VALIDATION OF A BIOMEME SMARTPHONE BASED-DNA REAL-TIME PCR ASSAY FOR DIAGNOSIS OFHUMAN MALARIA AT THE POINT-OF-CARE

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Validation of a Biomeme smartphone Based-DNA Real-Time PCR Assay for Diagnosis ofHuman Malaria at the Point-of-Care

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DECLARATION

This thesis is my original work and has not been presented for a degree award in any other university.

Signature……Date ……

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.

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

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DEDICATION

I dedicate this work to all individuals who have provided me with advice and resources for this research and to the scientists who have supported me in carrying out this project.

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May glory and majesty be unto the Almighty God for giving me the opportunity, wisdom, knowledge and health to finish this project.

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LIST OF ABBREVIATIONS

ACC	Automated Blood Cell Counter
ABI	Applied Biosystem Instrument
BED	Biomeme Elution Buffer
BWB	Biomeme Wash Buffer
BPW	Biomeme Protein Wash
BLB	Biomeme Lysis Buffer
CRF	Case Record Form
DhP	Dihydrogenasepiperaqiune
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
ELISA	Enzyme-Linked Immunosorbent Assay
EDTA	Ethylenediamineteraacetic acid
GuSCN	Guanidium thiocynate
Hb	Hemoglobin
HRP	Histidine Rich Protein
LAMP	Loop Mediated Isothermal Amplification
LDMS	Laser Desorption Mass Spectrometre
KEMRI	Kenya Medical Research Institute
MS	Mass Spectrometer
MSP1	Merozoite Surface Protein 1
mM	milimolar solution
MoH	Ministry of Health
nM	nanomolar solution
PCR	Polymerase Chain Reaction
PLDH	Plasmodium Lactase dehyhrogenase
QBC	Quantity Buffer Coat
RDT	Rapid Diagnostic Test
RBC	Red Blood Cell

ABSTRACT

Malaria poses a diagnostic challenge in the majority of developing countries due to financial constraints. In 2017, there were approximately 219 million new cases of malaria, with over 435 000 deaths worldwide. Children under the age of five were the most affected, accounting for 70% of all cases. In some African countries with high transmission rates, a large proportion of the population is infected but asymptomatic, and the patients have developed enough immunity to protect them from malaria disease but not from infection. Cost and ease of diagnostic performance are major considerations in these countries. While microscopy and other modern malaria diagnosis methods are available, the development of an accurate, sensitive, and cost-effective rapid diagnostic tool would go a long way toward alleviating those challenges. This study evaluated the ability of a portable real-time PCR (smartphone-based real-time) assay tool from Biomeme Inc. to provide better diagnostic capability than existing methods. The study was conducted in Western Kenya from April 2016 to February 2018. Patients aged two and above who presented with malaria symptoms, such as an axillary temperature of 37.5°C or a history of fever 48 hours prior to admission to the health facilities, were recruited. The consent of the 315 respondents, including children who had their parents or legal guardians sign for them, was required. The initial parasitemia had to be in the range of 1,000 to 200,000 parasites per μL of blood. Patients with severe anemia and malaria were excluded, as were those with a hemoglobin level of 5 g/d, the presence of other diseases that cause febrile conditions, the presence of any other *Plasmodium* species other than *falciparum*, and patients witha history of adverse events with ACT and sulphonamide drugs. The Biomeme sample preparation kit was used to extract DNA, which was then compared to the Chelex method. The concentration, purity, and turbidity of the DNA obtained using the two extraction methods were measured using a NanoDrop spectrophotometer. Amplification of two *Plasmodium falciparum* gene markers, MSP1 and 18S rRNA, was used to determine the detection limits of the DNA obtained from Biomeme and Chelex. The Student's t-test was used to compare the means of the two methods, and the level of agreement was determined using a Kappa statistic value. The results of parasitemia densities effect on heamoglobin concentration showed that, the higher the parasitemia density the lower the heamoglobin concentration were. The age factor was also an influential indicator given that; the elderly were having comparatively lower heamoglon concentration with corresponding increase of parasitemia as compared to young ones. When compared to the Chelex method, the Biomeme sample preparation kit produced the highest yields and concentrations of DNA. Thus, the Biomeme yielded a score of (33.39mg±8.36), whereas Chelex yielded a score of (7.6mg±2.48). The 18S rRNA amplification detection results on Biomeme and Chelex amplicons were as follows: sensitivity (97.44±0.71 percent, 94.44±0.53 percent), specificity (50±0.71, 60±0.53 percent), and Kappa value (0.473, 0.544) respectively. MSP1 amplifications had the following results: sensitivity (97.370.49 percent, 91.67±0.43 percent), specificity (67±0.49 percent), and Kappa value (0.6401, 0.4755) respectively with $p<0.05$. On the other hand, the genetic diversity analysis showed that MSP1 and Biomeme amplicon had higher number of strains as compared the Chelex ones. In addition, 18S rRNA had lesser number of the predictive strains in comparison to MSP1 while using both DNA amplicons from Biomeme and Chelex. The amplicon intensities of Biomeme were

clearly higher than those of Chelex. Biomeme real-time PCR had relatively lower CT values as compared ABI 7500 real-time PCR. In this project study, it was incident that the method of DNA extraction, genomic marker and the platform of amplification had a considerable impact on the result. Despite the turbidity ratio, which indicated that it contained some chaoatrophic salts, these findings suggest that Biomeme was a rapid and accurate diagnostic technique for malaria that can be used at the point of care.

CHAPTER ONE

INTRODUCTION

1.1 Background information

There were approximately 384 000 deaths caused by malaria in the year 2015 on the African continent (World Health Organization, 2016). Children under the age of five are the most affected with mortality at 70% of the total malaria deaths (World Health Organization, 2016). In some African countries where transmission rates are high, a phenomenon has been noted whereby a large proportion of the population is infected but it still remains asymptomatic (Murphy *et al.,* 2014; Medeiros *et al.,* 2013). Such patients have developed sufficient immunity to protect them from malarial disease, but not from the infection (Oliveira-Ferreira *et al.,* 2010). In such situations, finding malaria parasites in a febrile patient does not necessarily mean that the disease is caused by the parasites (World Health Organization, 2016). Further investigation would be required to identify the cause of disease. In many malaria-endemic countries, the lack of resources is a major barrier to reliable and timely diagnosis. Therefore, there is a need for prompt, sensitive, specific and accurate diagnostic methods for proper patient management. These effective and practical diagnostic methods will be of great impact since they will reduce the number of cases that go undiagnosed (World Health Organization, 2006). Although microscopy is the gold standard for malaria diagnosis, it is relatively laborious when large quantities of samples are to be processed and requires expertise. Furthermore, with a detection limit of 20parasites/µl this method is not sufficiently sensitive. The low sensitivity of microscopic technique for malaria diagnostics has a considerable negative impact on malaria control (Alves *et al*., 2005).

Recent advances in technology have led to the development of rapid diagnostic tests (RTDs) (Moody, 2002; Markler *et al*., 1998), which are easier to use, but challenges concerning sensitivity, specificity and quantification still remain unresolved (Moody, 2002). One powerful diagnostic technique for malaria is use of Polymerase Chain Reaction (PCR). This technique can detect drug-resistant parasites, mixed infections, and is amenable to automation in processing large numbers of samples (Swan *et al.*, 2005). It is a more sensitive method in the diagnosis of malaria than quantitative blood

count (QBC), RDT assays and convectional microscopy (Morrasin *et al*., 2002)**.** However, some PCR diagnostic methods face a number of challenges namely; need for reliable electricity supply as well as the need for complex instruments and well-developed laboratory infrastructure (Yang and Rothman, 2004). It is also labor-intensive, is hazardous especially if ethidium bromide, a carcinogenic DNA intercalating agent is used as part of detection process. Moreover, DNA gel reading necessitates use of ultraviolet rays. Light of this non-ionizing wavelength can lead to DNA damage hence potential carcinogenesis. Fortunately, some of the shortcomings of PCR can be remedied by using quantitative real-time PCR (qRT PCR). The advantages of QRT PCR include potential use of follow-up patients for malaria re-infection and recrudescence studies. Here, we validated an improved, rapid, simpler, portable, genus-specific, cost-effective smartphone-based real-time PCR assay method based on the *P. falciparum* 18S rRNA gene that can be used at the point-of-care (Lee *et al*., 2002) for accuracy, sensitivity and specificity. This technology from Biomeme Inc. was compared to conventional PCR methods for limits of detection using the same set of primer sequences

1.2 Statement of the problem

Malaria remains a major cause of mortality and morbidity with about 229 million infections resulting into about 409 000 deaths globally every year with 94% of these infections in Sub-Saharan Africa (WHO, 2017). In the past decade, malaria control efforts have focused on scale-up successful interventions including use of insecticide treated bed nets, indoor residual spraying, rapid diagnostic tests and artemisinin-based combination therapy (Sutherland, C. J., *et al*. 2010; Noedl *et al*.,2009). Yet, there are still areas where malaria control is on the decline and risk of transmission increases (Beck *et al*., 1997). These areas are the major contributors to the overall increasing number of malaria cases. However, increasing prevalence of drug resistant strains of parasites, and in a relatively few cases, massive increases in international travel and migration also contribute to the increasing trend in global malaria infections (Pasvol, 2005). On the other hand, there has been increase in malarial infections much effort has been put to curb this situation. The WHO Global Technical Strategy for malaria 2015-2030 provides support for malarial endemic countries by providing guidance,

regional support and country programs towards malarial control and elimination. There are goals that have been setup by this body and these include: reducing malaria cases incidences by at least 90% by 2030, reducing malaria mortality rates by at least 90% by 2030, eliminating malaria in at least 35 countries by 2030 and preventing a resurgence of malaria in all countries that are malaria-free. Secondly, accelerating efforts towards elimination and attainment of malaria- free states and finally is transforming malaria surveillance into intervention. The WHO Global Malaria Program (GMP) coordinates WHO's global effort to control and eliminate malaria by; setting, communicating and promoting the adoption of evidence-based norms, standards, policies, technical strategies, and guidelines, keeping independent score of global progress and developing approaches for capacity building, systems strengthening, and surveillance and finally identifying threats to malaria control and elimination as well as new areas for action.

The need for effective and practical diagnostics for global malaria control is thus increasing. Effective diagnosis reduces both complications and mortality caused by malaria. Differentiating malaria from other tropical infections, based solely on patients' signs and symptoms or physicians' findings is difficult (WHO, 2006) and conclusive diagnoses cannot be made.

In remote settings, clinicians are forced to rely on clinical diagnosis based on symptoms rather than on the more accurate biological diagnostic tests. This is largely due to the high cost of adequate laboratory infrastructure and lack of trained personnel. Thus, in these resource limited settings there is a need to have a simple, rapid, accurate and portable diagnostic method. The success of malaria treatment depends on three factors namely identifying the infecting species, the clinical status of the patient and the drug susceptibility of the infecting parasite as determined by the geographical area where the infection has been acquired and previous use of antimalarial medicines, the latter provides information on the likelihood of drug resistance. This will enable the clinicians to choose an appropriate drug or drug combination and the treatment course.

1.3 Justification

Malaria diagnosis can be done with: microscopy, rapid diagnostic tests,

Immunofluorescence microscopy, Enzyme-Linked Immunosorbent Assay (ELISA), microarray, mass spectrometry, flow cytometry, automated blood cell counter, serological tests, Quantitative Buffy Coat (QBC), or loop mediated amplification (LAMP). On the other hand, Polymerase chain reaction (PCR) method (nested, real-time or reverse) has become available. However, these methods face a lot of challenges for example; expensive, unreliable in diagnosis, unpopular, need lab infrastructure and electrical power supply. The Biomeme smartphone-based DNA detection platform (Biomeme Inc. USA) is an assay for malaria diagnosis which is a real-time PCR. Since it combines the qualities of RTDs (affordability and ease of use) and the sophistication of molecular techniques of PCR, it is well suited for the clinical settings of the developing world. Thus, this assay for diagnosis of malaria was validated as a point-of- care application in resource limited areas.

1.4 Research questions

1. Is DNA extracted by Biomeme prep-kit superior to Chelex method?
2. Is the limit of detection influenced by the quality of the DNA and the method of amplification?
3. Is genetic diversity analysis affected by DNA extraction method, primers andamplification method?
4. Does parasitemia have an effect on Hb concentration?

1.5 Objectives

1.5.1 General objectives

To validate of a Smartphone-Based DNA Real-Time PCR Assay for Diagnosis of Human Malaria at the Point of Care

1.5.2 Specific objectives

1. To compare the quality and quantity of the DNA from of Biomeme sample preparatory kit to Chelex DNA extraction protocols.
2. To determine how variations in parasitemia densities affected the hemoglobin (Hb) mean in respect to different age groups.

3. To establish the limits of detection using Biomeme Smartphone-based DNA detection assay and compare them to those of conventional PCR, nested PCR and ABI 7500 real-time PCR assays for malaria diagnosis
4. To evaluate genetic diversity of the *Plasmodium falciparum* strains, presentin Nyando sub-county and to access the impact of genomic marker and DNA platform of extraction on the level of detection.

1.6 Hypothesis

Alternative hypothesis: Biomeme Smartphone-based DNA detection assay can be used to diagnose malaria parasite to the limit that is comparable to conventional and real-time PCR assays and can be used for detection of at point of care.

Null hypothesis: Biomeme Smartphone-based DNA diagnosis platform is inferior to the conventional and real-time PCR assays and cannot be used in the detection of malaria at point of care.

CHAPTER TWO

LITERATURE REVIEW

2.1 Burden of malaria disease

High malaria transmission in the world occur mostly in Africa south of the Sahara, where *P. falciparum* being the predominate with an estimated 90% of the deaths attributable to malaria worldwide. On the other hand, high transmission also occurs in other areas of the world for example Papua New Guinea, however, and not all endemic areas in Africa south of the Sahara are characterized by high rates of transmission. In 1999, it was estimated that there were approximately 261 million cases of malaria in areas with high transmission which translated into 87% of the global total of 300 million and 870 000 deaths (87% of the global total of >1 million) (WHO.,1998).

Malaria is currently considered to be a major global public health problem with high morbidity and mortality. Contrarily to being preventable and curable, malaria continues to have a distressing impact on people's health around the world. Almost half the world's population, living in nearly 100 countries and territories, are at risk of malaria (WHO, 2015)

Research showed that in malaria-endemic countries, severe malaria, infection associated with end organ damage, was more common in children under 5 years old compared with older children and adults, which affects local children's growth a lot.

This high burden may in fact be partly a result of misdiagnoses, since many facilities lack laboratory capacity and it is often difficult clinically to distinguish malaria from other infectious diseases. Nonetheless, malaria is responsible for a high proportion of public health expenditure on curative treatment, and substantial reductions in malaria incidence

Child mortality rates are known to be higher in poorer households and malaria is

responsible for a substantial proportion of these deaths. In a demographic surveillance system in rural areas of the United Republic of Tanzania, under-5 mortality following acute fever. Furthermore, it has been shown that repeated malaria infections make young children more susceptible to other common childhood illnesses, such as diarrhea and respiratory infections, and thus contribute indirectly to mortality (Molineaux L.). In addition, an overwhelming acute infection, which frequently presents as seizures or coma (cerebral malaria), is likely to kill a child directly and quickly. Likewise, repeated malaria infections is likely to contribute to the development of severe anaemia, which substantially increases the risk of death. Besides this, malaria burden is associated with low birth weight as frequently the consequence of malaria infection in pregnant women is a major risk (Steketee RW *et al.*, 2001).

2.2 Diagnostic methods for malaria

Malaria diagnosis process comprises; identification of malaria parasites, antigens or products in patient's blood. Even though this may appear modest, the analytical efficacy is subject to other many factors. Such factors are comprised of: various forms of the five-malaria species, dissimilar stages of erythrocytic schizogony, endemicity of various species, interrelatedness between levels of transmission, people's movement. In addition, parasitemia, immunity, signs and symptoms, drug resistance, problems of recurrent malaria are related factors to this problem. Furthermore; persistent viable or non-viable parasitemia, sequestration of the parasites in the subterranean tissues, use of chemoprophylaxis or unfluctuating presumptive treatment on the ground of clinical and biological diagnosis. All the above-mentioned factors can impact the identification, understanding and identification of malaria parasitemia in a diagnostic trial test (Bell *et al*., 2005).

The global harm of malaria has spurred interest in developing effective diagnostic strategies not only for resource-limited areas where malaria is a substantial burden on society. Delays in diagnosis and treatment are leading causes of death in many countries. Diagnosis can be difficult in areas where malaria is no longer endemic: healthcare providers may not be familiar with the disease. Clinicians may omit the use

of test necessary to diagnose malaria. And laboratory technicians may be unfamiliar with, or lack experience with malaria and fail to detect parasites when examining blood smears under a microscope (Reybum *et al*., 2007).

In some areas (such as Africa), malaria transmission is so intense that a large proportion of the population is infected but remains asymptomatic. Such carriers have developed sufficient immunity to protect themselves from malarial illness, but not from an infection. In such situations, finding malaria parasites in an ill person does not necessarily mean that the illness is caused by the specific type of the found parasites. In many malaria-endemic countries, the lack of resources is a major barrier to reliable and timely diagnosis. Malaria diagnostics can be classified into two groups, namely clinical and biological methods. Clinical methods are also known as the traditional methods and are based on symptoms and external conditions of the patient. These methods, however, are non-specific due to symptoms of malaria overlapping with other viral and bacterial infections. Diagnosis based on clinical methods therefore result in indiscriminate use of antimalarial drugs due to misdiagnosis and over- diagnosis. These methods of clinical diagnosis use early non-specific symptoms of malaria including, vomiting, abdominal pain, nausea, weakness, myalgia, headache and chills. The biological methods, which are the most-commonly used include microscopy, immunofluorescence antibody assay, ELISAs, Rapid Diagnostic Tests (RDTs) and PCRs.

Microscopy can be done in two ways: thin smear and thick smear. The thin smear method is used for morphological studies and thick smear is used for parasitemia diagnosis. The smear can be stained by Giesmsa, Wrights or Field stains (Bejon *et al*., 2006). This method is inexpensive, absolutely accurate, and field-friendly. Furthermore, the smear slides can be used to provide permanent diagnostic record.

However, the smear method requires experts and power source and has low detection limits. It is also important to note that usually, the washing process of thick smears affects the quantification and sensitivity of the results.

The RDT detection principles are based on the level of antigens produced through parasitic pathways. These antigens include histidine rich protein 2 (HRP–2),

plasmodium lactase dehydrogenase (pLDH) and plasmodium aldolase (Laurent *et al.*, 2010). Currently, there are about 86 RDTs produced by 28 manufactures (WHO, 2008). These tests are cheap, sensitive, simple to operate, field-friendly (no infrastructure is required), and also provide rapid results (Jelinek *et al*., 1999; Hanscheid, 1999). However, these tests are unstable in humid conditions, less sensitive also to *P*. *vivax* than other tests and affected by persistence of antigen. Most importantly, they do not give quantitative results and require constant monitoring (Wongsrichanalai *et al*., 2007; Wilson *et al*., 2008). In addition, mutation and deletions in HRP-2 may result into false negative.

Serological assays are based on the detection of antibodies against the asexual blood stage of the *Plasmodium*. The method is antigen-specific for immunoglobin-M (IgM) and IgG of the patient. One of the commonly used assays is immunofluorescence antibody testing, which is useful in epidemiological surveys and involves screening of the blood that normally discloses recent infections in non-immune patients.

The assay is simple, sensitive and suitable for large samples. However, it requires expertise and is not automated. Therefore, it is difficult to compare its results to those of other diagnostic methods. Further, the serological method cannot be used for an acute diagnosis where quantification is required (Eibach *et al*., 2013).

The polymerase chain reaction (PCR) method has been widely used for detection of malaria infection, determination of a therapeutic response follow-up and for identification of drug resistance of the parasite (Lima *et al*., 2010; Coleman *et al*., 2006). Polymerase chain reaction can detect as few as 1 to 5 parasites/µl of blood (≤ 0.0001% of infected red blood cells) compared with around 50-100 parasites/µl of blood by microscopy or RD. Moreover, PCR can help detect drug-resistantparasites, mixed infections, and may be automated to process large numbers of samples. It has been found to be more sensitive at diagnosis of malaria parasites than quantitative buffy coat, some RDTs assays and even, convectional microscopy (Morrasin *et al*., 2002)**.** Another technique that has been employed for diagnosis of the malaria parasite is quantitative buffy coat (QBC). This technique involves the use of fluorescence dyes for staining the DNA. During the staining process that uses acridine orange dye, the

parasite's nucleus becomes bright green while its cytoplasm glows yellow-orange. The QBC technique is rapid, field-friendly, reliable and sensitive. However, just like PCR, QBC is expensive, requires sophisticated equipment (in this case fluorescence microscopes) and does not give quantitative results (Ochola *et al*., 2006).

