QUANTITATIVE BUFFY COAT MALARIA TEST, QBC F.A.S.T.™ TEST AND SD BIOLINE™ MALARIA RAPID TEST IN MALARIA DIAGNOSIS AT AHERO SUB-COUNTY HOSPITAL, KISUMU COUNTY

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Quantitative Buffy Coat Malaria Test, QBC F.A.S.T.™ Test And SD Bioline™ Malaria Rapid Test in Malaria Diagnosis at Ahero Sub-County Hospital, Kisumu County

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DECLARATION

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

Signature…………………….. Date……………………………

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

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KEMRI, Kenya

DEDICATION

I dedicate this thesis to my parents Josephine and Richard Adera and siblings in appreciation for their encouragement and overwhelming support.

ACKNOWLEDGEMENT

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ACRONYMS AND ABBREVIATIONS

BCP: Benzothiocarboxypurine

GM: Giemsa microscopy

KAO: Kawamoto Acridine Orange

NPV: Negative Predictive Value

PCR: Polymerase Chain Reaction

P.f   *Plasmodium falciparum*

P.m   *Plasmodium malariae*

P.o   *Plasmodium ovale*

PPV: Positive Predictive Value

QBC: Quantitative Buffy Coat

QBC F.A.S.T. ™:   QBC (Fluorescence and Staining Technologies) Test

RBC: Red Blood cell

SD RDT: SD Bioline™ Malaria Antigen Pf Rapid Diagnostic Test

WBC: White Blood cell

WHO: World Health Organization

ABSTRACT

Malaria is one of the leading causes of morbidity and mortality in Sub Saharan Africa. Effective management of malaria requires rapid and accurate diagnosis and prompt treatment with efficacious anti-malarial drugs. Accurate diagnosis of malaria requires laboratory confirmation of the presence of malaria parasites in the blood of a febrile patient. Conventional Giemsa stained peripheral blood smear examination remains the gold standard for diagnosis of malaria in developing endemic countries. However, this technique is time-consuming and difficult to interpret for inexperienced microscopists. Due to this, there is need for redoubled effort in the development of highly sensitive and robust point of care malaria diagnostics. The aim of this study was to evaluate the performances of Quantitative Buffy Coat (QBC), QBC Fluorescence and Staining Technologies™ [(QBC F.A.S.T.™) - improved QBC system] and SD Bioline™ malaria rapid test in malaria diagnosis from children< 5 yrs (n= 385) at ahero sub-county hospital, Kisumu county. Thick and thin blood smears in two replicates each were prepared then stained independently with Giemsa and F.A.S.T. Malaria stains for viewing of any malaria parasite present. Quantitative Buffy Coat capillary tubes were prepared for malaria parasite examination and SD Bioline rapid test done on the blood samples. Real-Time PCR was conducted on 40 % (n= 52) of 131 samples that were found to be positive by QBC and not by Giemsa. The Sensitivity of QBC was 90% (95% CI: 85-94), QBC F.A.S.T.™ 77% (95% CI: 71-83) and SD Bioline ™ 91% (95% CI: 86-94). The Specificities were 30% (95% CI: 24-37), 83% (95% CI: 77-88) and 67% (95% CI: 60-73) $P < 0.01$ respectively. Positive Predictive Value (PPV) for the tests were 58% (95% CI: 52-63), 83% (95% CI: 77-88) and 74% (95% CI: 63-80) whilst Negative Predictive Values (NPV) were 74% (95% CI: 63-84), 78% (95% CI: 71-83) and 87% (95% CI: 81-92) respectively. Although the standard QBC malaria test and the SD Bioline™ malaria RDT showed better sensitivity relative to the improved QBC F.A.S.T. ™ test, the latter had a better specificity. The performance of these tests remains modest against microscopy. Quantitative Buffy Coat F.A.S.T. ™ test can be used as a point-of-care malaria device although further studies should be done to test the efficacy of the test in malaria diagnoses especially quantitative diagnoses on low parasitaemia and a distinct visual morphology of the *Plasmodium malariae*.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background Information

