.

(If subject is a minor, only parents/guardians > 18 years will be eligible to sign consent forms)



Name of Subject

Signature of Adult Subject (Thumb print if illiterate) Date

Name of Parent/Guardian (if subject is a minor):

Signature of Parent/Guardian (if subject is a minor) Date

Address

Thumb print if Volunteer is unable to sign

Name of Witness (if subject not able to read or write)

Signature of Witness Date

Address

Signature of Investigator/Study Clinician Date

Appendix 3: Research Approvals

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **October 28, 2014**

TO: **DR. BERNHARDS OGUTU, PRINCIPAL INVESTIGATOR**

THROUGH: **DR. VERONICA MANDUKU, AG DIRECTOR, CCR, NAIROBI**

Forwarded 25/11/2014

Dear Sir,

RE: SSC PROTOCOL No. 2008 *(REQUEST FOR 4TH AMENDMENT):* DEVELOPMENT OF STABLE FIELD DEPLOYABLE POSITIVE CONTROLS FOR VALIDATION OF MALARIA RAPID DIAGNOSTIC TEST

This is to inform you that at the 232nd meeting of the KEMRI Ethics Review Committee held on 21st October, 2014, the request for amendment to the above referenced research proposal was discussed.

The committee acknowledges receipt of the following documents:

- Sub-protocol version 4 dated 31 July 2014
- Currently approver SSC #2008 protocol version 9.3 dated 01 November 2012
- KEMRI ERC letter accepting expedited review request for addenda to this protocol dated 06 August 2012

The Committee noted that the Sub-protocol addendum seeks to:

1. Develop cheaper quality control materials from parasitized specimen in which:
   - Activity of the target markers (HRP-2, pLDH and Aldolase) remain stable at ambient conditions for long durations
   - Distilled water is the only requirement for reconstitution
   - Utility extended to a variety of RDTs
   - Operational utility sustained in resource limited settings

2. Preparation and cataloguing of sample repository of characterized dried blood spots and tubes of different parasite densities for use as reference or quality control materials for evaluation of RDTs.

DEPARTMENT OF THE ARMY
WALTER REED ARMY INSTITUTE OF RESEARCH
503 ROBERT GRANT AVENUE
SILVER SPRING, MD 20910-7500

REPLY TO
ATTENTION OF

MCMR-UWZ-C

19 December 2014

MEMORANDUM FOR Bernhards Ogutu, MBChB, Ph.D., Walter Reed Project/Kenya Medical Research Institute (KEMRI), P.O. Box 54, 40100, Kisumu, Kenya

SUBJECT: Project Qualifies as Research Not Involving Human Subjects, **WRAIR #1720.002**

1. A determination was made that the sub-study addendum, **WRAIR #1720.002**, entitled, "Development of Stable Field Deployable Positive Controls for Validation of Malaria Rapid Diagnostic Tests (RDTs)," (Version 4, dated 31 July 2014), being conducted under the approved minimal risk human subjects research protocol WRAIR #1720, entitled "Human Blood Collection for the Maintenance of Quality Malaria Diagnostics in Clinical and Research Settings," (Core Protocol Version 9.3, dated 1 November 2012), does not require review by the Walter Reed Army Institute of Research (WRAIR), Institutional Review Board (IRB), in accordance with WRAIR Policy Letter #12-09, as this project involves the analysis of anonymized malaria blood samples obtained under WRAIR #1720. The study personnel do not have access to the link and there is no identifiable human subjects data being provided with the samples. Therefore, this project does not meet the definition of research involving human subjects, and 32 CFR 219 does not apply.

2. The main objective of this sub-study is to develop cheaper quality control materials from parasitized specimens in which: (1) activity of the target markers (HRP-2, pLDH, and Aldolase) remain stable at ambient conditions for long durations; (2) distilled water is the only requirement for reconstitution; (3) utility extended to a variety of RDTs; and (4) operational utility sustained in resource limited settings. In addition, a sample repository of characterized dried blood spots and tubes of different parasite densities will be prepared and catalogued for use as a reference or quality control materials for evaluation of RDTs.

This sub-study will use 25-50 samples of *P. falciparum*, *P. malariae*, *P. ovale*, and mixed infections of *P. falciparum* and *P. malariae* and *P. falciparum* and *P. ovale*.

A database with samples only identified by study identification number, the age of the volunteer, the date and time the sample was collected, RDT results, lot numbers, expiration dates, Giemsa microscopy results, and polymerase chain reaction results where necessary, is to be maintained by the KEMRI/U.S. Army Medical Research Unit – Kenya (USAMRU-K).

3. The KEMRI Ethical Review Committee (ERC) approved this sub study addendum (Version 4, dated 31 July 2014) as minimal risk on 28 October 2014.

4. Per the WRAIR Education Policy Letter #11-49, all individuals covered under the WRAIR Human Research Protection Program (HRPP) are required to complete Collaborative Institutional Training Initiative (CITI) training; documentation of completed CITI training (modules 1-4) have been provided for the Principal Investigator (PI) and key research personnel.

5. Funding for this project will be provided by the Military Infectious Diseases Research Program L0324_13_WR_OC "Development of Stable Field Deployable Positive Controls for Validation of Malaria Rapid Diagnostic Tests."