Loop-mediated amplification of DNA (LAMP) is another technique used for routine screening of malaria in endemic regions. It is normally employed to detect *P.falciparum* by using 18S rRNA for detection of conserved and variable genomic region of this parasite. This method is sensitive not only to *P.falciparum* but also to other species, for example *P.vivax, P.malariae* and *P.ovale.* Even though this method is simple, inexpensive, reliable and rapid, the reagent storage requires cooling (Poon *et al*., 2006).

Southern blotting hybridization, in this method the DNA fragments are separated by gel electrophoresis. DNA which has been denatured is transferred to the nitrocellulose paper and then labeled with probes for the hybridization to take place. Washing off the unbounded probe is done and finally autoradiography. This method has the following advantages; able to detect multiple homologous genes in a genome, able to detect orthologous or paralogous genes in a similar or distant species, easier to multiplex/ detect multiple products and lastly time effective (Devrim, 1975). However, on the other hand this method has the following shortcomings; more expensive, labor-intensive, time consuming and requires large amount of the target DNA complex. Microarrays is another diagnostic method that is completely parallel to the traditional Southern blotting hybridization and this method is done by extracting and purifying of DNA from cells. Its application principle is based on labeling a target gene and subsequentlyseparating it from the nucleus in the sample. The nucleus is caught onto probes on thearray and enables probing of a number of genes in a single experiment. One of the microarray technique commonly used is the pan-microbial oligonucleotides techniquefor diagnosis of infectious diseases and the *P.falciparum* parasite (Palacios *et al*., 2007).This technique is accurate, automated and sensitive, but also expensive and requires expertise.

Another malaria diagnostic method is flow cytometry which detects hemozoins which

is usually produced when intra-erythrocytic plasmodium digest host's hemoglobin and subsequently crystallizes hemozoin into the acidic vacuole. This method has sensitivity of 49-98% and specificity of 82-97% (Evers *et al.,* 2008).

Mass spectrometry is a novel method for plasmodium detection with sensitivity of 10 parasites per µl of blood. During the diagnosis the sample is washed and then illuminated by laser desorption mass spectrometry (LDMS). It uses specific bio-markers which are equivalent to hemes from hemozoin of the parasite.

Some of the above-mentioned methods are not suitable for malaria diagnosis in resource- limited environments, because they are either equipment-intensive and/or require qualified personnel, which is currently not available in these areas. Rather than training personnel and obtaining the equipment, it is more cost-effective to use a simpler and more affordable method, such as the Biomeme smartphone-based DNA real- time PCR assay (Gadia *et al.,* 20082010).

2.3 Biomeme Smartphone-based DNA real- time PCR assay

Biomeme assay apparatus (Philadelphia U.S.A.) is a portable real-time PCR instrument. The assay has three components: hardware, sample prep-kit and a docking slot for a Smartphone. The hardware is connected to an iPhone (through Bluetooth technology. This system weighs 0.45Kg and has specific measurements (7.7x7.7x17.8cm) or (3.03 x3.03x7.01 inches). The sample prep kit is corded red, yellow and green for easy operation. It has a syringe for collecting the sample that works under the Boom technology (Boom *et al.,* 1990).

2.3.1 The blood DNA sample kit

This extraction kit does not require: alcohol precipitation, incubation, phenol, or chloroform. However, the kit uses Boom technology to bind the RNA/DNA (Boom *et al.,* 1990). It has the following solutions: Biomeme Lysis Buffer (BLB), Biomeme Protein Wash (BPW), Biomeme Wash Buffer (BWB) and lastly Biomeme Elution Buffer (BEB). The kit has also single-use-1mL syringe. The whole testing is done within 2-5 minutes with an RNA concentration of 5µg/ml (www. biomeme.com,

2015).

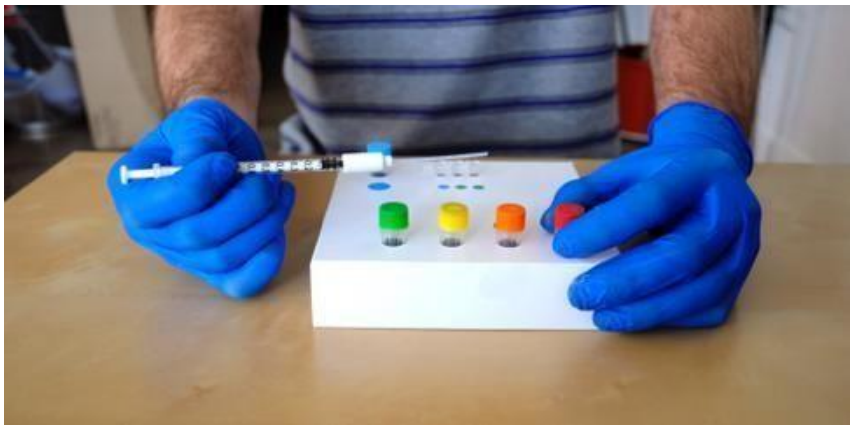


Figure 2.1: Biomeme sample preparation kit

The above shows the DNA preparation kit which is coded with different colours on the buffer tubes (Biomeme Inc. Philadelphia US).

2.3.2 Biomeme boom technology

The Biomeme syringe works on the principle of the boom technology: the nucleic acids bind to the silica particles or the diatoms in presence of chaotropic agents. Chaotropic agents' anion in aqueous solution disturbs the water structure and weakens the hydrophobic interactions (Boom *et al.,* 1990).. The agents are able to alter the secondary, tertiary and quaternary structures of the proteins and nucleic acids with no or minimal alteration ofthe primary structures. The agents increase the entropy of the system and thus interferewith the molecular interactions that are mediated by the non-covalent bonds forexample hydrogen bonds and the van der Waal forces of attraction (Boom *et al.,* 1990).

2.3.3 Biomeme one$_3$ control application systems

Biomeme application systems allow the operator to control the Smartphone- based DNA detection platform assay. When any steps are missed during the diagnostic test,

the audio and visual system of the application reminds the operator/user what to do next. The application system is dual – user and developer. The user application works with field tests and this provides the diagnostics in the following forms: positive, negative, inconclusive and incomplete results. The developer application has advanced features that help in programming protocols and assessment of raw data (in the form of amplification plots, amplification curves and CVS files (www. biomeme.com, 2015).

2.3.4 Biomeme settings and application system

Settings on the machine have the following components: "account management" which enables one to log into Biomeme account, "account information" is used for allowing the assessment of the user's results in the web portal, "protocol management" is used for seeing and cycling the parameters of the protocols stored by the user, "GPS locations" helps in adjusting the desired geological settings, the "data recovery" allows the user to recover lost results from the previous last 3 experiments performed on the phone (www. biomeme.com, 2015).

Figure 2.2: Biomeme settings and application system

2.3.5 Starting an experiment application

To start an experiment, the application allows the user to select the test protocol and also add a new protocol for the experiment being done. This screen has the following parts: Experiment ID (which allows the user to have unique cording system for the identification of his/her experiment from the rest), project (that allows having an identifier from a group of experiments) and location (this can be done automatically by the GPS when enabled, or set manually). The "note" part allows one to make further comments on the experiment or write any other subsidiary information. The protocol part contains the previously experiments protocols loaded on the app.

2.3.6 Adding a new test protocol: multiplex

To add this option, one needs to click on the new protocol above and then choose "multiplex." This will assign a label and a colour channel to each target. To enter the unknown sample, type the word "unk" on "enter target name. To assign the positive control, select the appropriate positive and type "pos" for colour channel. Similarly, to select a negative control, select the negative and type "neg" for colour channel(www. biomeme.com, 2015).

2.3.7 Experiment and Results application

This application screen has the following components: "experiment ID," "time remaining, "current step of reaction, "Two$_{3,}$" "device battery remaining" and "number of cycles completed." the "result application screen" will automatically appear when the experiment is over. Plotting screen will show raw data, baseline data, quantification cycles and additional notes. The application screen will at first display the baseline data to view the raw data. To switch to the desired data section, user has to tap the appropriate circle on the left-hand side(www. biomeme.com, 2015).

2.4 DNA extraction

The choice for nucleic acids extraction method is one of the most significant steps influencing the accuracy and sensitivity of parasitic infection diagnosis and parasitemia determination. One of the important factors while selecting and determining an extraction method for DNA extraction is its reproducibility (Tan and Yiap2009). Regardless of the type of method deployed for DNA extraction, one or more of the following processes are included: chemical lysis, physical interference and/or enzymatic lysis (Miller *et al.*, 1999). These processes should guarantee that sufficient amounts of high molecular weight DNA are extracted with minimal inhibitors and the extract should reflect an accurate depiction of the total parasite diversity within the sample (Yeates *et al.*, 1998).

The utility of DNA analyses in molecular research depends largely on its purity, stability and genomic integrity (Aliyu *et al*., 2013). The purity of DNA is important

because impurities compromise accuracy, consistency and reproducibility of results. Using alternative approaches for purifying DNA may lead to improved sensitivity and accuracy of surveillance and diagnosis data, which may promote more timely treatment and the overarching goal of eradication of malaria. DNA extracted for molecular biological research should be pure (Aliyu *et al*., 2013). Moreover, to be broadly applicable, the protocols for isolating DNA from the cellular matrix should be simple, affordable and produce DNA of good yield and quality. The assay method should also be rapid and reliable (Aliyu *et al*., 2013). Other desirable qualities are that the assay method should be practical, free from contamination and toxicity, and lead to minimal DNA fragmentation (Barea *et al*., 2004; Aidar and Line, 2007; Yang *et al.*, 2008). Some DNA extraction protocols, while meeting a number of the above criteria, are undesirable as they utilize hazardous chemicals, such as phenol, chloroform, cetyltrimethylammonium bromide (CTAB) and isoamyl alcohol (Ahmadikhah, 2009; Sun *et al.*, 2010; Ferdous *et al*., 2012; Roychowbury *et al*., 2012; Mutou *et al*., 2014).

In addition, to this procedure is that it is time-consuming. Moreover, DNA extracted by this requires further purification to avoid inhibition of PCR analyzes (Demeke and Jenkins, 1977-1990, 2010).

One of the methods for extraction of plasmodium DNA extraction is chemical- and matrix-based which can be either in the form of silicate matrix or cellulose matrix. The silicate method is in-expensive and has an increased sensitivity for multiple infections (Foleny, 1922; Sultan *et al.*, 2009; Henning *et al*., 1999). This technique also provides high-purity DNA, is easy to perform, and also is able to reproduce quantitatively as well as qualitatively. In addition, the technique has a demerit of being unable to recover small fragments of DNA efficiently, as small fragments bind tightly within the silica matrix (Green and Sambrook, 2012). Furthermore, with this method DNA deteriorates gradually if stored for a long period of time (Sultan *et al*., 2009). On the other hand, the cellulose method is easy to use and storage but its disadvantageous in that the process is complex while dealing with dilute sample which results into errors (Burgoyne, 1996). The Instant Gene method is perceived to have an advantage over the Chelex method, in that it can use 20 µl of the DNA template while the Chelex method uses 10 µl, above which it inhibits PCR amplification. In addition, the Chelex

method requires two heating cycles, making it a more labor-intensive method (Cox-Sigh *et al*., 1997; Strøm *et al*., 2014). The Instant Gene method gives good DNA yield (Verom *et al*., 2006).

The guanine isothiocyanate method of DNA extraction is lengthy and, therefore, time-consuming, with an added risk of DNA cross-contamination owing to the high number of steps involved (Henning *et al*., 1999). The major drawback associated with this

method is that phenol and chloroform are both hazardous chemicals (Chomczynski and Sacchi, 2006). The Tris-EDTA method produces good-quality DNA with sensitivity ranging from 93% to 100%; however, the DNA has been found to have poor performance (Berezkyl *et al*., 2005; Miguel *et al*., 2013; Strøm *et al*., 2014). Tris-EDTA may not be compatible with the host of nucleases and other enzymes and compounds released during lysis that do not normally come into contact with DNA and may react with it. It is vital to realize that inactivation is an equilibrium condition, and that Mg^{2+} will switch between being chelated to Tris-EDTA and being made available to the nucleases. DNA extraction from parasite-infected red blood cells can be done using the isotachophoresis method, which is good for the production of microfluidic parasite DNA (Manshall *et al*., 2011). One of the demerits associated with this method is its low limit of detection of 500 parasites/µl, which is not suitable for clinical diagnosis when the parasitemia level is very low. The microwave irradiation method has been found to be very efficient; the DNA extraction process takes fewer than 3 minutes and is easy to perform, fast and cost-effectively (Port *et al.*, 2014). However, this method cannot be used in the field whereby resources like electricity are lacking thus making its less suitable when compared to the Biomeme extraction kit method.

Other commercial extraction kits have also been deployed in DNA extraction, e.g., the QIAamp DNA mini kit is widely used (Strøm *et al*., 2014). This kit has been found to produce better results than the Chelex (Cnops *et al.*, 2010). The GentraPuregene Blood Kit has been shown to produce good-quality DNA, (Qiagen, 2015). The Promega Wizard Genomic Purification Kit has been used as the standard for other protocols for DNA extraction from *plasmodium* (Miguel *et al*., 2013).

2.5 Genetic diversity of *Plasmodium falciparum*

A number of factors affect genetic diversity, namely: mutation; natural selection; gene flow; and genetic drift (Hartl *et al*., 1997). Studies carried out in Western Kenya on *Plasmodium falciparum* using eight microsatellite loci indicated that the level of diversity was high (Gabriel *et al*., 2010). The same study showed that after insecticide spraying, a drastic decrease in the disease prevalence occurred but there was no effect on the level of multiple infections. Studies done in the Amazon region of Peru showed that multiple infections increased, in contrast, to a decline in disease prevalence after spraying with insecticides (Sutton *et al*., 2011).

Gene flow, which is defined as the genetic successfulness stray rate into a population, and renders the two populations similar. The exchange of genetic material is brought by the movement of individual animals, spores or gametes. The gene flow can be between (horizontal gene transfer) or within the species, that is, antigenic shift and reassortment (Lum *et al.*, 2007). Gene flow analysis can help in understanding the gene spread in the parasitic population in different geographical regions, thus helping in the management of the disease. Mechanisms have been put forward to explain gene flow, including climatic changes in Africa after the last glaciation event, which resulted in optimal warming and humid conditions within the equatorial regions (Olago, 2001).

Increases in the human population in Africa due to the spread of slash and burn agriculture (Salamini *et al*., 2002), proliferation and rapid diversification in the highly anthropophilic *Anopheles* mosquito vectors (Coluzzis *et al*., 1999) are also considered to be a possible mechanism. Human migratory patterns play a great role in the gene flow, in that any dramatic changes in the host gene can cause the global extinction of the parasites through a process called demographic compensation, which shifts the parasite density. During this process, the shift can occur with little or no changes in the parasites' host (Brandt *et al*., 2007), leading to reciprocal adaptation between the host and the parasite, which can uncouple the rate of the gene flow.

Another mechanism put forward to explain gene flow is migratory mosquito patterns; about 30 to 40 *Anopheles* mosquito species have the ability to transmit malaria (Kiszeski *et al*., 2004), but their ability varies from one species to another (Alavi *et al*.,

2003) and further studies have shown that even within genotypes in the species, these differences exist (Lambrechts *et al*., 2005). It has been shown that there is a strong co-adaptation between the host and the mosquitoes, in that there is high infectivity for local hosts compared with a foreign host. This means that there is a probability that when a parasite has an evolutionary advantage over the co-evolving host, within such a shorter generation time, larger population sizes or recombination rates were observed (Kaltz and Shykoff, 1998; Brandt *et al*., 2007).

2.6. Chelex method of DNA extraction

Chelex 100 (Bio-Rad Laboratories, CA, USA) is based on a styrene-divinylbenzene copolymer containing paired iminodiacetate ions method. It works on the principles of chelating transition metal ions and selectivity of which depends on iminodiacetic acid. This cation exchanging ability of the resin is functional at neutral or weakly acidic of pH (> 4.0). Furthermore, at very low pH, the resin begins to function as an anion exchanger. In addition, Chelex is categorized as a weakly acidic cation exchanger with high affinity for divalent metal ions in its mode of action. The pioneer protocol for DNA extraction using Chelex 100 was developed by Walsh et al. (Wash et al.,1991). This method, is application mostly in forensics, which involves heat denaturation of cells which may be attached to paper or fabric, in a solution containing Chelex 100 resin. High temperatures normally result in the release of DNA into the solution as well as facilitate the binding of Chelex resin to magnesium ions. Magnesium ions present in this matrix solution serve as cofactors to deoxyribonucleases and aid in their activation. Given that magnesium ions are rendered unavailable to bind to deoxyribonucleases; DNA degradation is averted. Therefore, this protocol was established, the Chelex 100 resin became the method of choice for protocols requiring the rapid extraction of DNA from trace amounts of biological samples Soderstrom *et al.,* 2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

Samples were obtained from a cross-sectional field study carried out in five wards namely Ahero, Kabura, Kabonyo, East Kano and Awasi in Nyando-sub-county located in the lake region in western Kenya characterized with high prevalence of malaria (Ng'ang'a *et al*., 2008) high poverty rates and poor community medical services, these are risk factors for high mortality and morbidity rates.

Nyando has a river basin with a coverage area of 3517 km^2 which promotes agriculture and fishing as their economic activities in the area (Swallow *et al.,* 2008). It is situated within the Winam Gulf between longitudes 34°47" E and 35°44" E, and latitudes 0°07" N and 0°20" S. The main land use activities in the catchment include indigenous and plantation forests, agriculture and shrub land.

The long rains occur March-May and short rains September-November. Nyando experiences an average relative humidity of 65% with an average temperature of 17.32°C and rainfall of between 1000 and 1800mm annually. The main vectors of the common parasite, *Plasmodium falciparum* are; *A.gambie, A.arabiensis* and *A.funestus*. *P.falciparum*is the most common species with an annual sporozoite inoculation rate of 90 to 410 infection bites. The Sub-County is endemic for malaria and has a prevalence of 27% which is above the average national prevalence for the disease (Kenya Malaria Indicator Survey, 2015). This study was conducted at various selected health facilities (District hospitals and the health centers).in malaria endemic and epidemic zone of Nyando Sub-County in Kisumu County.

3.2 Ethical approval and consenting

Ethical approval to undertake this study was sought from Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit (SERU), protocol number

KEMRI/SERU/CBRD/183/3757. This was part of the previous parent case study. Informed consent form was administered prior to collection of a blood sample. In the informed consent form participants were appraised about the benefits, risks, confidentiality, discomforts and their rights. The risk to participating for children under five was minimal, since it was limited to temporary discomfort associated with finger prick for blood collection. No participant was forced to participate in the study. Furthermore, procedures, information, and purpose of the study were explained to the participants by the community health volunteers (CHV) in a language they were most comfortable with. After agreeing to all the provisions in the consent form, the consent forms were filled by the clinician and signed by the participant and an independent witness. Strict confidentiality was maintained and all personal identifiers were removed from the data during analysis. The parasitological data for each patient was recorded in their medical cards while demographic data included date, name, next of kin, relationship, gender, age, weight, residence and symptoms recorded as case record form (CRF) (Appendix I).

3.3 Inclusion and exclusion criteria

In the parent study, subjects who were two years old and above and were willing to consent to take part in the study were enrolled. For children, consent was sought from the parents or legal guardians. All subjects were required to have taken drug under the period of 42 weeks. This was confirmed by urine test for presence of chloroquine, quinine or sulphonamides. Finally, all the subjects presented with malaria symptoms including axillary temperature of 37.5^0C or history of fever 48 hours prior to recruitment. The initial parasitemia had to be from 1,000 to 200,000 parasites per µlof blood. The following were exempted from the study; patients who hadnt taken anti-malarial drugs within a period of 42 weeks; those who had declined to consent to participate in the study; patients showing signs of severe anemia and malaria; those with a hemoglobin level of <5 g/d; presence of other diseases that causes febrile conditions; presence of any other Plasmodium species other than *falciparum*; patients with a history of adverse events against ACT (artemisinin-based combined therapy)and sulphonamide drugs.