People in more than 97 countries around the world remain at risk of malaria, with nearly 212 million estimated cases and nearly 429,000 of deaths reported in 2015 (WHO, 2016). Over 90 percent of these deaths occur in African region (WHO, 2016). Over the years clinical diagnosis of malaria and presumptive treatment of fever with anti-malarial has been the mainstay of malaria control in Africa (Gosling, 2008). However, these are not recommended because of signs and symptoms that overlap with other febrile diseases (White, 2005). On the other hand, presumptive anti-malarial treatment for any fever with no obvious alternative cause is still widely practiced and has been promoted and adopted by the Integrated Management of Childhood Illness (IMCI) as a policy in many developing countries (Gosling, 2008). This has led to significant overuse of anti-malarial drugs throughout Africa. An example is seen with the increase in treatment with artemisinin combination treatment (ACT; artesunate/mefloquin [Artequine®] or artemether/lumefantrine [Riamet®]) (Barat *et al*., 1999; Amexo *et al*., 2004; Mwangi *et al*., 2005). Increased non-rational use of anti-malarials has been shown to exert drug pressure on circulating parasites leading to the emergence of malaria parasite drug resistance (Amexo et al., 2004). In this regard, World Health Organization (WHO) currently recommends that all suspected malaria cases be parasitologically confirmed before initiating treatment (WHO, 2010b). By 2013, the WHO African region reported the largest increase in the number of suspected cases being tested (62%) compared to 47% in 2010 when this was initiated (WHO, 2014).

Laboratory confirmation of malaria infection requires the availability of affordable, rapid, sensitive and specific tests. Currently, the conventional method in malaria diagnosis is by microscopy examination of stained blood films with the ability of detecting 20-40 parasites/µL of blood in a standard blood film that allows identification

of *Plasmodium* parasites and species in good hands (Schindler *et al*., 2001). Microscopy also allows estimation of parasite density. However, the technique is labour intensive, time-consuming and requires well-trained microscopists for accurate results and is not readily deployable in remote areas lacking power source for light microscopy (Pinto *et al*., 2001). Due to these limitations the development of alternative diagnostic methods has remained imperative.

In the recent years, Quantitative Buffy Coat (standard QBC) test has been developed and found to be sensitive, easier to use and accurate being able to detect 1-2 parasites/µL of blood (Bhandari *et al*., 2008). Immunochromatographic tests such as the Rapid Diagnostic Tests (RDTs) have also been proposed as alternatives to microscopic diagnosis of malaria (WHO, 2013). They detect malaria antigen in whole or peripheral blood. Most RDTs available in the market target *P. falciparum* specific histidine-rich protein II (HRP-2) and *Plasmodium* lactate dehydrogenase enzyme (p-LDH). Some tests detect pan specific pLDH or Adolase from the parasite glycolytic pathway found in all *Plasmodium* species (Wongsrichanalai *et al*., 2007). A well-known limitation of RDTs is the occurrence of false positive results caused by persistent antigenemia even after effective anti-malarial treatment. This is especially to those tests that detect the parasite antigen histidine-rich protein 2 (HRP-2) specific to *Plasmodium falciparum* (Gitonga, 2012). Molecular techniques such as polymerase chain reaction (PCR) and flow cytometry among others have also been explored in laboratories to enhance sensitive detection of malaria parasites at very low parasite densities although they are expensive, limiting their use to reference laboratories (Tangpukdee *et al*., 2009a). All these techniques have their own limitations with respect to sensitivity, specificity, turnaround time, cost effectiveness, and ease of performance of the procedures.

Despite the reduction of malaria prevalence from 70% to about 40%, the disease still remains a major public health problem in Kenya causing 20% of all deaths in children under 5 years of age (Hay, 2010). For complete control and possible elimination of malaria there is need for a robust diagnostic tool hence need to evaluate other diagnostic techniques, compare and assess their effectiveness in malaria diagnosis. The last 10

years has seen a major drive towards the deployment of RDTs to increase accessibility to malaria diagnosis but performance remains variable (WHO, 2013). Even though there has been increasing use of these RDTs as part of national monitoring and evaluation efforts, just like other biological tests , malaria RDTs are prone to deterioration through exposure to humidity and through manufacturing faults hence laboratory and field trials are imperative in order to determine the suitability for the use of the available tests (Gitonga, 2012). This study evaluated the performance of the QBC malaria test, QBC F.A.S.T.™ test (improved QBC) and the SD Bioline™ malaria rapid test against microscopy using Giemsa stained blood slides for malaria diagnosis in young children aged < 5 years residing in a malaria holoendemic area of western Kenya.

1.2 Literature Review

1.2.1 Aetiology of malaria

Malaria is caused by an obligate intracellular protozoan parasite of the genus *Plasmodium*. Four distinct species have long been recognized to infect humans: *P. falciparum,P. vivax, P. ovale* and *P. malariae* (CDC, 2012). These malaria parasites are typically transmitted to people by mosquitoes of the genus *Anopheles*. In addition to the four species, a fifth one *P. knowlesi* which is a zoonotic parasiteusually infects has been documented to cause human infections in many countries of Southeast Asia causing severe quotidien malaria which infects macaque monkeys may even result in death (Kantele & Jokiranta, 2010).

1.2.2 The parasite

Of the four main species of *Plasmodium* that infect humans, *P. falciparum* causes the most severe illnesses and deaths and is the most common species (WHO, 2010b). Attachment of the erythrocytes to *P. falciparum* mediated by the *P. falciparum* erythrocyte membrane protein 1 on the red blood cell membrane to the receptors of the microvasculature creates a major contribution to the pathology and morbidity associated

with malaria (Chaisavaneeyakorn *et al*., 2005). Studies suggest that the mortality levels are greatest and mostly in children under 5 years of age accounting for 90% of the deaths due to malignant tertian malaria, caused by *P. falciparum* (WHO, 2011). *P. falciparum* is the most predominant species while the prevalence of *P. vivax* is also high but usually rare in sub-Saharan Africa whereas *P. ovale* is common in West Africa as well as *P.malariae* (Cook *et al*., 2008).

1.2.3 Life cycle of the parasite

The sporozoite, the infective stage of plasmodia, is injected from the salivary glands of infected mosquitoes during feeding (Strickland, 1991). After about 30 minutes the sporozoites disappear from the bloodstream and invade the hepatocytes where they remain for 9-16 days and undergo asexual replication and exo-erythrocytic schizogony (Mota *et al*., 2001). This leads to thousands of uninucleate merozoites which displace the nucleus of the liver cells. The invaded liver cells then rupture releasing the merozoites into the bloodstream (Gilles, 1993). This occurs 6-16 days after initial infection depending on the infecting *Plasmodium* species sporozoites form. *P. vivax* and *P. ovale* differentiate into hypnozoites that remain dormant for weeks, months or years hence have two exo-erythrocytic forms and hence the relapses caused by these two species. In the erythrocytic phase, the released merozoites invade RBCs where they further develop into trophozoites. The immature trophozoites are the ring forms (Tuteja, 2007). The trophozoites then develop into merozoites after some period of growth. The host RBCs ruptures releasing merozoites, waste products, haemozoin which are associated to the symptoms such as fever associated to malaria at this point (Krogstad, 1995). The released merozoites then invade fresh erythrocytes and this occurs repeatedly. The length of the development cycle differs according to the two species of the parasite, varying from 48 hours in vivax, ovale and falciparum malaria to 72 hours in *P. malariae* infections. Some merozoites then differentiate to micro and macro gametocytes (Carter, 1980) as shown in Figure 1.1.