3.4 Sample size

The sample size was obtained from the previous parent study, whereby it was calculated using the single population formula;

$n = z^2 pq/$ whereby n is sample size; z^2 is the abscissa of the normal curve at e^2 1−a= 0.95, e is the desired precision, p is the estimated proportion requiring knownN(population), and q is $1 - p$ (Cochran *et al*., 1963). The following assumptions were taken into consideration; 95% confidence interval, 5% margin of error, and disease prevalence of 29%. The resultant sample size was 315. This calculated sample size factored in non-response of 0.9%. To obtain the number of samples to be collected from each site, 315 were divided by five so as to ensure the sampled wards were equally represented in the study. Thus, 65 study participants were obtained per ward.

3.5 Sample collection, parasite speciation, parasitemia, and hemoglobin determination

Patients were finger pricked to get the blood samples for parasite speciation and parasitemia determination (Bejon *et al*., 2006). At the time of sample collection, all slides were read by two independent microscopists. If counts were in discordant, the slides were examined and verified by a third microscopist. In brief thick and thin blood smears were carried out as follows. For the thick blood smear, parasitic density was determined by counting parasitemia in every 2000 white blood cells (WBCs) while for the thin blood smear, parasitemia percentage was calculated in every 1000 WBCs then compared with the initial reading of the samples that is the field clinician figures. Thin blood smear was done by using the edge of another slide that was held at 45^o to the first one. The spread was made spreading the cells across the width and then made along the length with a swift smooth spread. The blood films were air-dried and fixed with absolute methanol and then stained with 5% Giesma stain for 10 minutes. These slides were then rinsed with tap running water; air dried and then observed underoil emersion at 1000X magnification was done. For hemoglobin determination, HemoCuehemoglobin system (HemoCue AB, Sweden) was used whereby

approximately 10 µL of undiluted blood was taken up in a capillary cuvette containing a reagent consisting of sodium deoxycholate, sodium nitrite and sodium azidenitrite reagent which lyses the blood and converts the hemoglobin to haemiglobinazide. The absorption was then read photo- metrically at 570 nm for determining hemoglobin and at 880 nm for turbidity compensation.

In addition, for further lab analysis approximately 500µl of the blood were collected by venipuncture using EDTA vacutainer tubes or citrate tubes (Becton Dickinson) from each patient. The samples were spun (350g, 10 min) and the pellets containing packed red blood cells (RBC), and white blood cells (WBC) were frozen with an equal volume of cryopreservation solution (0.9% NaCl, 4.2% sorbitol and 28% glycerol) and transported in liquid nitrogen container to KEMRI. The samples were stored at -20°C and thawed at 4°C prior to testing. Dried blood spots from the finger-pricks were prepared on 3 MM paper, Whatman-FTA-cards (Whatman, Florham Park, NJ) from blood samples collected from patients who attended the malaria clinic. Filter papers were dried at ambient temperature in the field, shipped to KEMRI and stored in plastic bags at -20°C containing silicate as desiccant.

3.6 DNA extraction and quantification

The DNA was extracted from the blood samples by Chelex Resin 100 as describedby (Plowes *et al.*, 1995) and Biomeme preparation sample kit (Biomeme, In. Philadelphia. USA) from equal volume (25µl) from each sample. Briefly in Chelex method, scalpels and forceps were immersed in 5M HCl for a few seconds to get rid of DNA contamination while glass plates or tiles were wiped with tissue paper followed by neutralization in 5M NaOH and final washing in sterile water. Approximately 4mm^2 piece of Whatman filter with 25 µl of dry blood spot was cut using a sterile scalpel blade. This was then incubated in 0.5% saponin in 1X phosphate buffered saline (PBS) which had the following constituents; 3.2mM Na_2PO_4, 1.3mM KCL, 1.35mM, pH 7.4 left for an overnight at 4^0C. Brown solution was discarded and replaced with 1X PBS, then incubated for 30 minutes at 4^0C.The solution was discarded and 100 µl of DNAse free water was added, this was followed by addition of 50µl of 20% of Chelex solution.

The solutions in the tubes were placedon a heated block at 99^0C and vortexed every

two minutes for a total of ten minutes. The solution was then centrifuged at 10,000xg for two minutes at room temperature. The supernatant containing the DNA was removed and then aliquoted into units of 40 µl in order to avoid freezing and thawing which would have led to degradation of the DNA. The DNA aliquots were stored at -20^0C.

In the second method, DNA was extracted from the blood samples by Biomeme prep sample kit following the manufacturer's instructions (Biomeme Inc. Philadelphia. USA). Initially 25µl of whole blood was picked using Biomeme syringe and this was then transferred into a tube containing 0.5 ml of Biomeme lysis buffer and the mixture was pumped once using the same syringe. Using the syringe again, this was then transferred into another tube containing 0.5 ml of Biomeme Protein Wash Buffer. Thereafter, the contents were picked using the syringe and dispensed into a tube containing1 ml of Biomeme Salt Wash buffer and having done this, the syringe was air dried by pumping till no droplets were seen coming out at the syringe's spout. Elution of the DNA was done by pumping the syringe thrice into syringe 0.5 ml of Biomeme Elution Buffer. The eluted DNA was stored at -20^0C for future lab analysis. The respective DNA extracted from each method above was quantified using a spectrophotometer NanoDrop 2000 (Thermo Scientific Inc.US) according to the manufacturer's instructions with the absorbance measurements being taken at 230, 260 and 280 NM. Control sample was DNAse free water that was used for blanking the spectrophotometer.

3.7 Determination of detection limits of the extracted DNA using conventional and Nested PCRs

Limits of detection were done using extracted DNA from the two extraction methods. The respective equal volumes of the DNA from each method of extraction were serially diluted ten-fold as follows (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}). Conventional PCR was done using the 18S rRNA gene primers while the nested PCR amplification was performed using the Merozoite Surface Protein1(MSP1) gene primers. PCR primers used were as described by Lee *et al.* (2002) as shown in table 3.1 below. A PCR reaction volume of 30 µl was constituted; 1XKEM® PCR Buffer

(KEMRI, Nairobi, Kenya), 500nM for each primer FAM™ (Applied Biosystems, Foster City, USA),250nMKEM® dNTPs (KEMRI, Nairobi, Kenya), 1µl of DNA and finally 1unit KEMTAQ®(KEMRI, Nairobi, Kenya). The amplification was carried on GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA) under the following conditions: denaturation at 94℃ for 3 min for1 cycle; 30 cycles of 94℃ for 60 s, annealing at 50℃ for 2 min and 72℃for2 min; and a final elongation step at 72℃for 10 min.

Each experiment included control tubes corresponding to a serial dilution of (i) a positive control consisting of *P. falciparum* (3D7) genomic DNA and (ii) a negative control containing no target DNA. For the determination of the DNA fragment size, 8µL of the amplicon was electrophoresed in a 1.5% agarose (Sigma) in 1xTAE (40 mM Tris acetate, 1mM EDTA pH8.0) as running buffer containing 0.1 µg of ethidium bromide (Promega, USA) per ml. This was left to run for 40 minutes at 80 volts on horizontal electrophoresis tank (Bio-Rad). The amplicons were visualized under

Ultraviolet light against a 100 base pair DNA molecular weight marker (Promega, USA) on UVP transillumination machine (An Analytik Jena, Cambridge, UK).

Limits of detection using the nested PCR was performed on GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). The initial reaction was carried out using a volume of 30µl reaction mixture containing;1µL template DNA,1X KEM® PCR Buffer (KEMRI, Nairobi, Kenya), 400µMKEM® dNTPs(KEMRI, Nairobi, Kenya), 100nM of each primer FAM™ (Applied Biosystems, Foster City, USA), and 1-unit KEMTAQ®(KEMRI, Nairobi, Kenya).The thermocycler conditions used were as follows, denaturation at 94℃ for 3 min for 1 cycle; 30 cycles of 94℃ for 25 s, annealing at 50℃ for 35 s and 68℃ for 120 s; and a final elongation step at 72℃ for 3 min. DNA amplified in the initial PCR was vortexed gently to mix and 0.2 µ used as a template in a nested PCR with the same conditions. The inner nest had similar reaction volume and constituents except 0.1 µl template DNA was the product of the outer nest PCR. Negative and positive controls were both included for outer and inner nested PCRs as described above for conventional PCR. The primers used in both outer and inner nested PCRs were as illustrated in table 3.1 below.

Table 3.1: Primers for conventional PCR and Nested PCR targeting 18S rRNA and MSP1 genes

18S rRNA primer sequences
Forward5ˈACA TGG CTA TGA CGG GTA ACG 3`
ReversE 5`TGC CTT CCT TAG ATG TGG TAG CTA3
MSP1 primer sequences
Nest 1 Forward Primer (1F) 5ˈCGCCCGTACTATGAAGAAGATC3`
Nest 1 reverse primer (1R) 5ˈGGCTTTTACCTGAACTGTTCAG 3`
Nest 2 forward primer (2F) 5ˈCGTAAACAGAATATTCAGGATTGC 3'
`Nest 2 reverse primer (2R) 5ˈCTAGCCCTTTATTATCATTATCG 3'

3.8 Determination of the genetic diversity

The micrographs of the gel were taken on VisionWorks®LS Image Acquisition and Analysis Software (Analytik Jena Company, UK). The bands analysis was done as per the manufactures' instructions. In brief, all the bands and the lanes were selected under multiple dendrogram alignment. The "ID Analysis Action" was opened which was followed by find "Lanes and Bands menu". The region was defined and an automatic search for lanes and bands was performed. Sensitivity for lanes and bands window was adjusted by the slider either to the right for more or on the left for fewer lanes and bands. The repeatedly linkage rule was used for merging similar groups into larger clusters, until all clusters joined into one. The lanes that were similar to each other appeared in clusters near the bottom of the hierarchy. Background correction was added to account for variable illumination or over-exposure during image capturing.

This was performed in the following order: ID analysis → Lane profiles → Background correction and the options for these corrections can be done as follows; no background correction, using straight line, joined valleys, rolling disc and area between the areas. No background correction was when the lane profiles graphs were not corrected, in the straight-line correction the software removed the area between the graph and the straight line and the remaining values we reemphasized thus it tended to correct over-exposure and variable illuminationthat were formed at the edge or the image corners. The dendrograms of the defined lanes and bands were captured and saved in the desired file locations.

3.9 Comparison of the limits of detections using Biomeme Smartphone DNAand ABI 7500 real-time PCR assays and field evaluation of Biomeme assay

Six serial dilutions of, 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} of equal volume of the DNA sample for each protocol were made in triplicates. These serially diluted DNA from the respective protocols above were used for these two assays for comparison. In the case of Biomeme, the reaction volume of 50µl was constituted as per manufacturer's instructions. In brief, the primer and the probes concentrations were 400nM (Biomeme Inc. Philadelphia, USA) and 200 nM (Biomeme Inc. Philadelphia, USA) respectively, 15µl of2X Biomeme LyoRNA™ Master Mix (buffer, magnesium ions, dNTP nucleotides, Taq DNA polymerase and a thermostable MMuLV RNA-dependent DNA polymerase) (Biomeme Inc. Philadelphia, USA) and 5µl of the DNA. The reaction volume was 30µl after topping up was done with primers and probe. The reaction was performed on the Biomeme one$_3$ (Biomeme Inc. Philadelphia, USA) thermocycler conditions were as follows; 95℃for 10 min, (95℃ for 10 seconds, 50℃ for 5 seconds) X 40 cycles and 72℃ for 10 seconds. The amplification curves were computed withinthe Smartphone's web portal. On the hand, ABI 7500 real PCR was carried out using the 18S rRNA gene primers of *Plasmodium spp* (Singh *et al.,* 1999; Afonina *et al.,* 2007) and the probes were as described by Lukhtanov *et al.* (2007) and their sequencesare as shown in table 3.2 below.

Table 3.2: The primers and probes sequences used for *Plasmodium spp* 7500ABI real time PCR assay targeting 18S rRNA gene

Primers and probe sequences
Forward primer5´-AATAAATCATAAGTATTCAGATGTCAGAGGTG-3´
Reverse primer 5´- AATAAATCATAAGRCAAATGCTTTCGCAGTTG -3´
Probe 5´- MGB-FAM- TTCTGGAGACG*A*G*CAA*CT -Quencher -3´.

A reaction volume of 30µl consisting of primers at a concentration of 200nM (Coriel Institute, USA), probe 100 nM (Coriel Institute, USA) and 5µl of DNA was used. The reaction was performed on Applied Biosystems 7500 Fast Real-Time PCR System

(Applied Biosystems, CA, USA) under the following conditions; Initiation denaturation temperature was 95^{o}C for 10 minutes followed by 40 cycles of 95^{o}C for 10 seconds, 50^{0}C for 5 seconds and 72^{o}C for 20 seconds with fluorescence acquisition at the end. For checking the recrudescence and re-infection melting curves the program was set as follows 2 minutes at 95^{o}C, 2 minutes at 68^{o}C then a stepwise temperature at 0.2^{o}C/second until 90^{o}C was attained with fluorescence acquisition at each transition temperature

3.10 Data analysis

One-way ANOVA test and Levene-type tests for a trend and homogeneity in the group variances were performed using SPSS statistics software version 22 (IBM, NY, USA). This was done to examine whether or not there was significant difference in the average quantification values between the extraction methods. Comparison of sensitivity and specificity between 18S rRNA and merozoite surface protein 1 (MSP1) in the detection of *Plasmodium* spp. in clinical samples was examined with Chi-square and Cohen's kappa coefficient tests using SPSS statistics software (IBM, NY, USA). Cohen's Kappa values (k) calculation were performed manually and the following guideline for degree of agreement were used: poor k <0.00, slight $0.00 \leq k \leq 0.20$, fair $0.21 \leq k \leq 0.40$, moderate $0.41 \leq k \leq 0.60$, substantial $0.61 \leq k \leq 0.80$ and almost perfect k>0.80. VisionWorks®LSImage Acquisition and AnalysisSoftware (Analytik Jena Company, UK) were used to analyze the genetic diversity amongst plasmodium parasites. The numbers of true positive (TP), true negatives (TN), false positive (FP) and false negatives (FN) were used to calculate: Sensitivity = TP/(TP + FN) × 100; Specificity = TN/(TN + FP) × 100; The Positive Predictive Value (PPV) = TP/(TP + FP) × 100; The Negative Predictive Value (NPV) = TN/(FN + TN) × 100 and Diagnostic Accuracy (DA) = TP + TN/Total No. of patients × 100.

CHAPTER FOUR

RESULTS

4.1 Parasitemia and hemoglobin results

The patients from whom samples were collected blood were aged 2-79 years. Parasitemia ranged from 1120/µl to 992000/µl (appendix IV), which were reflected for the age group$\geq 30 < 40$ years. The lowest and the highest mean parasitemia per microliter values were 29368 (SD±22604) and 45495 (SD±22580) in age groups $\geq 60 < 70$ and $\geq 50 < 60$respectively. The Hb concentrations ranged from 4.7 g/dl to 15.6g/dl as in age-group of $\geq 2 < 10$. The lowest and highest mean Hb values were 8.1 (SD±2.46), and 11.56 (SD±2.47) respectively. These results indicated that the $\geq 70 < 80$ years old age group had the lowest mean Hb concentration as shown in Table 4.1. The $\geq 20 < 30$ years old age group had the highest number (100 [31.7%]) of patients registered for this study while the $\geq 60 < 70$ years old age group had the fewest(5 [1.6%]). In terms of normality in their anemia conditions, it was clear that the mean value of the following age groups; ≥20<30, ≥40<50 and >60 < 70 age groups had a normal mean Hb concentration of>11 g/dl, while the remaining age groups all had a mean value that indicated moderate anemia, with mean values ranging from >7.0 to 10.9 g/dl. The lowest and highest range of hazard ratios were $0.01 - 0.49$ and $0.02 - 1.00$ in age groups $\geq 60 < 70$ and $\geq 20 < 30$ respectively.

The Pearson's correlations for parasitemia/age, parasitemia/Hb concentration, and age/Hb concentration were 0.105, 0.681, and 0.040, respectively. These correlation coefficients indicated that there were very minimal relationships between the factors as shown in table 4.1 below. The parasitemia concentration was calculated as the number of parasites per microlitrewhile the hemoglobin density was taken as g/dl and the baseline parasitemia of $> 100000\ parasites/\mu l$was used to calculate range of hazard ratio.

Table 4.1. The comparative analysis of parasitemia (parasites/µl) and hemoglobin(Hb) concentration in g/dl)

Age(years)	Number/ % patients	Mean parasitemia/µl	Parasitemia range/ µl	Range of Hazard Ratio	Mean (Hb) in g/dl	Hb con. Range in g/dl
$\geq 2 < 10$	59(18.7)	318678 $\pm$ 21440	1160 $-$ 78800	0.01 $-$ 0.78	10.8 $\pm$ 2.4	4.7 $-$ 15.6
≥ 10 < 20	40(12.7)	38578 $\pm$ 24906	1520 $-$ 76000	0.02 $-$ 0.76	10.1 $\pm$ 2.6	6.5 $-$ 13.6
≥ 20 < 30	100(31.7)	42144 $\pm$ 23286	1920 $-$ 99200	0.02 $-$ 1.00	11.1 $\pm$ 2.5	5.6 $-$ 14.6
≥ 30 < 40	70(22.2)	42430 $\pm$ 23271	1120 $-$ 96400	0.01 $-$ 0.96	10.5 $\pm$ 2.5	6.2 $-$ 15
≥ 40 < 50	25(7.9)	36571 $\pm$ 22850	1200 $-$ 63200	0.01 $-$ 0.63	11.3 $\pm$ 2.5	7.1 $-$ 14.5
≥ 50 < 60	8(2.5)	45495 $\pm$ 22580	1640 $-$ 77600	0.02 $-$ 0.78	10.7 $\pm$ 2.5	6.6 $-$ 12.9
≥ 60 < 70	5(1.6)	29368 $\pm$ 22604	9000 $-$ 49200	0.01 $-$ 0.49	11.6 $\pm$ 2.5	10.4 $-$ 14
≥ 70 < 80	8(2.5)	44255 $\pm$ 22526	3640 $-$ 80400	0.03 $-$ 0.80	8.1 $\pm$ 2.5	6.5 $-$ 10.7

4.2 DNA quantification results

4.2.1 Assessment of the DNA extracted

The two methods of extraction generated a pellet of DNA at the end of each extraction. However, the color of the precipitated DNA varied between the methods. The Chelex DNA extraction method produced pellets that were light yellow, yellow or dark brown, whereas the Biomeme sample prep kit method generated clear pellets for the same samples. The DNA suspension had low viscosity with both methods. The DNA concentrations for both Chelex and Biomeme extraction methods had mean values of 42.93 ng/µl (SD±32.88) and 66.74 ng/µl (SD ±16.73), while the DNA concentrations ranged from 30.3ng/µl to 77.7 ng/µl and 13ng/µl to 96 ng/µl, respectively. The A_{260}/A_{280} (purity) and A_{260}/A_{230} (turbidity) ratios for Chelex ranged from 1.19 to 3.44 and 0.17 to 5.66 while those of the Biomeme kit were from 0.23 to 2.85 and 0.008 to 0.01, respectively. According to a t-test, the average A_{260}/A_{280} and A_{260}/A_{230}

differences were statistically significant between the two methods (p <0.001) and negative control was DNAse free water. as shown in Table 4.2 below.

Table 4.2: T- Test for; Concentration, purity, turbidity and yield of DNA extracted by two protocols

$Sample$		$Con.$	$A260/A280$	$A260/A230$	$Yield$
BO	$Mean$	66.74 ± 16.73	1.97 ± 0.48	0.01 ± 0.01	33.36 ± 8.4
	$c.o.v$	0.25	0.24	1.00	0.25
CO	$Mean$	42.93 ± 32.88	2.86 ± 0.67	2.35 ± 1.76	7.69 ± 2.48
	$c.o.v$	0.77	0.23	0.75	0.32

From the table above; BO, CO, Con. were Biomeme, Chelex and concentration respectively. The coefficient of variation (c.o.v) was calculated is shown.

Levene's test for equal variances from that homogeneity and trends in the means above were significantly different. The Ftest (F), degree of freedom (df) and significance value (p) for concentrations, $A260/A280$, $A260/A230$ and yields were; (0.54, 76, 0.001), (3.57, 76, 0.001) and (29, 76, 0.001) respectively. There were significant differences (p<0.0001) for concentration, purity, turbidity and the yield between the two DNA extraction platforms.