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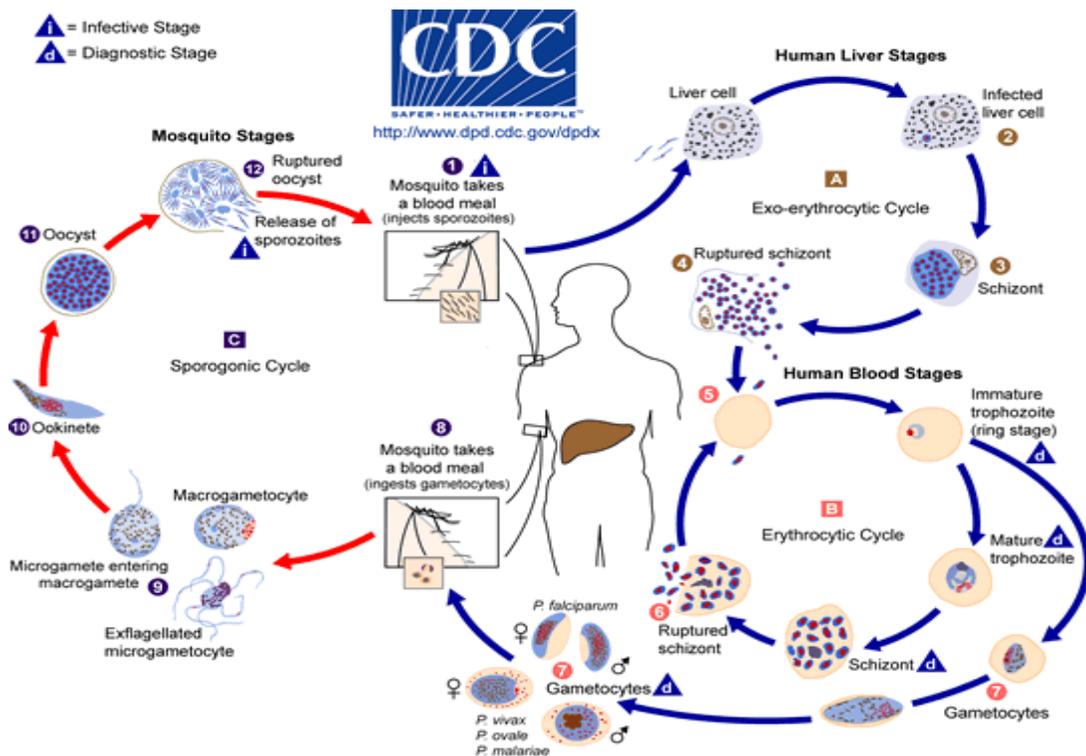
Figure 1.1: Life cycle of Plasmodium species,

Source: *www.cdc.gov/malaria/about/biology/* (2012)

1.2.3.1 Mosquito phase (Gametogony and sporogony)

The female *Anopheles* mosquito now becomes infected by ingesting the sexual forms while taking a blood meal. The micro and macro gametocytes form micro and macrogamete which then fuse to form a zygote which undergoes mitosis to form ookinetes. The ookinetes force themselves into the epithelial of the outer surfaces and develop to oocysts (Gilles, 1993). The oocyst now enlarges as the nucleus divides and ruptures releasing thousands of sporozoites (White, 1994). The sporozoites migrate to

the salivary glands, making the female mosquito infective. When the female mosquito bites a susceptible host the cycle begins again (Strickland, 1991).

1.2.4 Diagnosis of malaria

Microscopic examination of blood smears has been the most relied method in malaria diagnosis (Krafts *et al*., 2011). However in resource-poor areas, microscopic diagnosis has been shown to be insensitive and non-specific, especially when parasitaemia is low or mixed infections are present (Coleman *et al*., 2002; Amexo *et al*., 2004).