There was a strong relationship between the DNA yield and concentration, for example the DNA extracted by Biomeme prep sample kit had a significant higher amount of DNA yield as compared to the Chelex. To sum, the results showed that Pearson correlation coefficient ($\sqrt{R2}$) was 0.8672 which meant that the two methods had a strong correlation as showed in figure 4.1 below.

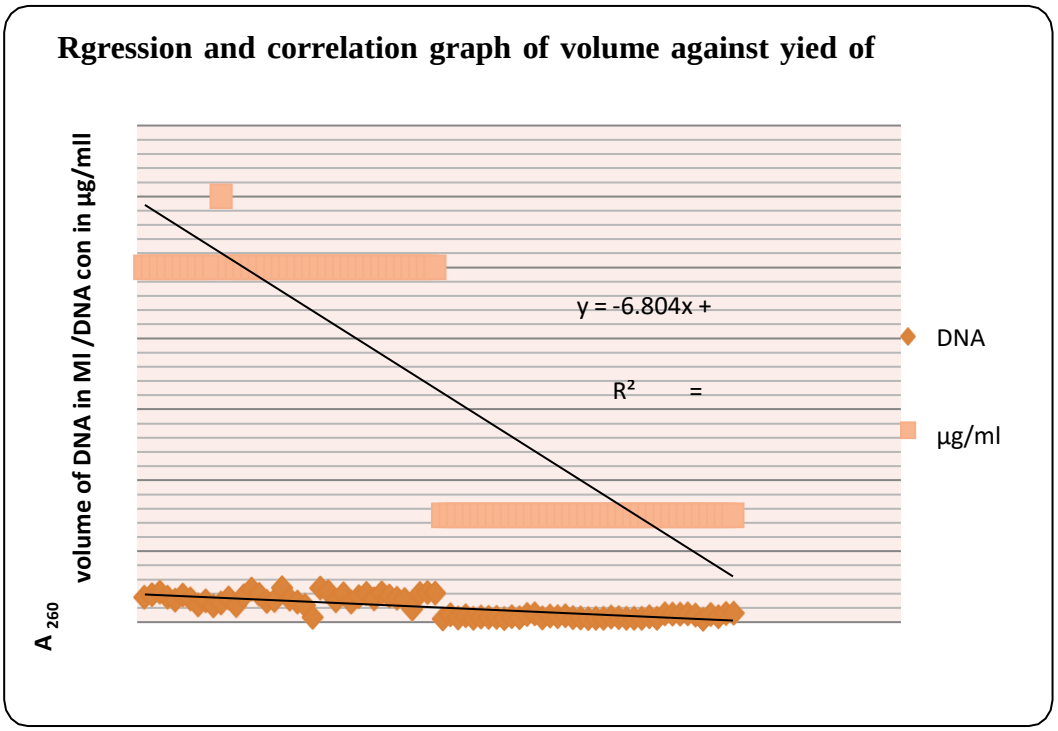


Figure 4.1: Correlation between the two methods of extraction of volume against yield.

The first 40 sample results were from the Biomeme kit while the rest (41 to 80) were from the Chelex DNA extraction.

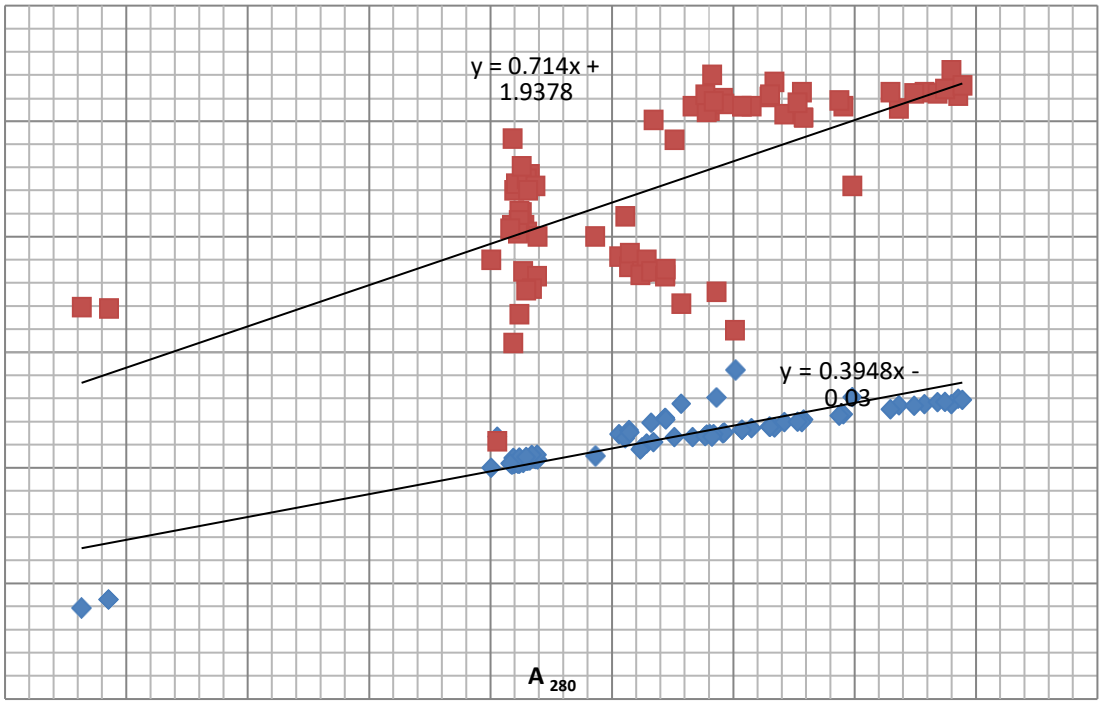


Figure 4.2: Scatter graph of A260 against A280 of Biomeme and Chelex

From fig. 4.2 above, blue scatter graph represented Biomeme while the red one was for Chelex. The Biomeme graph showed that there was a strong collinearity between the A_{260} and A_{280} and this meant the mean deviation in the purity ratio (A_{260} / A_{280}) were below the standard values. However, from the Chelex scatter graph it was clear that there was less collinearity and this could be seen by a larger margin of (±0.67) within the standard deviation in the purity ratio. Therefore, from the above two graphs, the Biomeme one had nearly a perfect correlation since most of the scatters lied on the straight line as compared to the Chelex ones.

The ratio of absorbance at 260/230 nm was used to determine contamination by aromatic compounds, phenols and carbohydrates (Roh *et al.*, 2006). Ratios between 1.5 and 1.8 were taken as an indication of DNA without aromatic compound contamination (Weiss *et al.*, 2007) while protein contamination was measured using

the ratio of absorbance at 260/280 nm. A ratio between 1.8 and 2.2 was indicative of no protein contamination (Weiss *et al.*,2007).

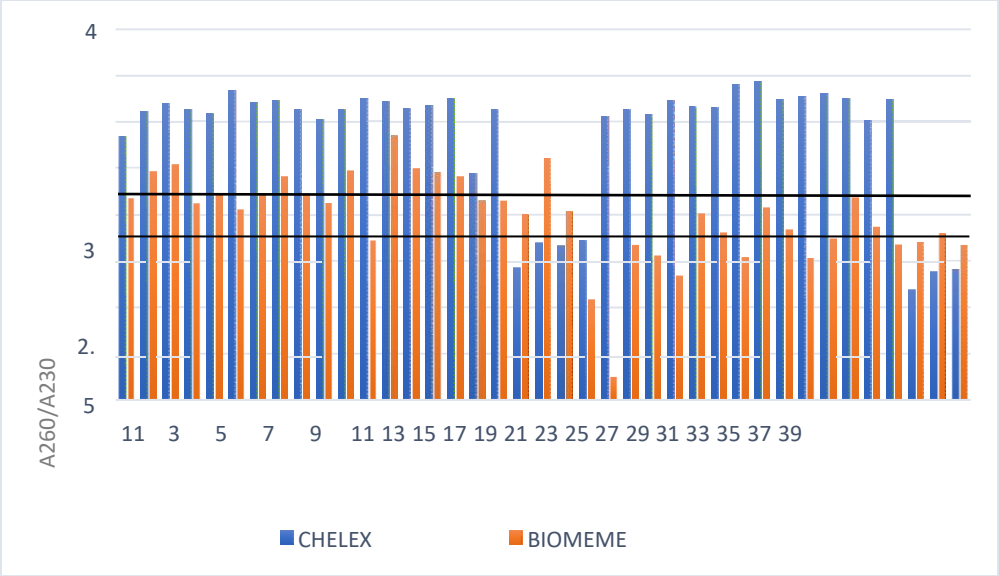


Figure 4.3: The absorbance ratios for Biomeme and Chelex DNA extract at 260/280 nm

The area between the lines is indicative of pure DNA, i.e., DNAs with no protein contamination. The y-axis represented the absorbance ratios at A_{260}/A_{280} nm.

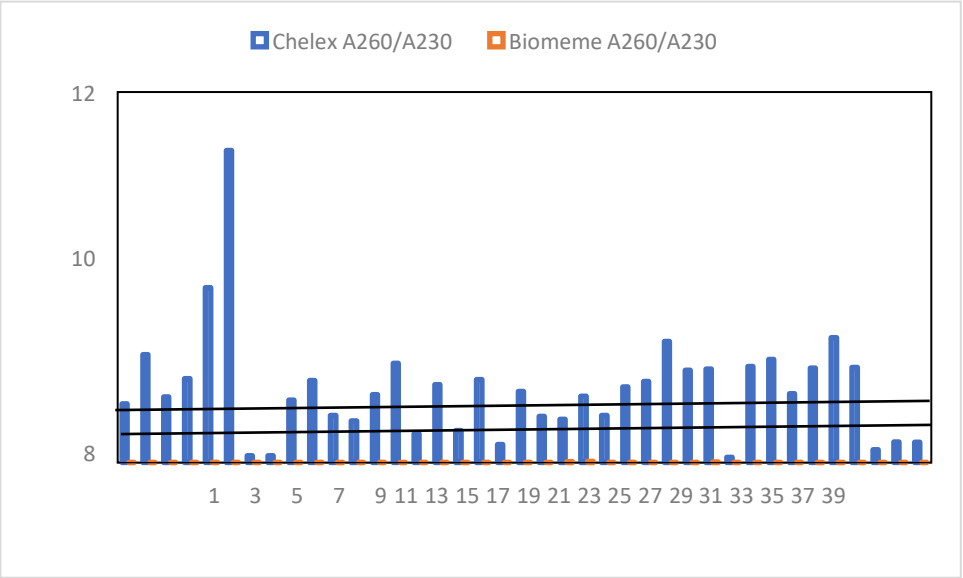


Figure 4.4: The absorbance ratios at 260/230 for both Biomeme and Chelex DNA extract

The area between the lines was an indicative of pure DNA, i.e., DNA with no contamination by aromatic compounds, phenols and carbohydrates (ratios between 1.5 and 1.8). The Y-axis represented the absorbance at A_{260}/A_{230}.nm while X-axis represented the respective DNA from Biomeme and Chelex extraction methods. From the figure 4.4 above none of the DNA extract from the Biomeme had the expected range of free phenolic and carbohydrates. Chelex method had 7 (39) which represented 17.9%. The Biomeme had none of such values within the range.

4.3 Limits of detection assays

According to table 4.4 below, it was evident that the Biomeme DNA amplicons had a good precision in the respective ratios as compared to the Chelex ones thus a promising as a reputable extraction method for provision of molecular DNA. The respective Kappa values (k) and standard error of Kappa values SE (k) for the Biomeme $k = 0.473$, SE $(k) = 0.363$ and Chelex $k = 0.544$, SE $(k) = 0.272$ amplicons analysis were calculated at confidence interval of 95%. Therefore, was indicative enough across the two DNA platforms that the level of agreements was moderate ($0.41 \leq k \leq 0.60$).

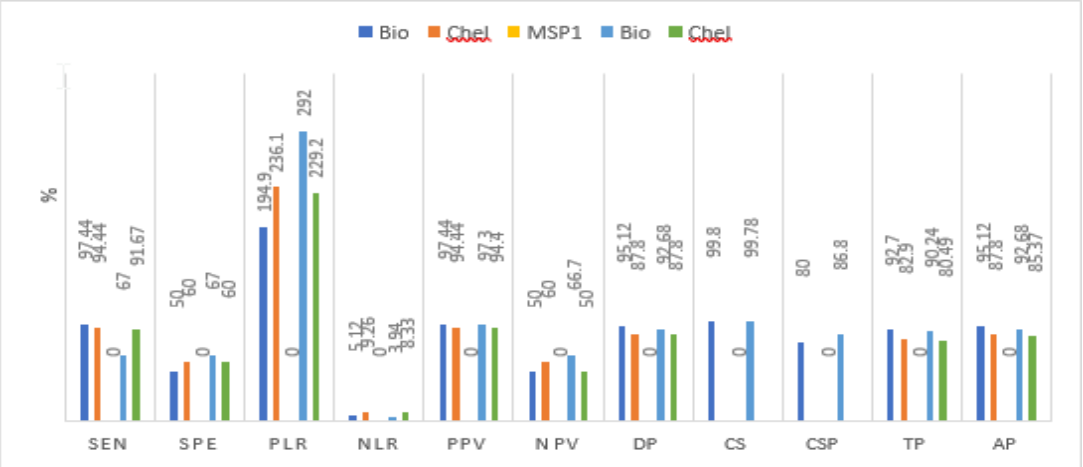
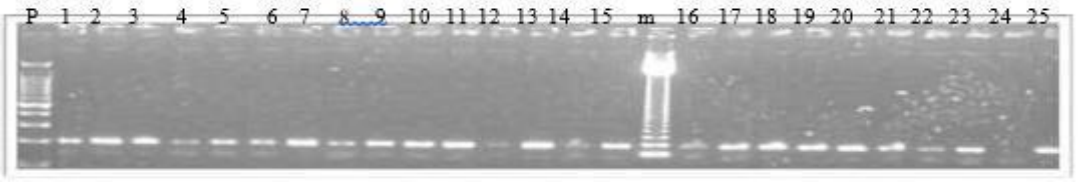
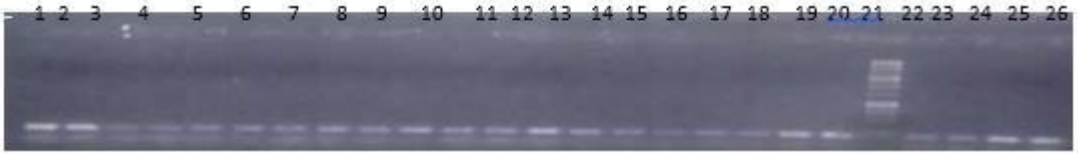


Figure 4.5: Likelihood ratios of the diagnostic on both MSP1 and 18SrRNA gene markers.

The 18S rRNA and MSP1analysis showed that the results were dependent on the DNA extraction platform and the gene marker used in the amplification. Combined sensitivity and specificity ranged from 99.78% to 99.80% and 80% to 86.8%, on 18S rRNA and MSP1 gene markers respectively. The sensitivity and positive predictive values were approximately equal while using 18S rRNA as the gene marker. For the Biomeme kit and the Chelex method, amplification sensitivity and positive predictive. The likelihood ratios of 100%, 115%, 30% and 145% meant that there was none, slight increase, moderate increase and large increment within the diagnostic ratios respectively. values were 97.44% and 94.44%, respectively. The correlation coefficients for 18S rRNA and MSP1 were 0.975 and 0.980, respectively, and those for the Biomeme kit and the Chelex method for 18S rRNA and MSP1 were 0.925 and 0.986, respectively.The combined (sensitivity and specificity) results had a correlation coefficient of 0.989. This indicates that there was a strong correlation in the results of amplification across the two methods. The above data analysis (Figure 4.5) was done on free version of WPS 2016 office spreadsheet (Hong Kong Kingsun Computer.CO.LTD).



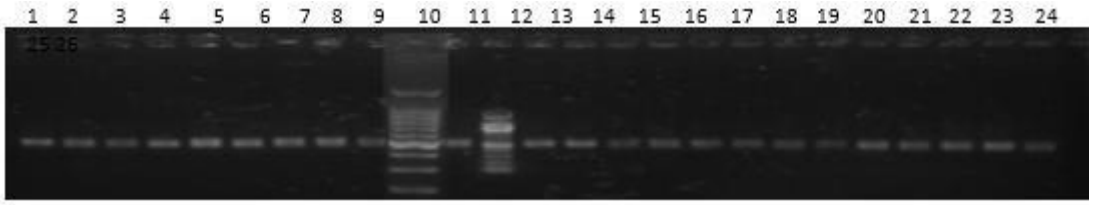
Panel A



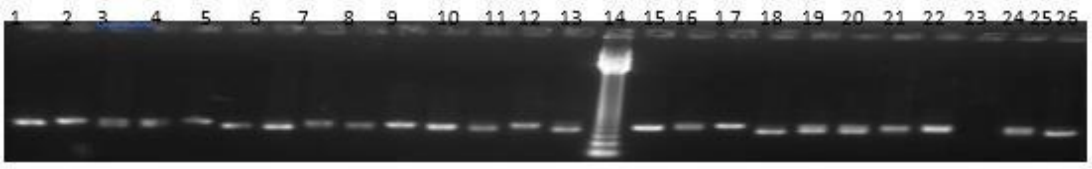
Panel B

Figure 4.6: Micrographs of amplicons under 18S rRNA as the primers

The upper gel (Fig.4.6 panel A) micrograph was from Biomeme amplicon while the lower one (Fig.4.6 panel B) was from the chelex. The molecular weight markers (m&21) in both gels were of 100 base pairs (bp) while p was 1kb. Lanes 25 and 24 in panel A represented positive control (3D7) and negative control (free DNAse water) respectively while in panel B, lanes 25&26 represented positive control (3D7) and negative control (free DNAse water) respectively.



Panel A



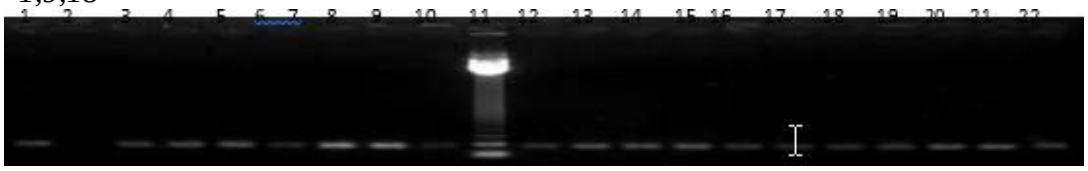
Panel B

Figure 4.6: Gel pictures of MSP1amplicons

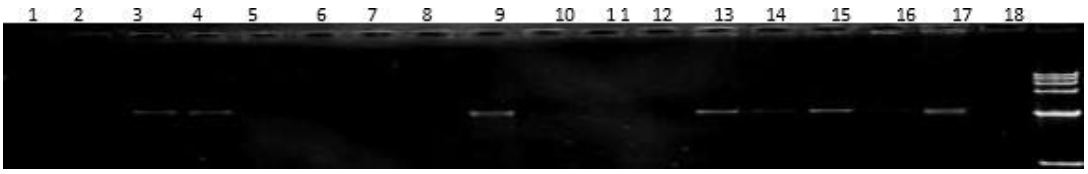
A, Biomeme kit extracted DNA and (B) the Chelex extracted DNA. The DNA fragments in both panels were 400bp. Lanes 10 and 12 represents 1000bp and 100bp DNA weight markers respectively, In Panel A, negative (DNase-free water) and positive (3D7) controls were in lanes 26 and 25, respectively. On Panel B, negative (DNase-free water) and positive (3D7) controls were in lanes 24 and 25, respectively.

Table 4.2: Serial dilutions of DNA for limits of detection on both conventional and nested PCR amplification

10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
For Figure 4.6 18S RNA gene marker							
1,9,17	*2,10,18*	*3,1,19*	*4,12,20*	*5,13,21*	*8,15,22*	*7,15,23*	*8,16*
1,9,17	*2,10,18*	*3,11,19*	*4,12,20*	*5,13,22*	*6,14,23*	*7,15,24*	*8,16*
For Figure 4.7 MSP1 gene marker							
10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1,9,19	*2,11,20*	*3,13,21*	*4,14,22*	*5,15,23*	*6,16*	*7,17*	*8,18*
lanes	*2,10,19*	*3,11,20*	*4,12,21*	*5,13,22*	*6,14,23*	*7,16,26*	*8,17*
1,9,18							



Panel A



Panel B

Figure 4.7: The micrographs of dilution factor of 10^{-2} using 18S RNA primer

Panel A (Biomeme amplicons) and Panel B (Chelex amplicons). DNA was serially diluted to 10^{-2} and amplified using 18S RNA as the gene marker. The gene fragment sizes were 200bp and the molecular marker (m) was 100bp.The limits of detection were dependent on the method of DNA extraction, the primers used and the PCR

method used. The primers used for theMSP1 gene marker had better performance than those of the 18SrRNA gene marker.