Modern techniques utilizing antigen tests such as the Rapid Diagnostic Tests (RDTs) and molecular techniques such as Polymerase Chain Reaction (PCR) have been discovered in an attempt to enhance the detection of the malaria parasite; though they require adequate laboratory facilities and so are not used as a routine option in the diagnosis of malaria in malaria endemic regions (Ling *et al*., 1986; Johnston *et al*., 2006a).

1.2.4.1 Giemsa microscopy

The current "gold standard" for malaria diagnosis in most clinical laboratories remains microscopic examination of Giemsa-stained thick and thin blood smears, but this method requires a reader with experience and well-developed pattern recognition skills to provide an accurate diagnosis (Coleman *et al*., 2002; McKenzie *et al*., 2003).

Giemsa microscopy is regarded as the most suitable diagnostic instrument for malaria control because it is inexpensive to perform, able to differentiate malaria species and quantify parasites (Jonkman *et al*., 1995). The detection threshold in Giemsa-stained thick blood smear has been estimated to be 4-20 parasites/µl (Dowling and Shute, 1966; Bruce-Chwatt, 1984; Payne, 1988).

1.2.4.2 Rapid diagnostic tests

Rapid diagnostic tests (RDTs) are slowly replacing microscopy everywhere in endemic countries at the Primary Health Care level. They detect malaria antigen in whole or peripheral blood by immunochromatographic assay. Most commonly used RDTs based on the Histidine-rich protein II (HRP-2) and on the lactate dehydrogenase enzyme (p-LDH) only detect *P. falciparum* however there are those that are pan-specific (Wongsrichanalai *et al*., 2007).

Histidine-rich protein II (HRP-2), the most common malaria antigen targeted specific for *P. falciparum* is a water soluble protein usually produced by the asexual stages of the parasite and is usually expressed on the surface of the red blood cell membrane. It is known to remain in blood for a number of days; other tests target the genus-specific aldolase enzyme and parasite lactate dehydrogenase enzymes (p-LDH) (Moody, 2002). The aldolase enzyme is an enyme in the parasite glycolytic pathway which is expressed by the blood stages of the *P. falciparum* and the other human malaria species (Milne *et al*., 1994).

1.2.4.2.1 Test performance of RDTs

The performance of RDTs crucially depends on the underlying prevalence and the type of RDT used (WHO, 2011). Several RDTs are virtually 100% sensitive at comparatively low parasite density (200 parasites/µl) albeit they may miss very low parasite densities that are of no clinical significance (WHO, 2010a). However specificity is a more complex matter in the use of RDTs. Due to their sensitivity RDTs should limit double prescription done when an antimalarial and antibacterial treatment is administered in case of a positive RDT test for the diagnosis of malaria attributable fever. Examples of the RDTs are shown in Figure 1.2.4.2. In addition to that RDTs may be useful to identify pockets of infected people, regardless of the presence of fever and treat them, both to their benefit and that of the community (Bisoffi *et al*., 2012). Hence evaluations of more RDTs should be done in the field so as to enhance malaria parasite detection. SD Bioline

Malaria Ag 05FK50 (Standard Diagnostics Inc, Hagal-Dong Korea), referred to as SD Fk50 is a one-step three-band RDT targeting histidine-rich protein II (HRP-II). The study evaluated the performance of SDFk50 in routine diagnosis.

Dipstick   Cassette   Card

Figure 1.2: Example of RDT formats

Source: Malaria Diagnostic Centre of Excellence (MDCoE), Kisumu Kenya (2012).

1.2.4.3 Fluorescence microscopy

In fluorescence microscopy, the samples are subjected to fluorescent microscope which emits energy detectable as visible light when irradiated with light of a specific wavelength. The samples can either fluoresce by itself or with the aid of fluorochromes which improves miscroscopic diagnosis though they have limitations such as cost (Moody, 2002). Quantitative Buffy Coat test (QBC), the Kawamoto that uses acridine orange and an interference filter placed in a standard microscope and the recently developed QBC F.A.S.T.™ test (QBC Diagnostics, London) are some of the many methods developed using fluorescence microscopy.

The centrifugal QBC recombines an Acridine Orange-coated capillary tube and an internal float to separate layers of WBCs and platelets using centrifugation. The Acridine Orange stains the nucleic acid of all malarial parasites although it stains nucleic acid of all cell types making it non-specific (Moody, 2002).

According to the manufacturer, QBC F.A.S.T. ™ malaria stain is a fluorescent stain that revolutionizes malaria detection with features that provide a fast clear alternative to standard Giemsa stain through improving clarity, simple training requirements and malaria staining in minutes.

Malaria parasites stained with F.A.S.T. ™ malaria stain glow bright gold or orange against a dark background with morphology typical of malaria parasites maximizing contrast during review with the aid of a fluorescence microscope. In the absence of a fluorescence microscope, a QBC Paralens Advance LED fluorescence microscope attachment which is more portable for use in the field can be attached to any standard light microscope so as to upgrade it to fluorescence microscopy through the use of a bright, durable and easy-to-power LED light source.

According to the manufacturers, F.A.S.T.^{TM} stain is sensitive, specific and have simple training requirements. This has not been validated on many clinical settings leading to the aim of this study to validate the performance of the stain based on fieldwork conditions.

1.2.4.4 Molecular techniques

Molecular malaria techniques such as PCR on blood or more recently, even on saliva samples, the loop mediated isothermal amplification (LAMP), microarray, mass spectrometry (MS) and flow cytometry (FCM) assay techniques are all new developments mainly utilized in research settings rather than during routine patient care (Tangpukdee *et al*., 2009b).

1.3 Statement of the problem

Greater access to early diagnosis and prompt treatment coverage in remote areas is one of the strategies of the National Malaria Elimination Programme 2010-2020. Moreover, World Health Organization (WHO) recommends that malaria case management be based on parasite-based diagnosis in all cases, with the exception of young children in

areas of high transmission and where lack of resources or need for urgent response temporarily limits its application. Giemsa microscopy has remained the gold standard for the laboratory diagnosis of malaria although the technique is laborious, time consuming and requires a well-trained microscopist for accurate identification. Due to these limitations there has been an increase in development of alternative diagnostic tools which are sensitive, specific and affordable. This study evaluated the performance of the Quantitative Buffy Coat test, QBC F.A.S.T. ™ test and the SD Bioline™ RDT for the detection of *Plasmodium species* causing human malaria in human blood and compare the results with the Giemsa microscopy, the gold standard.

1.4 Justification of the study

Despite recent progress towards elimination, malaria continues to affect over 219 million people per year with an estimated 660,000 deaths therefore rapid and accurate diagnosis is very important for good malaria control. Microscopy works best with well-maintained equipment, uninterrupted supply of good-quality reagents, trained staff and good quality monitoring and supervisory systems and it is the most commonly used technique in district hospitals because it is inexpensive and an easy method. To achieve complete elimination of malaria then there is need to evaluate other techniques, compare and assess their effectiveness in malaria diagnosis. Despite the development of new diagnostic technologies, microscopic examination remains the method commonly used to diagnose malaria. This study evaluated some malaria diagnostics, rather than replace methods of diagnosis.

1.5 Hypothesis

1.5.1 Null Hypothesis

The performance of Quantitative Buffy Coat, QBC F.A.S.T. ™ and SD Bioline™ RDT tests is not different from that of Giemsa microscopy the gold standard for malaria diagnosis in endemic areas.

1.6 Objectives of the Study

1.6.1 Broad objective

To evaluate the performance of Quantitative Buffy Coat malaria test (QBC), Q.B.C
F.A.S.T. ™ test and the SD Bioline™ malaria rapid test relative to Giemsa microscopy
the gold standard in malaria diagnosis at Ahero sub-County hospital, Kisumu County.