4.4 Genetic diversity results of the Plasmodium falciparum parasite

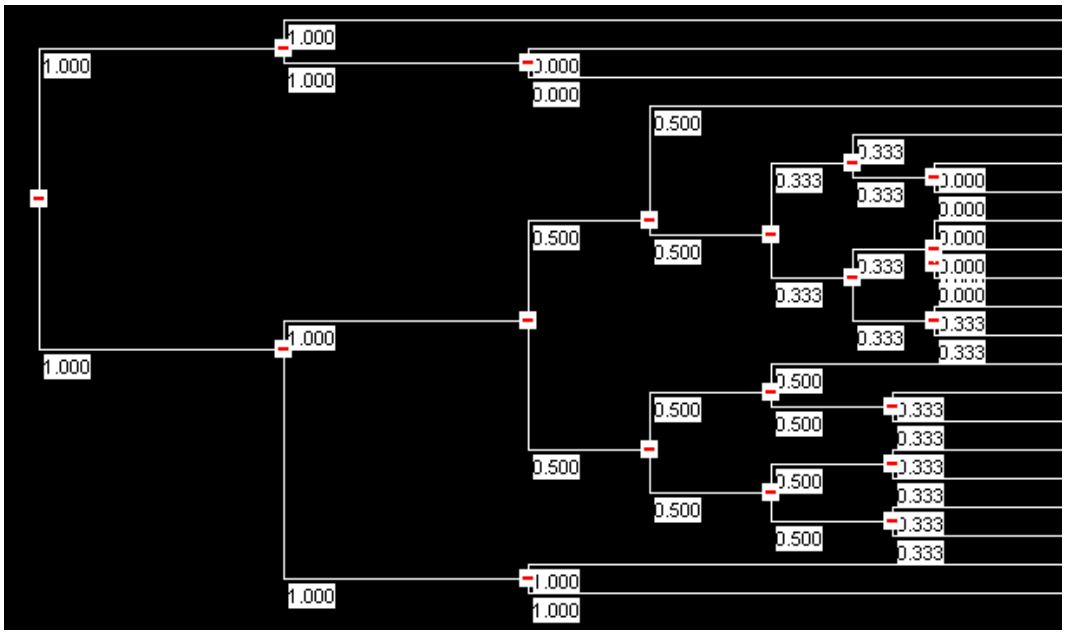


Figure 4.8: Dendrogram for the Chelex method 18S rRNA amplicon analysis

The dendrogram showing the amplicon analysis for 18S rRNA for the Chelex method is shown in Figure 4.9 above had two main clusters within the analysis, with the lower one having a higher number of strains than the upper. The lower cluster had 18 strains (85.7%), while the upper cluster had 3 strains (14.3%). The genetic diversity relatedness ranged from 0.00 to 1.00. The pair-wise band ratios for inter-population genetic distances were 0.0(7), 0.33(9), 0.5(2) and 1.00 (3), which translated to 33.3%, 42.9%, 9.5% and 14.3%, respectively.

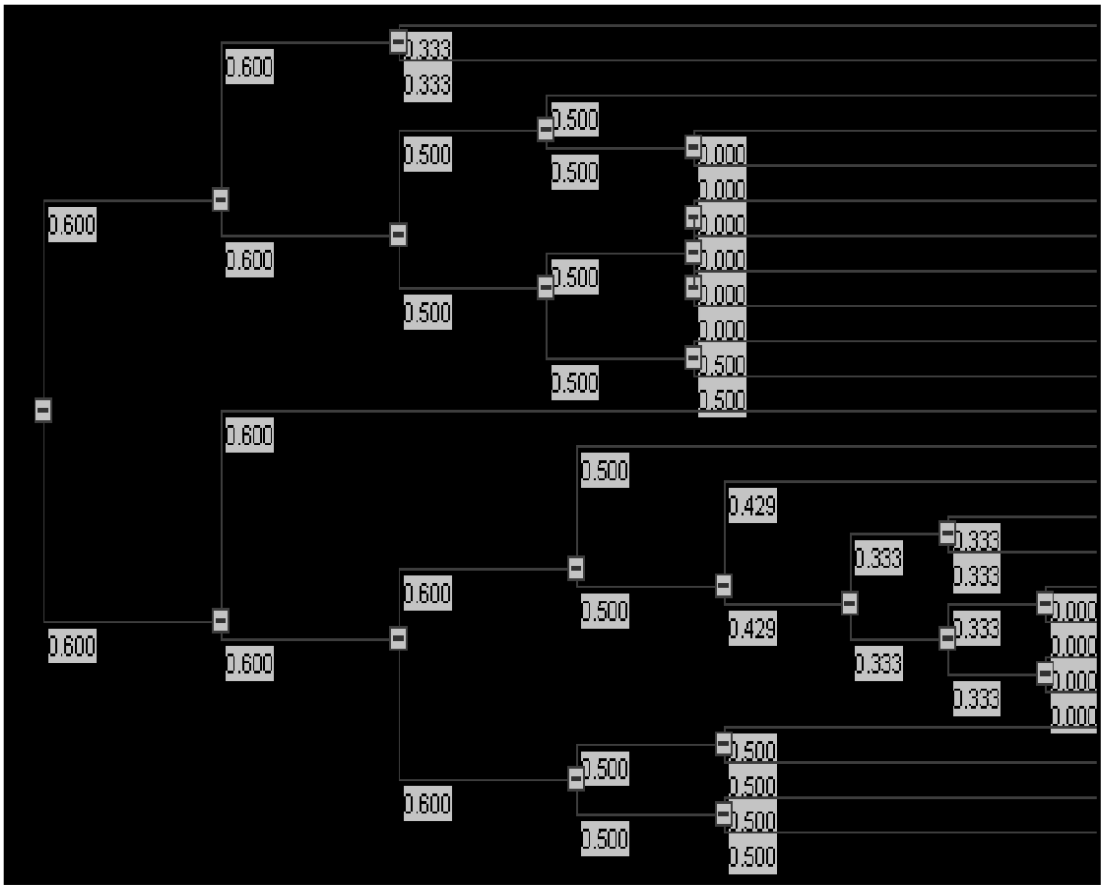


Figure 4.9: Dendrogram for the Biomeme kit 18SrRNA amplicon analysis

Figure 4.10 shows the results of the Biomeme kit 18S rRNA amplicon analysis. The dendrogram had two main clusters of which the numbers of strains were evenly distributed. The upper cluster had 12 (48%) while the lower had 13 (52%). There was no statistical correlation between the dendrograms of Figures 4.9 and 4.10, despite the same gene marker (18S rRNA) being used. The platform used for DNA extraction had an impact on the dendrogram analysis. We noted that the DNA qualities (purity and concentration) were different between the two extraction methods and this could have been one of the contributing factors to these differences. The pairwise inter- population genetic distance of the strain's relatedness was as follows: 0.00 (11) 0.33(4), 0.429 (1), 0.5 (8) and 0.6 (1), which translated to 44%, 16%, 4%, 32% and 4%, respectively.

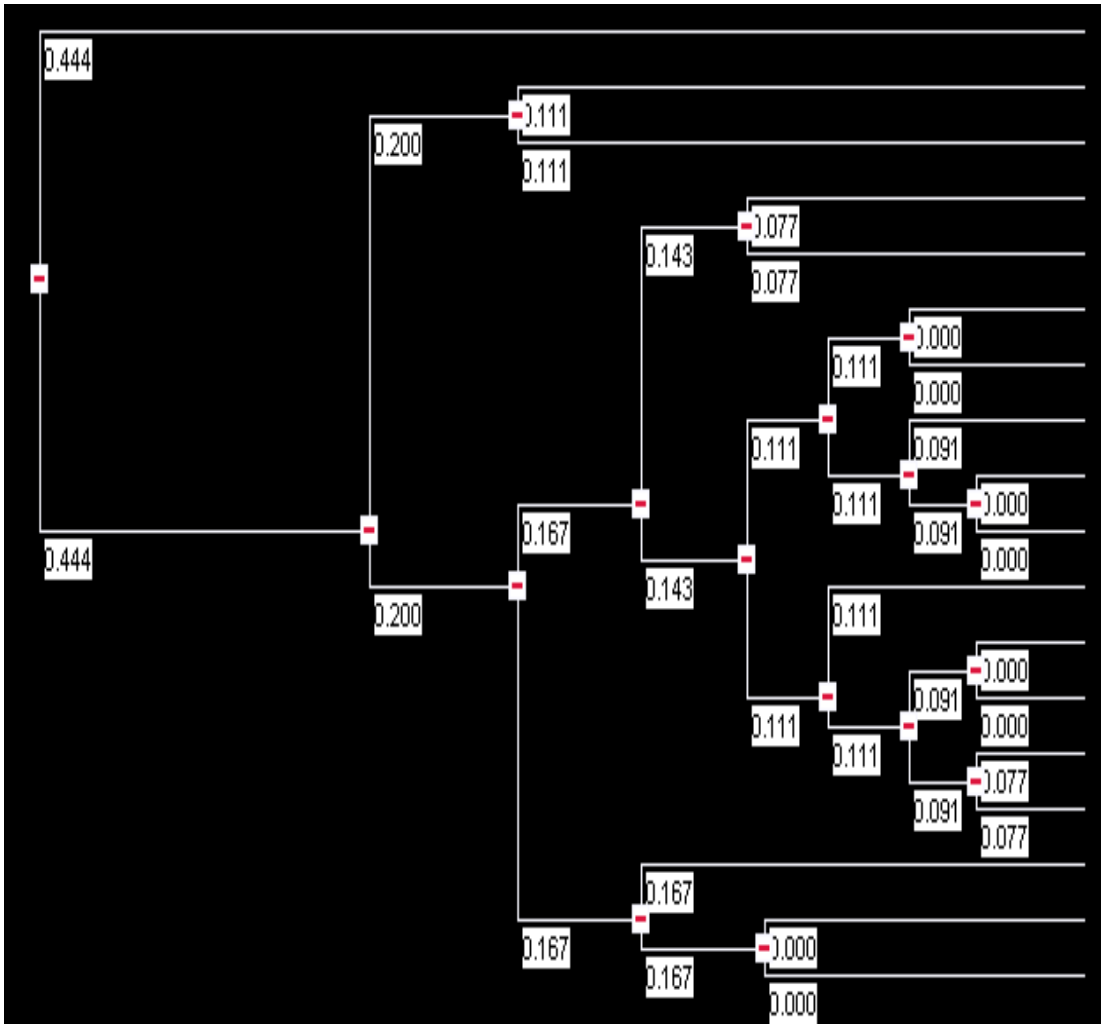


Figure 4.10: Dendrogram for Biomeme kit MSP1 amplicons analysis

The Biomeme kit MSP1 amplicon analysis dendrogram in (Figure 4.11) shows that the lower cluster had 17 strains while the upper cluster had only 1. A genetic diversity pairwise distances of relatedness for the whole data set ranged from 0.077 to 0.444. The pairwise inter-population genetic distance of the strain's relatedness was: 0.00 (8), 0.077 (4), 0.091(1), 0.11 (3), 0.167 (1) and 0.44 (1), which translated to 44.5%, 22.2%, 5.6%, 16.7%, 5.6% and 5.6%, respectively. The MSP1 gene marker showed more intra-specific diversity within the parasite.

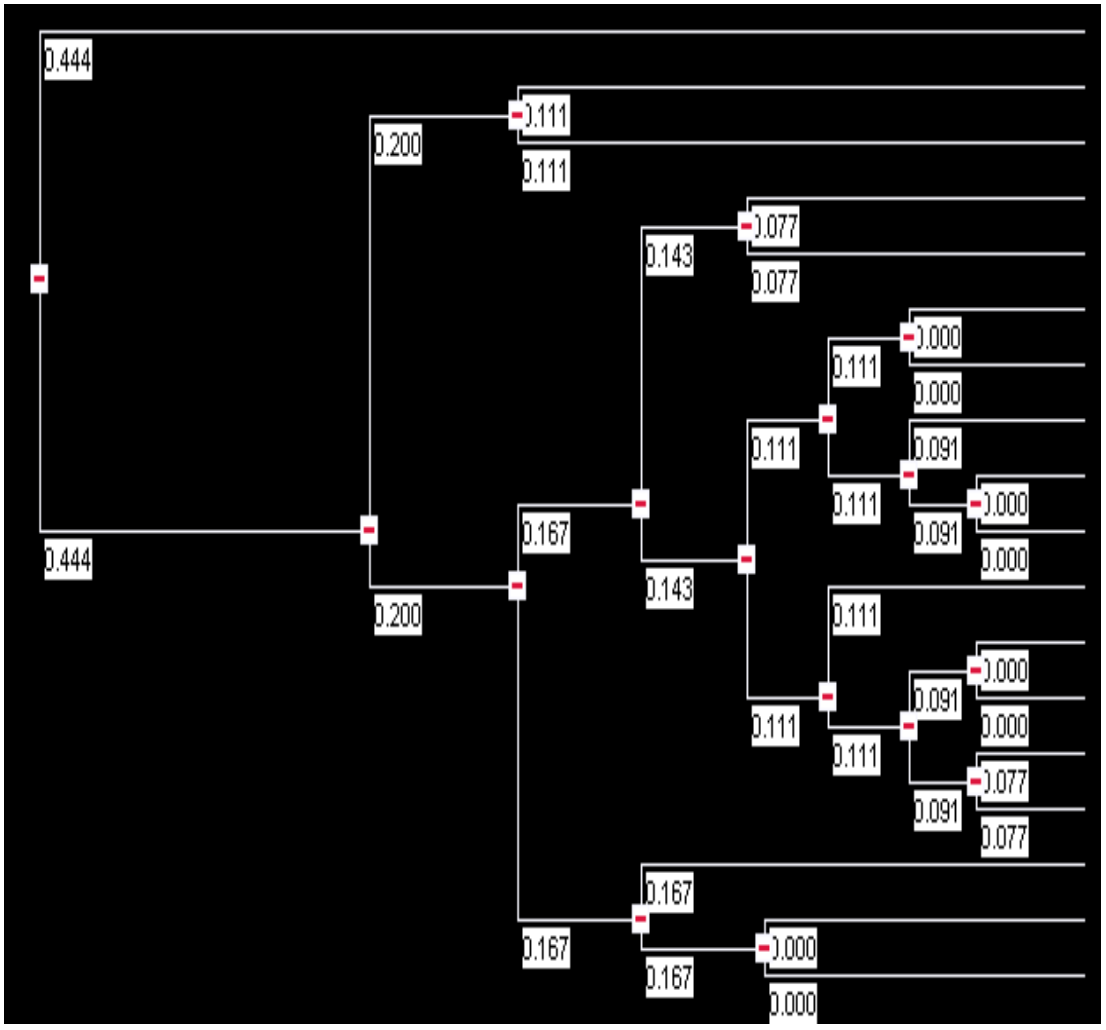
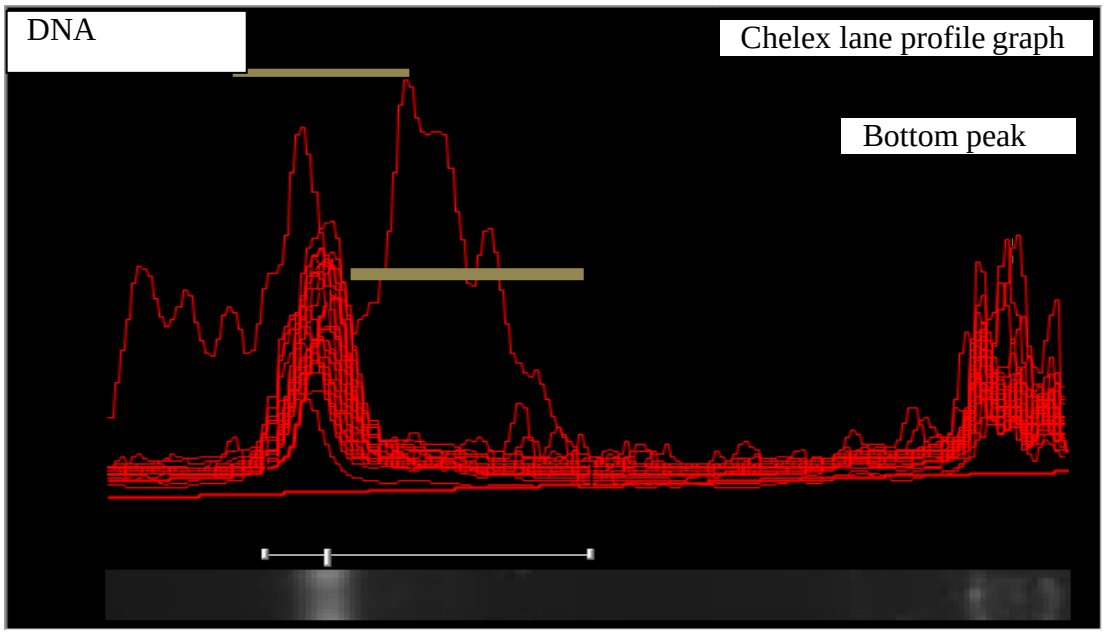


Figure 4.11: Dendrogram for the Chelex method MSP1 amplicon analysis

The dendrogram in Figure 4.12 shows that there were 21 strains of the *plasmodium* in the study site. The clusters scores were: 0.00, 1.00 (23.8%), 0.33(42.9%), and 0.5(9.5%). The pairwise genetic distance of strain relatedness ranged from 0 to 1. There was no statistically significant correlation between the dendrograms in Figures 4.11 and 4.12. There were no significant correlations between the independent sets of genetic markers, which is strong evidence for linkage disequilibrium. This was an indication of bias between 18SrRNA and MSP1.



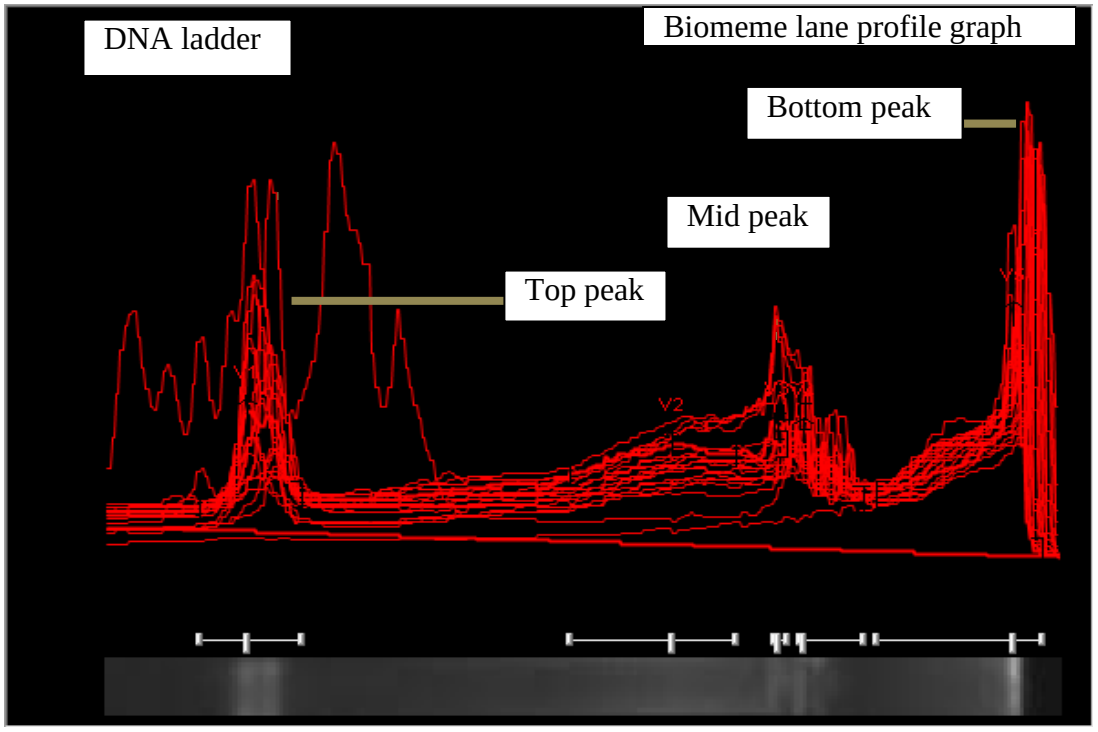


Figure 4.12: The lane profile graphs from MSP1 amplicons analysis

The lane profile graphs were measured as band intensity against the pixels. The number

of peaks represented the number of strains present. The height of the peak was an indication of its fragment size. There were three strains from the Biomeme amplicons and two predominant strains in the Chelex amplicons. For both methods of extraction post PCR amplicons were shown as heterozygous (Figure 4.13). However, the genetic patterns obtained by the different methods of DNA extraction and the use of MSP1 in this study did not show a correlation in the grouping of the strains as seen in (Figure 4.13). Panels A and B were lane profile graphs for Chelex and Biomeme amplicons respectively. In (figure 4.14) below, the presence of two peaks in both graphs suggests that there were two strains present that were dominant. The height of the peak signified the sizeof the amplicon fragments.

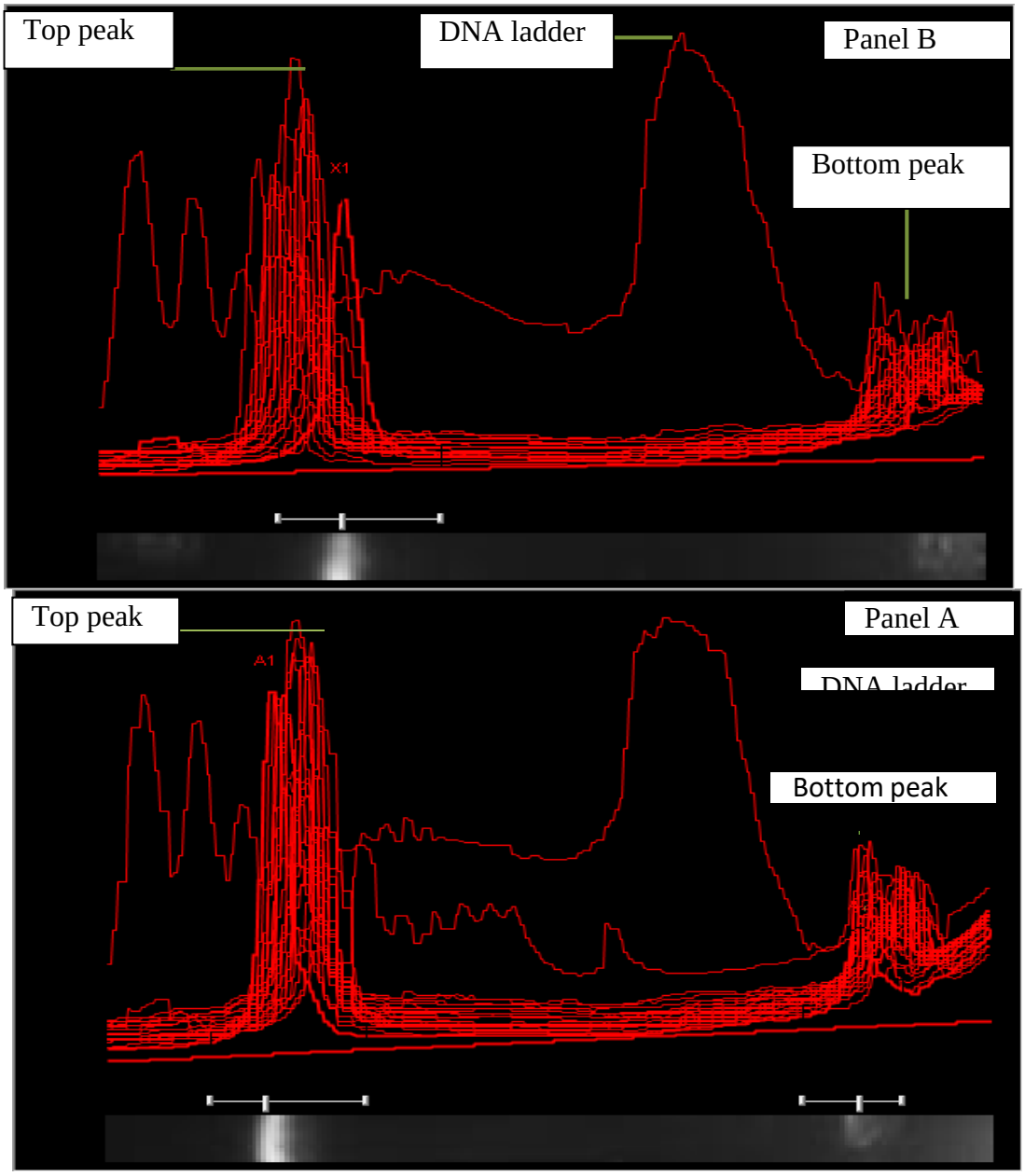


Figure 4.13: The lane profile graphs from MSP1 amplicons analysis

Panels A and B were lane profile graphs for Chelex and Biomeme amplicons respectively.

Table 4.3: Band score analysis for MSP1 amplicons

	Rf	Top	Peak	Bottom	Left	Right	I-M	I-Vol	I-%	I-Ma
BO	38.78	26217	27722	28572	22511	23111	6954	1072647	2600	2600
CO	34.8	23556	2469	25419	18520	19264	8644	1431213	2500	2500

Key; RF_ Retardation Factor, I-M_ Intensity Maxima, I-Vol_ Intensity Volume, I-%_ Intensity %, I-Ma_ Intensity Mass, BO_Biomeme summation, CO_Chelexsummation

Band scores for MSP1 amplification using DNA from the two extraction methods were almost identical (correlation = 0.999991). There was no significant difference in their values (p >0.001) as measured by a *t* test and F-test, which resulted in p-values of 0.848 and 0.39592, respectively. This was also seen in the case of the variance and covariance values, which were similar at values of 15.02 and 14.67, respectively on the *t* test.

4.5 Detection limits results from ABI 7500 real time PCR

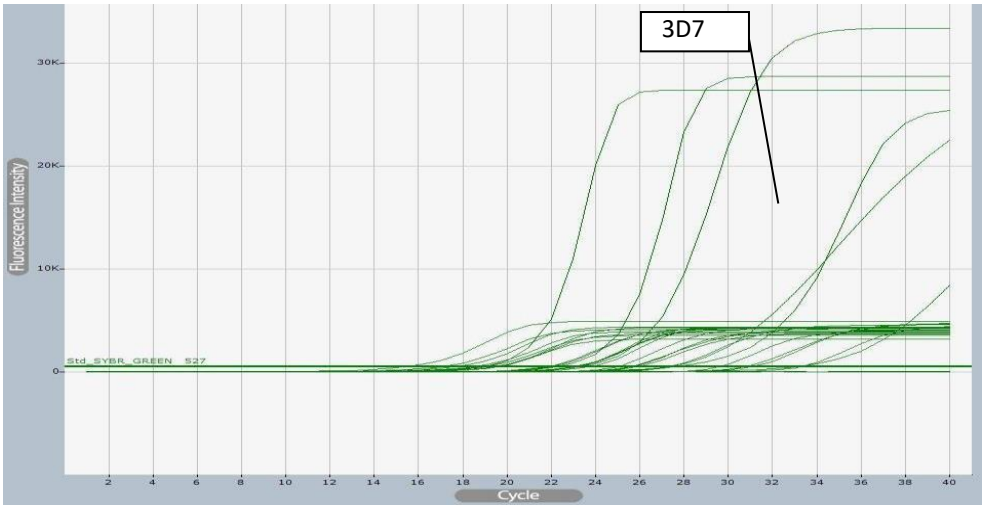


Figure 4.14: Real-time amplification with SYBR Green fluorescence detection for Chelex DNA patient samples with varying parasitemia levels.

Wild-type 3D7 was used as the positive control. The remaining curves were of patient

specimens with various parasitemia levels. The cycle threshold (Ct) values ranged from 16 to 34, which gave a dynamic range of 18.

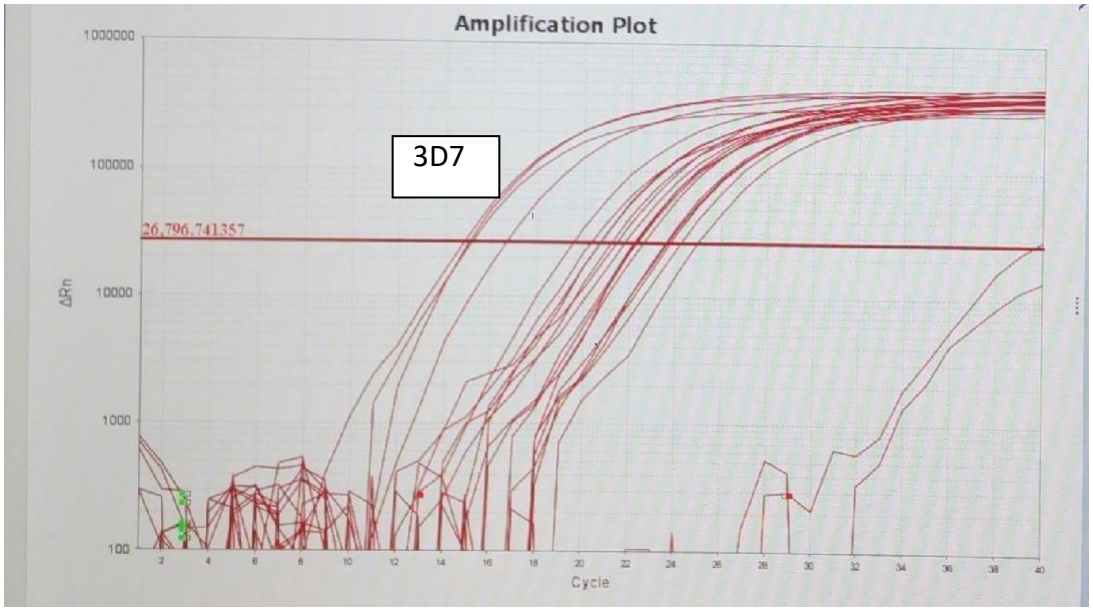


Figure 4.15: Real-time amplification curves for Biomeme DNA patient samples with varying parasitemia levels

Biomeme had low number of cycles and the Ct values with later indicating that it had high DNA

Yield as compared to the Chelex one. The Biomeme Ct ranged from 8 to 17 with a dynamic range of 9.

CHAPTER FIVE

DISCUSSION

Malaria can remain in the human body for a long time without manifestation of clinical symptoms, normally caused by partial immunity. The lack of symptoms and low parasitemia during diagnosis is a challenge (Bottius *et al*., 1996). There remains a lack of awareness concerning these atypical manifestations, which are often diagnosed late or not at all, ultimately resulting in severe complications or death. Thus, optimization of current techniques, along with the development of new technologies, is essential. Ultimately, the creation of rapid, specific, cheap, user-friendly and accessible assays that can facilitate the diagnosis of subclinical cases in the field is needed to reduce malarial transmission, morbidity and mortality.

Microscopy remains the gold standard for the diagnosis of malaria because it is comparatively less a expensive diagnostic assay and gives both qualitative and quantitative data. However, the use of microscopy requires a highly trained expert to give accurate diagnosis and quantification of parasitemia densities. As such, microscopy has limitations of sensitivity, quality control, quantity control, standardization and poor specificity (Payne, 1988; Ohrt *et al*., 2007; O'Meara *et al.*, 2006).

Molecular techniques are becoming more commonly used in malarial diagnosis (Johnston *et al*., 2006; Sauerewein *et al*., 2011; Padley *et al* 2008). However, there are differences and variability in the sensitivity of the assays, which are likely attributed to intrinsic variability in assay sensitivity or a consequence of calibration using different reference reagents, which may be poorly standardized.

In the current study, comprehensive validation tests were conducted on the Biomeme smartphone-based real-time PCR assay for the detection of human malaria at point of care. This was to investigate an alternative novel diagnostic platform that could suit resource-constrained settings e.g., field medical hospitals, dispensaries and mobile-clinic.

The quality and the integrity of the nucleic acid extracted are directly influenced by the choice of the extraction method that is deployed (Carrigg *et al.,* 2007). Inefficiencies at various stages in the extraction process could negatively affect the quality and the quantity of the final product. Such inefficiencies include incomplete cell lysis, DNA adsorption to the surface of diverse particles in the sample, damage of the extracted DNA, the loss of DNA at different stages in the extraction process and the co-extraction of assorted enzymatic inhibitors that could impede with downstream processing of the DNA, for example PCR inhibitors (Miller *et al.,* 1999; Claassen *et al.,* 2013).

The Chelex method has been found to be simple, fast, effective and cheap. It involves fewer steps than other methods and does not employ hazardous organic solvents (Walsh *et al.,* 1991; Siminato *et al*., 2007; Fernades *et al.,* 2004; Karthikeyan, 2010). However, in this case study it was evident that this method of extraction was not sufficiently fast enough as opposed to the above previous studies. For example, this extraction method needs two days for it to be done successful. When applied to the study of malarial parasites, the Chelex method has a sensitivity of 30 parasites/µl and is, therefore, suitable for the detection of low levels of parasitemia in the field (Sigh *et al.,* 1996; Morris *et al*., 2013). In addition, this method had an indication of not been sensitive to very low parasitemia level during this case study. Biomeme extraction kit proved to superior as compared to the Chelex since it was able to detect the presence of malaria at very low parasitemia of the respective samples. In as much Chelex method involve few steps, it was evident that Biomeme extraction method was more rapid as in comparison to this method.

However, it is a labor-intensive method, and the purity of the DNA extracted is low compared with commercial kits (Hawang *et al*., 2012). Another issue associated with the Chelex method is that the DNA obtained is exposed to many cycles of preheating and thawing after storage (GreensSpoon *et al*., 1998). In addition, Chelex chelates polyvalent metal ions, which may work in the breakdown of DNA and PCR inhibition (Sepp *et al.,* 1994; Barea *et al.,* 2004). In the present study, there is correlation of this sentiment, in that the method indeed was labor-intensive (14 hours). Contrary to this, Biomeme extraction was less labour intensive (50 minutes for 45 smaples) thus

making it more rapid as opposed to this extraction method. In addition, there were no pre-heating cycles which could denaturethe DNA thus making it have superior DNA quality.

From the current study, the Biomeme extraction platform has proved to be more efficient than the Chelex method. The Biomeme extraction had higher DNA yield and DNA concentrations, and the DNA purity was better than that obtained using the Chelex method. However, the Chelex method had a better turbidity ratio at absorbance A_{260}/A_{230} than the Biomeme extraction. The turbidity ratio obtained from the Biomeme samples may indicate that some chaotropic salts were not eliminated from the DNA preparation. Overall, the Biomeme extraction kit was fast, not labor-intensive, user friendly (i.e. ease of sending the data to another web portal and thereafter the phone could be used for normal uses), portable and little skill was needed to complete the extraction. In comparison, the Chelex method was relatively labor-intensive and time-consuming (at least 14 hours), and required a higher degree of technical analytical knowledge. Protein contamination was measured using the ratio of absorbance at 260 and 280 nm. Ratiosof values between 1.5 and 1.8 were taken as an indication of DNA free from aromaticcompound contamination (Weiss *et al.*, 2007).

Another important molecular parameter that was investigated in this study was genetic diversity of the *plasmodium* strains. Monitoring of the dynamics in the parasite transmission, diversity in its virulence, the potential for re-infection, recrudescence and the mechanisms of the strain immunity system can be understood in different environments (Anderson *et al.*, 200; Mu *et al*., 2005; Joy *et al*., 2003). MSP1 and MSP2 antigenic markers can be used to test for moderate and high levels of genetic diversity (Schultzs *et al*., 2010; Falk *et al*., 2006; Schoepflin *et al*., 2009; Takala *et al*., 2006). MSP1 is normally divided into seventeen blocks: seven are highly variable, five are conserved and five are semi-variable (Snounou *et al*., 1999).

The other merozoite surface protein markers used for diversity analysis in *Plasmodium falciparum* are: circumsporozoite protein (Escalante *et al.*, 2002); apical membrane antigen-1 (Oliveira *et al*., 2009); and glutamic-rich protein (Mwingira *et al*., 2011). The dendrograms for the Biomeme amplicon showed that Jaccard's coefficient ranged

from 0.00 to 1.00. The dendrograms also revealed that the Biomeme amplicon had more genetic strains than the Chelex amplicons, which may be an indication that the DNA quality (purity and turbidity) had a great impact of on the effective amplification and the overall analysis of the bands using the dendograms. It was also evident that the type of primers had an influence on the number of the strains analyzed. Merozoite Surface Protein 1 (MSP 1) gave a predicate of three dominant strains as opposed to 18S rRNA (had two strains) thus indicating the former is better in analysis of recrudescence. Possibilities of a fewer strains within our samples might be as a result of gene flow which is brought about by genetic materials exchange among the population resulting into more similarity. The platform of DNA extraction suggests to have an influence too, for example there were three predominant strains from the analysis of Biomeme amplicon while Chelex ones had only two by using MSP1 primers. This might be associated with the better quality of DNA extracted by Biomeme prep-kit as compared to Chelex method.

Molecular methods have been developed for the diagnosis of or screening for malaria in asymptomatic individuals, and have been deployed at the point of care. These include the PCR-NALFIA, which uses lateral flow as a readout. The sensitivity and specificity ranges of this method are higher than those of the Biomeme method reported in this study (Mens *et al*., 2012). However, the PCR-NALFIA method is expensive. The Biomeme method may find wider applicability as a point-of-care method owing to its cost-effectiveness, despite some loss of specificity and sensitivity.

In the present study, a comparison of conventional PCR and nested PCR using DNA extracted using two different extraction methods (Biomeme sample prep kit and Chelex method) was performed. Furthermore, the Biomeme amplifications had superior values

sensitivity and specificity) to the Chelex amplifications, which may be attributed to the superior quality and quantity of the DNA obtained using the Biomeme platform. The low values for the Chelex samples may be due to PCR inhibitors, which are normally associated with this method (Butler, 2005; Sepp *et al.*, 1994; Barea *et al.*, 2004). Interestingly, the nested PCR results obtained in this work were precise than those from the conventional PCR, and this was contrary to early findings (Snounou *et al*.,

1993; Snounou *et al*., 1996).

Analysis of Ct values during for the current study showed that real-time PCR sensitivity and specificity were influenced by the extraction method and primers used. Furthermore, results from these plotting curves show that the Biomeme amplicons Ct values were comparatively lower than those of the Chelex method. This may be attributed to DNA quality and quantity, as well as the presence or absence of enzyme inhibitors. The Ct levels are inversely proportional to the amount of target nucleic acid in the sample (the lower the Ct level, the greater the amount of target nucleic acid in the sample). The Biomeme Ct values showed a strong, positive reaction indicative of abundant target nucleic acid in the sample (Ct <29) while the Chelex Ct values demonstrated a mixture of strong and moderate positive reaction, with Ct values of 30 to 37, which indicated that the amount of the target nucleic acid was moderate. Our results from both cases demonstrated that there were no incidences of Ct values of 38 to 40, which are normally weak reactions with an indication of minimal amounts of target nucleic acid that could represent an infection state or environmental contamination. No statistical differences were revealed between the Ct pairs from each DNA sample ($p > 0.05$) and all Ct values generated from each DNA parasite sample indifferent experiments were positively associated ($p < 0.05$). Although it has been reported that in real-time PCR assays increasing cycle numbers are tentatively/apparently related to an augment of variation at the threshold cycle (Klein,2002), in our study this happened only with two clinical blood samples (1120 parasites/µl, Hb 12.6 g/dl and 1640 parasites/µl, Hb 11.0 g/dl). As a result, it was concluded that low parasitemia values were not necessarily associated with an increased Ct coefficient of variation.

Taken together, the Biomeme DNA extraction platform offered a cheap, rapid and a simple technique for molecular DNA extraction, which performed well for the detection of malarial parasites. The Biomeme smartphone- based DNA real-time PCR assay for malaria has been shown to detect low parasite concentrations and to be successfully applicable in asymptomatic *Plasmodium* infections. With the ambitious goals of improving malaria control, eradication and elimination, simple and easy-to-perform diagnostic methods need to be employed to screen patients with asymptomatic

malaria.