1.6.2 Specific Objectives

1. To determine the sensitivity of the QBC, QBC F.A.S.T.™ and SD Bioline™
   RDT tests relative to Giemsa microscopy the gold standard.

2. To determine the specificity of the QBC, QBC F.A.S.T.™ and SD Bioline™
   RDT tests relative to Giemsa microscopy the gold standard.

3. To determine the parasite densities from the QBC F.A.S.T.™ test relative to
   Giemsa microscopy the gold standard.

4. To compare the accuracy in malaria species identification from the QBC
   F.A.S.T.™ Test relative to Giemsa microscopy the gold standard.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study Site

The study was carried out at Ahero sub-County Hospital in Nyando District, Kisumu County which is about 24 km from Kisumu city in western Kenya as shown in Figure 2.1. The study area a high malaria transmission area because of the warm weather and swamps from the rice irrigation schemes creating suitable conditions for the growth of malaria transmitting mosquitoes (Bukhari, 2011). The hospital has antenatal and child welfare clinics from Monday to Friday from where potential study participants were screened.
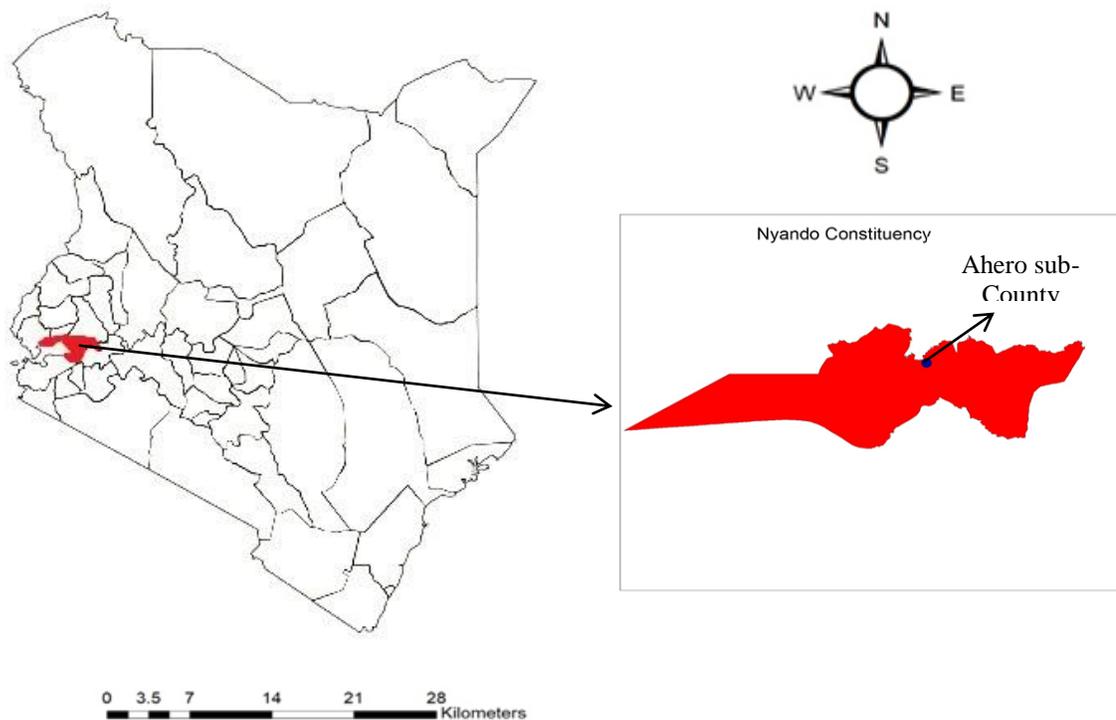
Figure 2.1: A map showing the study area, Ahero sub-County hospital Kisumu County

Source: Anastasia Wanda

2.2 Study Design

A hospital-based cross-sectional study was conducted at the Ahero sub-County Hospital, Kisumu County. Children under 5 years of age, who are among the most vulnerable group, were recruited after fulfilling the inclusion criteria.