However, PCR, the most sensitive molecular diagnostic tool currently available, is limited by its technical requirements, particularly in resource-limited field settings. A robust, low-cost, sensitive, specific and high-throughput assay for the rapid detection of malarial parasite DNA from the finger-prick blood samples would meet this need. The Biomeme Smartphone assay has demonstrated its suitability in such settings. The present study found a number of advantages coupled with the Biomeme sample prep kit and the assay at large: first, DNA contamination was minimized as a syringe was used, and therefore, the chances of direct contact were eliminated. Post-amplification DNA contamination among samples was dramatically reduced. Second, while the Biomemesmartphone assay platform has been used here with *P. falciparum* primers, it could beadapted to detect any other infectious disease pathogens of interest, thereby broadeningthe potential applications of the platform beyond the diagnosis of malaria at point of care or in the laboratory set-up. Notable examples where this has been applied include, but are not limited to, Ebola, Gonorrhea, cancer, Syphilis and Influenza.

The turnaround time needed for the completion of this assay using the Biomeme protocol, including DNA extraction, is 45minutes (Biomeme, 2015). Storage problem associated with other reagents that make them unsuitable for fieldwork deployment in other diagnostic platforms has been overcome by lyophilization of reagents. Portability and use of an in-built battery have been a great advantage, in that as opposed to most real-time PCR machines, which are not easy to transport from one station to another, this platform weighs approximately 0.45 kg and can easily fit in a small bag. As the in-built battery is rechargeable and can run for 8 hours while doing amplification, it is well-suited for resource-constrained areas. Data extrapolation has been made possible by the use of inter-phases on this assay platform and exchange of data from one data cloud to another, thus making this platform of great assistance in instantaneous response to the medical response between the patient and the physician.

5.1 Implications for further research

Studies have showed that PCR methods have a greater sensitivity and higher specificity but on the other hand they require highly trained personnel, well established infrastructure and incur high costs that is related to their implementation in rural set-up which are resource constrained. The Biomeme smartphone DNA-based real time PCR assay was shown to be a novel alternative diagnostic platform for such areas. This method has a DNA sample preparation kit which is rapid, simple to operate and portable. The amplification process is also rapid, accurate, sensitive, specific, reproducible, and less expensive (equipment, reagents, labor, training and maintenance).

For a successful diagnostic method, there is need to have a proper understanding of its intended use; i.e., clinical practice, or for malaria control or elimination, and its dependence on the experimental setting (point-of-care field based or laboratory). Clinical test should be accurate, fast and give robust results at various conditions in the field. Focus should be put on the relationship between clinical and analytical sensitivity as incidences of low-density parasitemia is likely to be in asymptomatic population and parasitemia below the detection might result into false-negative test results. In such a population, poor analytical sensitivity has higher chances of causing poor clinical sensitivity. The diagnostic method should be able to discriminate the five *plasmodiu*m parasites in human beings.

Due to high cost of molecular techniques deployment, it will be very hard to replace microscopy and RTDs in resource constrained settings. Biomeme smartphone assay platform may be an alternative in settings which have recorded a decline in malaria. This asymptomatic population needs a more sensitive screening diagnostic tool at point-of-care. One of the limitations in this study was the number of wells on the Biomeme platform and this might be a hindrance factor if the number of patients is too high even though the diagnostic time was 45 minutes. However, there is an opinion that by facilitating earlier patient treatment and household screening, potential time and cost saving in avoidingsending samples for PCR testing at reference laboratories and ease of interpretation ofthe visual results.

Therefore, this limitation would be balanced by the rapid turn-around time for the detection of parasites in microscopy negative samples. On the same note, there is a need to have further research using microsatellite markers for identification of the *Plasmodium* strains as shown in the dendrograms and the lane profile graphs. It is likely most established laboratories will continue using their laboratory developed real-time PCR assays for detection of malaria. Despite the application of various assays, it is very critical to reach a consensus or standardized method of performing assay to facilitate the evaluation and/or comparison of the qPCR assays reported by different authors and laboratories. This will be of great importance especially for a cross-study and/or cross-platform comparison with same reference reagent(s) such as the WHO International Standard for *P. falciparum* DNA.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Malaria remains one of the major killer diseases in the world and these exert immense health and economic burdens in many economic disadvantaged countries. One of the steps that will help in the eradication and elimination of malaria will largely depend on the prompt diagnostic methods.

During this research project, these were the main findings; the parasitemia densities has a great effect on the hemoglobin densities. The higher the parasitemia the lower the hemoglobin densities. Age was also a major factor in determining hemoglobin densities during various parastiemia infection levels. Children below age five years and elderly persons of age above 70 years had lower hemoglobin densities under higher parasitemia infections. Furthermore, the quality of DNA was largely affected by the platform of extraction. On quantitative aspect, Biomeme extraction had higher DNA yields and concentrations as compared to those of Chelex. Thirdly, Biomeme has a clear DNA while Chelex' ones were ranging in colour from light yellow, yellow to brown in colour. In addition, Biome had a better quality DNA as compared to Chelex' in terms of purty while on contrary the latter had superior DNA on aspect of its turbidity ratios.

Sensitivity and specificity analysis using DNA amplicons from Biomeme on both MSP1 and 18S rRNA was higher than the Chelex ones using the same gene markers. The number of plasmodium strains noted, Biomeme had three while Chelex showed only three using MSP1 as the gene marker in profile lanes analysis while the using 18S rRNA in both case there were two predominant strains. The Ct values using Biomeme real-time PCR were lower as compared to ABI 7500 possibly indicating it could detect low concentration of DNA as compared to the later.

Biomeme smartphone assay has proven to be a novel diagnostic platform which incorporates accuracy, rapidity, sensitivity, specificity, reproducibility, robustness,

cost-effectiveness, user friendliness and simplicity in its operation. Besides, it is portable and highly connected via web portal system for the transfer of data from one point to another. The technology described in this novel study has demonstrated the potential to facilitate major advancements by supporting key areas of malaria control: as a diagnostic for malaria, for surveillance in elimination settings, and as a tool in clinical evaluations of genetic diversity.

6.2 Recommendations

The following were the recommendations based on our research findings and the suggestions from the panel of investigators. First, the technology can be recommended for adoption by bodies that deal with malaria prevention and cure. Secondly, the Biomeme DNA extraction kit has better quality DNA thus making it suitable for the molecular diagnostic studies. On the other hand, this technology suits the areas which are resources constrained, therefore, it highly recommendable for such areas. Furthermore, there is a need to further research on identification of the predominant strains of *P. falciparum* that were present among our samples by application of other microsatellites markers.

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APPENDICES

Appendix I: Case Record Form

Date……

Name……..…

Next of kin ……..

Relationship……

Gender……

Age……

Weight……

Residence……

Symptoms……

……
……

Appendix II: Ethical Clearance Forms

Informed Consent form agreement

Study title: Validation of a smartphone based-DNA real time PCR assay for the detection of human malaria at point-of-care.

Date of consent_____Month_Year__

By signing this consent to participate in the survey, I as a head of household, confirm I have been explained and have understood the above-given information regarding the survey objectives, method and benefit of in detail.

The investigator/survey team has answered all of my questions clearly and I am satisfied with the answers. I, as a head of household, give my consent to implement the study. The investigator ensures that he/she will keep my personal information confidential and will only be disclosed as part of the survey results or disclosed to the people who support or monitor the survey. The investigator ensures that if I have any questions, I can contact: Lukoye John Kedogo, Institute of Biotechnology and Research, Jomo Kenyatta University of Agriculture and Technology. P.O Box 62000-00200. Nairobi. Tel no. 254-(0)67-52711. Phone number +254724399316I, as a head of household, have read the survey description, including the consent form, and I have been answered all of my questions and understand clearly. I as a head of household, sign this form voluntarily.

Signature/thumbprint Person giving consent

Date……. (…) Name (type or print)

Signature ……. (Investigator).

Date…..…… (…) Name (type or print)

Signature ……Assigned staff Date ……...…….

(…) Name (type or print)

Signature ……Witness

Date ……...……... Name (type or print)

Additional consent for samples and shipment

Thereby also accept the blood sample taken be stored for future approved studies and further analysis out of the country if need arises.

Parent's/guardian's signature……Date Thumb print……..

Parent's/ guardian's printed name……Date Thumb print……..

Witness's signature……Date… Thumb print……

Witness's printed

name……

PI's Name……

PI's signature……

1. Information sheet for head of household

Study title: Validation of a smartphone based-DNA real time PCR assay for the detection of human malaria at point-of-care.

Sponsor: Biomeme Inc. and Japanese International Corporation Agency {JICA}

Principal investigator: Mr. Lukoye John Kedogo

Office: Institute of Biotechnology and Research, Jomo Kenyatta University of Agriculture and Technology. P.O Box62000-00200 Nairobi. Tel no. 254-(0)67-

52711.Phone no. +254724399316.

Background to this study

Malaria eradication and elimination in pandemic and endemic success depend majorly on prompt, rapid, sensitive, specific, affordable and can be applied at point of care. In order to ensure that our program helps people most at point of care, we will conduct validation trial using the new technique using Biomeme smartphone assay in areas where malaria has been reported, and you live in such an area. You and your village were randomly selected to participate in this study.

Objectives

This survey was conducted to compare the efficiency of Biomeme sample preparation kit to Chelex's DNA extraction standard protocol and secondly to compare the limits of detection by using the DNA extracted from the two methods above and lastly to assess and compare the level of performance of Biomeme smartphone assay in the lab and in the field settings.

What the survey involves

The study team visited randomly selected households in your village and interviewed the head of the household, thereafter we carried out the diagnostic test using the new technology

Study duration

The validation took approximately 42 days.

Procedures to be followed

We took the medical history, examine your child or the adults and then obtain a small amount of blood obtained by finger prick. Microscopy test for thin and thick blood was carried out and counter confirmed by WHO certified microscopist and the DNA

was extracted by Biomeme prep kit for five minutes and then validation assay of the smartphone was used to detect the presence of malaria. The same portions of the blood were tested for malaria using the conventional PCR and the real time PCR standard method in the lab. For repeatability and reproducibility, the child or the adult patients were asked to come back on the following days:1, 2, 3, 7, 14, 21, 28 and date 42. If on any of these visits the clinician thinks that you of the household member requires additional health care to cure malaria, the clinician provided the malaria treatment. No charge for this malaria treatment. If you accepted your child or the household member to the study, you can bring him/her to the hospital for free malaria diagnostics using the Biomeme smartphone assay or there and there at your house during the time you participate in the research. The transport cost and food were reimbursed by giving you 200 Ksh for using the public transport means.

Risk and Benefits to You and others

We do not believe that there are any risks to your participating in this study. Participation in this survey cost you or your family anything, and the validation assay findings were too used to improve efforts to decrease malaria in the community. There was a possibility of mild discomfort and bruising at the site the blood was been obtained. The risks to your child from participating in this study are very minimal.

Confidentiality

You or your families were not to be identified with any information that we collected from you. The information was only to be disclosed as part of overall survey results. In this way, no one person's information can be identified. You received a copy of consent form.

Refusal/withdrawal

You were completely free to participate, or not participate, in this study. After you received all information about the survey, you were free to make that decision, with no risk or harm to you. If you decided to participate in the validation trial assay, the trial

assay staff asked you to sign a form that gave us permission to interview you, and you were given a copy for yourself. Additionally, if you changed your mind during the interview and no longer wanted to participate in the study, you could stop at any time without any risk of harm to you or your family. You and your family were to continue

have access to medical care as usual at the nearest health facility.

Compensation

You received free malarial treatment and 200 Ksh as transport for participating in this validation trial.

Medical care for injuries or illness

Your child was entitled to free malaria diagnosis and treatment during your participation in this research project. Circumstances under which your child ‘s participation may be terminated without your consent: The health of your child participating may be in dangerOther conditions which might have occurred that would make continued participationdetrimental to his/her health.

Contact Information

If you could have any further questions about this study you could contacted: Mr. Lukoye John Kedogo, phone number +254724399316. If you have further questions about your rights and benefit as a participant, you could contact: The KEMRI Scientific Ethic Review Unit (SERU). P. O Box 54840-00200, Nairobi, Kenya.Tel no. (020)2722541, 2713349, 0722-205901, 0733-40003.

IF THERE ANY PORTION OF THIS CONSENT EXPLANATION SHHET THAT YOU DON’T UNDERSTAND, ASK THE INVESTIGATOR BEFORE YOU SIGNING

I acknowledged receipt of this informed Explanation,

Child's/adult'sname……

Parent's/guardian's
signature…….Date……

Witness's signature…….Date…….

2. Consent to participate in the survey for ommunity leader/headof village

Study title: Validation of a smartphone based-DNA real time PCR assay for the detection of human malaria at point-of-care.

Date of consent_____Month_Year__

By signing this consent to participate in the survey, I as a community leader, confirm I have been explained and have understood the given information in detail regarding the survey objectives, method, and potential benefit of participation in the survey.

The investigator/survey team has answered all of my questions clearly and I was satisfied with the answers. As a community leader, I was pleased to allow the survey to take place in my community.

The investigator/survey team ensured that he/she were to keep participants' personal information confidential and was only be disclosed as part of the summary results or disclosed to the people who supported or monitored the survey.

The investigator ensured that if I had any questions, I could contact: Lukoye John Kedogo, Institute of Biotechnology and Research, Jomo Kenyatta University of Agriculture and Technology. P.O Box 62000-00200. Nairobi. Tel no. 254-(0)67-52711. Phone no. +254724399316. I, as a community leader, I had read the survey description, including the consent form, and I have been answered all of my questions and understand clearly. I signed this form voluntarily as a community leader.

Signature/thumbprint……. Person giving consent

Date…… (…) Name (type or print)

Signature ……. (Investigator).

Date ……. (…) Name (type or print)

Signature……Assigned staff

Date …….. (…) Name (type or print)

Signature ……(Witness).

Date ……. (…) Name (type or print).

3. Assent form to participate in a research study for 13-17 years old

Title of Study*:* Validation of a smartphone based-DNA real time PCR assay for the detection of human malaria at point-of-care**.**

Sub-group:"13–17-Year-Old"

My name is Lukoye John Kedogo. I am astudentin the Department of Biotechnology at the University of Jomo Kenyatta University of Agriculture and Technology. I was doing a research study under supervision of Dr. Fredrick Eyase and Professor Bulimo Wallace. I would like to tell you about this study and ask if you would take part in it.

What is a research study?

It can also be defined as a scientific and systematic search for pertinent information on a specific topic. In fact, research is an art of scientific investigation. So please read this form carefully. You could discuss it with your parents or anyone else. If you have any questions about the research, you were free to ask me.

Why we were doing this study?

I and the co-investigators we were doing this study to find out more about how we can use a smartphone for the diagnosis of malaria at any place at any time and how rapid, sensitive, specific, user friendly and cost effective.

Why we talked to you about this study?

Our main goal was to learn about new diagnostic method for malaria diagnosis, we would like to study in people of around your age. We were doing this by comparing some tests of people who have malaria with tests of people who did not. We invited you to participate because you were going to know whether you have it or you do not.

What would happen if you took part in this study?

If you agreed to be in the study and your parents give permission, we would ask you to: On the first day, you and your parents would come to our out-clinic health centre. We would ask you and your parents to answer some questions about the way you thought, acted, and felt about things. You were free not answering any question at your liberty. This part would about one hour.

Blood sample

We would ask you to give a sample of your blood. This was done by a trained health technician or researcher by injecting a needle into a vein in your arm and drawing the blood into a small tube. It would take about 10 to 15 minutes at the most

Would you get healthier if you were in the study?

This was not a study about getting healthier or a treatment to make you better. Butwe hoped to learn more about advancement in molecular diagnosis which would suite rural settings with resource constraint.

Would any part of the study be uncomfortable or hurt?

Blood drawing**:** Getting your blood drawn could hurt for a few seconds from the needle stick injection, like when you got a shot at the doctor's office. Afterwards, you could have gotten a little bruise. Sometimes an infection could develop there, but that hardly ever happens in most cases.

Who would have known about your study participation?

Besides you and your parents, the researchers were the only ones who would have known about your study participation. If we were to publish reports or gave talks about this research, we would only discuss group results. We would not use your name or any other personal information that would identify you.

To help protect confidentiality, we would give your study data a code number, and kept in a file with a password that only the researchers knew. The file would be on a computer that only the researchers were allowed to use.

We planned to keep this information for ten years, in case we or other researchers want to use it later for other studies. But we would follow the same steps we have just described to keep it as confidential as possible.

Would you get paid for being in the study?

You would not be paid for being in this study. But only what happened is that your parent/guardian was given amount equivalent to your public fare and the food bought to reimburse.

Did you have to be in the study?

No, you needed not. Research is something you do only if you wanted to. No one was to get mad at you if you did not want to be in the study. And remember, you could always change your mind later if you decided you did not want to be in the study anymore

Did you have any questions?

You could ask questions about this study at any time, now or later. You could talk to

me, or your parents, or someone else at any time during the study. You can contact me, phone 0724399316 or via email lukesjohn@yahoo.com

If you could have any questions or concerns about your rights and treatment as a research subject, you could contact, KEMRI, P.O Box 54840-00200. Nairobi.

Assent form of adolescent (13–17 years old)

If you decided to participate, and your parents agree, we would give you a copy of this form to keep for future reference.

If you would like to be in this research study, please sign your name on the line below.

Subject's Name/Signature (*written by adolescent*) Date

Signature of Investigator/Person Obtaining Assent Date

Appendix III: Quantification values for Biomeme DNA

ID	x	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}/A_{230}	Factor	b	c	d
B022	69	0.131	0.065	2.15	0.01	50	20	34.5	500
B042	76	0.151	0.061	2.46	0.01	50	20	38	500
B099	83	0.161	0.065	2.54	0.01	50	20	41.5	500
B017	69	0.139	0.066	2.1	0.01	50	20	34.5	500
B3D7	61	0.121	0.055	2.21	0.01	50	20	30.5	500
B298	75	0.15	0.064	2.04	0.01	50	20	37.5	500
B040	65	0.129	0.061	2.21	0.01	50	20	32.5	500
B034	48	0.097	0.034	2.4	0.01	50	20	24	500
B217	59	0.11	0.036	2.22	0.01	50	20	29.5	500
B254	44	0.088	0.027	2.1	0.01	50	20	22.5	500
B288	53	0.105	0.043	2.46	0.01	50	20	26.5	500
B267	67	0.134	0.041	1.7	0.01	50	20	33.5	500
B216	46	0.091	0.032	2.85	0.01	50	20	23	500
B106	74	0.149	0.06	2.5	0.01	50	20	37	500
B125	92	0.184	0.075	2.44	0.01	50	20	46	500
BO	77	0.154	0.064	2.4	0.01	50	20	38.5	500
B049	58	0.116	0.054	2.14	0.01	50	20	29	500
B059	60	0.119	0.056	2.14	0.01	50	20	30	500
B150	96	0.193	0.071	2.003	0.01	50	20	48	500
B206	64	0.127	0.049	2.61	0.01	50	20	32	500
B002	56	0.112	0.035	2.03	0.01	50	20	28	500
BO17	47	0.093	0.087	1.08	0.03	50	20	23.5	500
B298	13	0.027	0.27	0.23	0.04	50	20	6.5	500
B252	95	0.191	0.115	1.66	0.01	50	20	47.5	500
B191	85	0.171	0.11	1.55	0.01	50	20	42.5	500
B282	59	0.118	0.089	1.33	0.01	50	20	29.5	500
B076	78.6	0.43	0.103	2.002	0.008	50	20	39.3	500
B180	55.6	0.003	0.001	1.8	0.006	50	20	27.8	500
B262	70.56	0.146	0.095	1.537	0.02	50	20	35.28	500
B246	79.8	0.081	0.039	2.07	0.01	50	20	39.9	500
B274	64.8	0.53	0.29	1.8278	0.007	50	20	32.4	500
B250	80.8	0.93	0.61	1.5246	0.005	50	20	40.8	500
B303	72.4	0.57	0.328	1.738	0.009	50	20	36.2	500
B191	66.8	0.555	0.255	2.176	0.01	50	20	33.4	500
B070	63.4	0.574	0.309	1.858	0.006	50	20	31.7	500
B079	36	0.72	0.43	1.667	0.01	50	20	18	500
B116	80.1	0.623	0.178	1.7	0.0065	50	20	40.05	500
B197	82.2	0.643	0.212	1.8	0.01	50	20	41.1	500
B155	80.9	0.617	0.158	1.67	0.0078	50	20	40.45	500

The concentration(x) units were ng/µl. “B” stand for Biomeme DNA extracts, ID was DNA identity code, (x) was DNA concentration, (b) was dilution factor, (c) stands for DNA yield in mg/ml while (d) was DNA volume in the elution buffer in ml.

Appendix IV: Appendix IV: Quantification values for Chelex DNA

ID	x	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}/A_{230}	Factor	b	c	d
C022	30.3	0.758	0.267	2.84	1.9	50	8	4.545	150
C042	67.3	1.682	0.54	3.11	3.49	50	8	10.095	150
C099	38.3	0.957	0.299	3.2	2.13	50	8	5.745	150
C017	58.1	1.453	0.464	3.13	2.72	50	8	8.715	150
C3D7	36.1	0.902	0.292	3.09	5.66	50	8	5.415	150
C298	46.7	1.169	0.35	3.34	10.11	50	8	7.005	150
C040	45.9	1.148	0.358	3.21	0.22	50	8	6.885	150
C034	46	1.151	0.356	3.23	0.22	50	8	6.9	150
C217	33.2	0.831	0.265	3.13	2.02	50	8	4.98	150
C254	51.5	1.289	0.418	3.03	2.65	50	8	7.725	150
C288	41.4	1.036	0.33	3.13	1.53	50	8	6.21	150
C267	71.5	1.788	0.55	3.25	1.35	50	8	10.725	150
C216	77	1.926	0.598	3.22	2.2	50	8	11.55	150
C106	38.5	0.962	0.305	3.15	3.21	50	8	5.775	150
C125	57.5	1.436	0.452	3.18	0.93	50	8	8.625	150
CO	51.5	1.282	0.396	3.25	2.52	50	8	7.725	150
C049	59.6	1.49	0.611	2.44	1.04	50	8	8.94	150
C059	43	1.075	0.344	3.13	2.69	50	8	6.45	150
C150	39.3	0.786	0.554	1.42	0.58	50	8	5.895	150
C206	33.1	0.661	0.39	1.7	2.3	50	8	4.965	150
C002	35.9	0.718	0.433	1.66	1.49	50	8	5.385	150
CO17	36	0.721	0.419	1.72	1.4	50	8	5.4	150
C298	48.3	1.209	0.396	3.06	2.15	50	8	7.245	150
C252	41.4	1.036	0.33	3.13	1.53	50	8	6.21	150
C191	35.6	0.89	0.289	3.08	2.45	50	8	5.34	150
C282	35.4	0.885	0.274	3.23	2.62	50	8	5.31	150
C076	36.8	0.919	0.29	3.17	3.92	50	8	5.52	150
C180	50.6	1.264	0.4	3.16	2.99	50	8	7.59	150
C262	36.5	0.912	0.268	3.4	3.03	50	8	5.475	150
C246	75.9	1.898	0.552	3.44	0.17	50	8	11.385	150
C274	73.6	1.841	0.569	3.24	3.11	50	8	11.04	150
C250	74.9	1.872	0.571	3.28	3.34	50	8	11.235	150
C303	77.7	1.944	0.586	3.31	2.23	50	8	11.655	150
C191	65.9	1.647	0.506	3.25	3.06	50	8	9.885	150
C070	26.9	0.672	0.223	3.01	4.04	50	8	4.035	150
C079	69.8	1.745	0.538	3.24	3.08	50	8	10.47	150
C116	50.3	1.006	0.846	1.19	0.42	50	8	7.545	150
C197	-78.7	-1.573	-1.14	1.38	0.66	50	8	11.805	150
C155	-84.2	-1.684	-1.213	1.39	0.65	50	8	12.63	150

The DNA concentrations were measured in ng/µl. “C” stands for the Chelex DNA extracts. DNA samples C197 and C155 had the lowest DNA concentrations.

Appendix V: Appendix IV: Quantification values for Chelex DNA

LD	BD	Rf	Top	Peak	Bottom	Left	Right	I-M	I-Vol	I-%	I-Mas
Z	Z1	0.12	258	283	321	622	634	146	25957	73.06	73.06
Z	Z2	0.78	384	428	432	622	634	36	8204	23.09	23.09
Z	Z3	0.82	435	436	438	622	634	36	1368	3.85	3.85
Y	Y1	0.03	258	263	273	596	608	35	5904	45.43	45.43
Y	Y2	0.81	417	433	438	596	608	87	7091	54.57	54.57
X	X1	0.11	258	281	357	572	584	33	19080	60.56	60.56
X	X2	0.81	384	433	438	572	584	102	12426	39.44	39.44
W	W1	0.11	270	280	294	548	560	109	12690	53.32	53.32
W	W2	0.8	387	432	435	548	560	65	11111	46.68	46.68
V	V1	0.1	270	279	288	524	536	48	6361	35.6	35.6
V	V2	0.76	408	422	429	524	536	45	5473	30.63	30.63
V	V3	0.81	432	434	444	524	536	59	6035	33.77	33.77
U	U1	0.1	258	278	288	500	512	94	13097	56.25	56.25
U	U2	0.8	384	431	435	500	512	43	10186	43.75	43.75
T	T1	0.09	267	277	288	477	489	111	13042	67.04	67.04
T	T2	0.77	390	425	426	477	489	43	6412	32.96	32.96
S	S1	0.09	264	277	294	454	466	127	17262	74.32	74.32
S	S2	0.75	405	420	426	454	466	60	5966	25.68	25.68
R	R1	0.09	267	276	285	430	442	80	9730	50.58	50.58
R	R2	0.77	378	424	426	430	442	40	7953	41.34	41.34
R	R3	0.79	429	430	432	430	442	37	1554	8.08	8.08
Q	Q1	0.08	258	275	288	408	420	191	23718	71.98	71.98
Q	Q2	0.78	381	426	429	408	420	63	9232	28.02	28.02
P	P1	0.08	258	274	288	383	395	184	23430	100	100
O	O1	0.08	264	274	288	359	371	60	8481	50.07	50.07
O	O2	0.78	408	428	435	359	371	97	8458	49.93	49.93
N	N1	0.03	258	263	270	336	348	199	20022	13.75	13.75
N	N2	0.08	273	275	282	336	348	149	11043	7.59	7.59
N	N3	0.13	285	286	291	336	348	82	4143	2.85	2.85
N	N4	0.17	294	295	297	336	348	58	2058	1.41	1.41
N	N5	0.2	300	301	303	336	348	61	2619	1.8	1.8
N	N6	0.29	309	320	330	336	348	87	20113	13.82	13.82
N	N7	0.51	351	368	405	336	348	203	79056	54.31	54.31
N	N8	0.73	411	416	420	336	348	97	3752	2.58	2.58
N	N9	0.78	423	427	438	36	48	64	2767	1.9	1.9
M	M1	0.07	264	273	285	311	23	77	9442	46.85	46.85
M	M2	0.73	366	416	423	311	323	27	6600	32.75	32.75
M	M3	0.79	426	430	438	311	323	34	4113	20.41	20.41
L	L1	0.07	258	272	285	288	300	117	16079	65.5	65.5
L	L2	0.76	375	422	426	288	300	47	8470	34.5	34.5
K	K1	0.07	258	272	357	264	276	120	27824	78.76	78.76
K	K2	0.76	387	423	426	264	276	40	7505	21.24	21.24

LD	BD	Rf	Top	Peak	Bottom	Left	Right	I-M	I-Vol	I-%	I-Mas
J	J1	0.06	258	271	282	241	253	131	16282	63.44	63.44
J	J2	0.75	387	421	423	241	253	42	7035	27.41	27.41
J	J3	0.79	426	429	432	241	253	33	2348	9.15	9.15
I	I1	0.06	261	270	282	213	225	87	10342	17.51	17.51
I	I2	0.74	384	419	423	213	225	58	7607	12.88	12.88
I	I3	0.78	426	427	432	213	225	73	3140	5.32	5.32
I	I4	0.97	435	468	471	213	225	201	37972	64.29	64.29
H	H1	0.06	258	269	282	189	201	73	10360	17.61	17.61
H	H2	0.72	378	413	420	189	201	29	6095	10.36	10.36
H	H3	0.79	423	429	432	189	201	48	3729	6.34	6.34
H	H4	0.98	438	470	471	189	201	203	38645	65.69	65.69
G	G1	0.06	261	269	360	166	178	88	21410	31.69	31.69
G	G2	0.73	387	417	420	166	178	27	5456	8.07	8.07
G	G3	0.97	435	469	471	166	178	204	40704	60.24	60.24
F	F1	0.06	261	269	282	144	156	89	12160	19.13	19.13
F	F2	0.73	378	417	420	144	156	32	6791	10.68	10.68
F	F3	0.77	423	424	429	144	156	74	3240	5.1	5.1
F	F4	0.97	435	468	471	144	156	205	41369	65.09	65.09
E	E1	0.06	261	269	285	124	136	114	12715	20.73	20.73
E	E2	0.72	384	413	417	124	136	27	4865	7.93	7.93
E	E3	0.77	423	424	432	124	136	46	3417	5.57	5.57
E	E4	0.96	435	467	471	124	136	208	40337	65.77	65.77
D	D1	0.06	258	269	279	102	114	137	14064	21.64	21.64
D	D2	0.76	384	422	429	102	114	49	10450	16.08	16.08
D	D3	0.96	435	467	471	102	114	210	40490	62.29	62.29
C	C1	0.06	261	269	333	86	98	113	12265	20.19	20.19
C	C2	0.72	387	415	420	86	98	39	5514	9.08	9.08
C	C3	0.77	423	424	429	86	98	37	2311	3.8	3.8
C	C4	0.96	435	467	471	86	98	212	40658	66.93	66.93
B	B1	0.06	258	269	282	69	81	99	12690	21.59	21.59
B	B2	0.72	402	415	417	69	81	46	4169	7.09	7.09
B	B3	0.96	435	467	471	69	81	212	41926	71.32	71.32
A	A1	0.97	372	468	471	53	65	215	46264	100	100

Key;

RF_ Retardation Factor, I-M_ Intensity Maxima, I-Vol_ Intensity Volume, I-% _ Intensity %, BD- Band ID, LD- Lane ID and I-Mas_ Intensity Mass.

From this table is clear that I-Max, I-Vol, I-% and I-Mass had a greater variation thus 130 a possible indicator of diversity within the strains of the parasite.

Appendix VI: Bands analysis for Chelex MSP1 amplicons

LD	BD	Rf	Top	Peak	Bottom	Left	Right	I-M	I-Vol	I-%	I-Ma
Y	Y1	0.26	330	344	360	586	598	226	27723	58.2	58.2
Y	Y2	0.95	441	469	474	586	598	97	19909	41.8	41.8
X	X1	0.08	297	310	318	565	577	51	8029	14.97	14.97
X	X2	0.27	330	345	363	565	577	233	27774	51.78	51.78
X	X3	0.95	441	468	471	565	577	101	17833	33.25	33.25
W	W1	0.27	327	345	360	541	553	103	21767	57.66	57.66
W	W2	0.93	441	465	468	541	553	82	15985	42.34	42.34
V	V1	0.26	327	344	360	523	535	137	21817	100	100
U	U1	0.24	327	339	351	477	489	216	18763	41.93	41.93
U	U2	0.43	372	374	381	477	489	45	3966	8.86	8.86
U	U3	0.94	435	467	471	477	489	99	22017	49.2	49.2
T	T1	0.23	306	338	354	451	463	227	35011	58.78	58.78
T	T2	0.96	438	470	474	451	463	98	24549	41.22	41.22
S	S1	0.25	327	342	366	427	439	122	26040	55.06	55.06
S	S2	0.92	438	464	465	427	439	88	17873	37.79	37.79
S	S3	0.97	471	472	474	427	439	79	3377	7.14	7.14
R	R1	0.24	312	340	351	406	418	116	24201	51.33	51.33
R	R2	0.93	438	465	468	406	418	86	19643	41.66	41.66
R	R3	0.97	471	472	474	406	418	88	3308	7.02	7.02
Q	Q1	0.24	321	340	357	382	394	226	32588	50.86	50.86
Q	Q2	0.96	429	470	474	382	394	126	31481	49.14	49.14
P	P1	0.23	321	338	351	360	372	230	32833	66.27	66.27
P	P2	0.95	450	469	471	360	372	103	16709	33.73	33.73
O	O1	0.21	321	335	357	337	349	142	27733	61.9	61.9
O	O2	0.91	447	461	465	337	349	90	13555	30.26	30.26
O	O3	0.95	468	469	471	337	349	99	3514	7.84	7.84
N	N1	0.24	321	339	348	313	325	175	25097	58.84	58.84
N	N2	0.91	447	461	465	313	325	90	14209	33.31	33.31
N	N3	0.95	468	469	471	313	325	90	3349	7.85	7.85
M	M1	0.2	303	333	354	291	303	186	38528	50.54	50.54
M	M2	0.93	414	466	471	291	303	119	37701	49.46	49.46
L	L1	0.04	297	303	309	271	283	231	26357	11.56	11.56
L	L2	0.1	312	315	321	271	283	226	19168	8.41	8.41
L	L3	0.16	324	325	330	271	283	187	10831	4.75	4.75
L	L4	0.21	333	334	336	271	283	159	5540	2.43	2.43
L	L5	0.26	342	344	390	271	283	186	67179	29.46	29.46
L	L6	0.66	399	416	447	271	283	255	84351	36.99	36.99
L	L7	0.88	456	457	471	271	283	198	14623	6.41	6.41
K	K1	0.21	321	335	354	246	258	247	34163	45.76	45.76
K	K2	0.91	405	462	468	246	258	106	40501	54.24	54.24
J	J1	0.23	321	337	369	223	235	137	30343	55.57	55.57
J	J2	0.89	429	458	462	223	235	100	21099	38.64	38.64

LD	BD	Rf	Top	Peak	Bottom	Left	Right	I-M	I-Vol	I-%	I-Ma
J	J3	0.93	465	466	468	223	235	79	3163	5.79	5.79
I	I1	0.23	309	338	357	200	212	223	31945	67.55	67.55
I	I2	0.88	441	457	459	200	212	111	12367	26.15	26.15
I	I3	0.93	465	466	468	200	212	76	2977	6.3	6.3
H	H1	0.19	306	331	345	178	190	209	27959	51.63	51.63
H	H2	0.87	426	455	468	178	190	115	26198	48.37	48.37
G	G1	0.2	306	332	357	154	166	201	36969	66.7	66.7
G	G2	0.88	444	457	468	154	166	101	18459	33.3	33.3
F	F1	0.19	318	331	351	129	141	182	28659	54.93	54.93
F	F2	0.88	426	457	465	129	141	91	23513	45.07	45.07
E	E1	0.21	306	335	393	106	118	155	43855	63.89	63.89
E	E2	0.87	426	455	465	106	118	107	24790	36.11	36.11
D	D1	0.2	306	333	354	83	95	207	34806	67.42	67.42
D	D2	0.88	441	456	465	83	95	93	16820	32.58	32.58
C	C1	0.21	321	334	345	63	75	72	14328	51.51	51.51
C	C2	0.86	441	453	462	63	75	96	13490	48.49	48.49
B	B1	0.2	306	333	348	40	52	149	27399	59.54	59.54
B	B2	0.86	426	452	462	40	52	86	18621	40.46	40.46
A	A1	0.18	318	328	342	18	30	208	21237	62.72	62.72
A	A2	0.87	441	454	462	18	30	81	12621	37.28	37.28

Key; RF_ Retardation Factor, I-M_ Intensity Maxima, I-Vol_ Intensity Volume, I-%

_ Intensity %, LD-L BD-Band ID and I-Ma-_ Intensity Mass.

Appendix VII: Sensitivity (SEN), specificity (SPEC), Positive likelihood ratio (PLR), Negative likelihood ratio (NLR), Disease prevalence (DP), Positive predictive value (PPV) and Negative predictive value (NPV) based on conventional PC

Conventional PCR using 18S r*RNA gen*OMIC MARKER *for undiluted DNAamplicons*

	SEN	SPEC	PLR	NLR	PPV	NPV	DP
B.DNA	97.4%	66.7%	292.5%	2.6%	97.4%	66.7%	92.7%
C.DNA	94.3%	66.3%	284.5%	7.8%	94.7%	50.0 %	92.7%

*Conventional PCR using 18S rRNA gen*omic marker *for serially diluted DNA amplicons*

	SEN	SPEC	PLR	NLR	PPV	NPV	DP
B.DNA	97.4%	50.0%	194.9%	5.1%	97.4%	50.0%	95.1%
C.DNA	94.4%	60.0%	236.1%	9.3%	94.4%	60.0%	87.8%

*Conventional PCR using MSP*1 *gen*omic marker *for undiluted DNA amplicons*

	SEN	SPEC	PLR	NLR	PPV	NPV	DP
B.DNA	97.4%	50.0%	194.9%	5.1%	97.4%	50.0%	95.1%
C.DNA	97.2%	60.0%	243.1%	4.6%	94.6%	75.0%	87.8%

*Conventional PCR using MSP*1 *gen*omic marker *for undiluted DNA amplicons*

	SEN	SPEC	PLR	NLR	PPV	NPV	DP
B.DNA	97.4%	67.0%	292.0%	3.9%	97.4%	66.7%	92.7%
C.DNA	91.7%	60.0%	229.2%	8.3%	94.3%	50.0%	87.8%

KeyB.DNA- Biomeme DNA amplicon and C.DNA-Chelex DNA amplicons

Appendix VIII: Kappa value, standard error, true prevalence and apparent prevalence

Conventional PCR using 18S rRNA genomic marker for undiluted DNA amplicons

	Kappa value	Standard error	True prevalence	Apparent prevalence
B.DNA	0.64	0.25	0.90	0.93
C.DNA	0.53	0.26	0.87	0.90

Conventional PCR using 18S rRNA genomic marker for serially *DNA amplicons*

	Kappa value	Standard error	True prevalence	Apparent prevalence
B.DNA	0.47	0.36	0.93	0.95
C.DNA	0.54	0.27	0.83	0.87

*Conventional PCR using MSP*1 *gen*omic marker *for undiluted DNA amplicon*

	Kappa value	Standard error	True prevalence	Apparent prevalence
B.DNA	0.47	0.36	0.93	0.95
C.DNA	0.63	0.21	0.85	0.90

*Conventional PCR using MSP*1 *gen*omic marker *for serially diluted DNA amplicon*

Kappa value	Standard error	True prevalence	Apparent prevalence	B.DNA
0.64	0.25	0.90	0.93	
C.DNA	0.48	0.22	0.80	0.85

KeyB.DNA- Biomeme DNA amplicon and C.DNA-Chelex DNA amplicons

Appendix IX: Amplicons score table

Biome 18*S RNA undiluted*		
$a = 37$	$c = 1$	$a + c = 38$
$b = 1$	$d = 2$	$b + d = 3$
$a + b = 38$	$c + d = 3$	$Total = 41$
Chelex 18*S RNA undiluted*		
$a = 36$	$c = 1$	$a + c = 37$
$b = 2$	$d = 2$	$b + d = 4$
$a + b = 38$	$c + d = 3$	$Total = 41$
Biomeme 18*S RNA serially diluted*		
$a = 38$	$c = 1$	$a + c = 39$
$b = 1$	$d = 1$	$b + d = 2$
$a + b = 39$	$c + d = 2$	$Total = 41$
Chelex 18*S RNA serially diluted*		
$a = 34$	$c = 2$	$a + c = 36$
$b = 2$	$d = 3$	$b + d = 5$
$a + b = 36$	$c + d = 3$	$Total = 41$
*Biomeme MSP*1 *undiluted*		
$a = 38$	$c = 1$	$a + c = 39$
$b = 1$	$d = 1$	$b + d = 2$
$a + b = 39$	$c + d = 2$	$Total = 41$
*Chelex MSP*1 *undiluted*		
$a = 35$	$c = 2$	$a + c = 37$
$b = 1$	$d = 3$	$b + d = 4$
$a + b = 36$	$c + d = 5$	$Total = 41$
*Biomeme MSP*1 *serially diluted*		
$a = 37$	$c = 1$	$a + c = 38$
$b = 1$	$d = 2$	$b + d = 3$
$a + b = 38$	$c + d = 3$	$Total = 41$
*Chelex MSP*1 *serially diluted*		
$a = 33$	$c = 2$	$a + c = 3$
$b = 3$	$d = 3$	$b + d = 6$
$a + b = 36$	$c + d = 5$	$Total = 41$