2.3 Study Population

This study involved children below 5 years of age presented to the Maternal Child Healthcare (MCH) department and met the enrollment criteria.

2.3.1 Inclusion criteria

The study participants aged 6 – 59 months presenting with fever or had a history suggestive of malaria were recruited into the study. Signed informed consent was obtained if the potential parent/guardian demonstrated understanding of the study and was willing to enroll. In case of a language barrier and an illiterate parent, the interview was done in Luo which is the local language in the study area and a thumb print by the parent in the presence of an impartial witness.

2.3.2 Exclusion criteria

Children aged > 5 yrs who attended the hospital for reviews, children aged< 5 yrs and had taken any anti-malarial drugs within fourteen days of reporting to the hospital were not included in this study. Children whose parents/guardian did not consent were also excluded from the study.

2.4 Sampling and Sample size determination

Consecutive sampling was used on children under 5 years of age presenting with fever and had not yet taken any antimalarials. The sample size for this study was 385 children as estimated by taking the prevalence 50% as there is no study reported in the area. The sample size was determined following the formula:

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$$n = \frac{z^2(pq)}{d^2}$$

Where *n* is the sample size, *z* the critical value of the standard normal distribution at the 5% level (1.96), *p* the malaria prevalence estimate (50%), *q=1-p* and *d*=precision level (0.05) (Munyekenye, 2005).

$= 1.96^2\, 0.5\, (1\text{-}\, 0.5)/0.05^2$

$= 3.8416 \text{ x } 0.25 \,/\, 0.0025$

$= 385$

2.5 Sample collection

Consecutive parents/guardians of patients aged 6-59 months presenting at the study site were approached for recruitment. The study participants were considered eligible if they had a fever of $\geq 37.5°C$ and their medical forms had a request for smear analysis. They were included in the study if written informed consent was provided by a parent or guardian. Using methylated spirit, the middle finger or heel (in the case of babies) were disinfected by a laboratory technician and pricked using a sterile lancet. A maximum volume of 0.25 ml blood was collected into the sterile microtainer and mixed well to prevent the blood from clotting. The pricked site was cleaned with a new swab where necessary. Blood samples and request forms were then taken for processing within 2 hours of collection. Processing of the samples and data collection was performed at the study site after a re-training on malaria diagnosis by standard operating procedures and supervised by the Malaria Diagnostic Centre, Centre Clinical Research, Kenya Medical Research Institute, Kisumu.

2.6 Laboratory Investigations

Blood samples with accompanying request forms were immediately taken for processing. Four tests: QBC, QBC F.A.S.T. ™ malaria test, SD Bioline™ RDT and Giemsa microscopy the gold standard were done independently.

2.6.1 Quantitative Buffy Coat test

The QBC capillary tubes were filled with blood (55-65µl) by tilting a well-mixed blood tube and placing the capillary tube nearest to blue lines in contact with the blood keeping the tube slightly above horizontal. The tubes were then rolled several times so as to mix blood with white anticoagulant coating. The tubes were then tilted to allow blood to flow to the opposite end of the orange reagent coating so as to mix the blood with the acridine orange coating. The tubes were then sealed with a closure and a float inserted at the unsealed end of the tubes then centrifuged at 12000 rpg for 5 minutes. The centrifuged QBC malaria tubes were then inserted into a paraviewer, 2-3 drops of fluorescence optical oil added for examination of the Buffy coat area at 1000x magnification using ParaLens Advance LED florescence attachment.

2.6.2 QBC F.A.S.T.TM Test

2.6.2.1 Preparation of thick film

Thick films preparation involved placing and spreading 6µl of blood on the slide template. The slides were then dried and fixed in a horizontal position with the help of a blower for 5-10 minutes.

2.6.2.2 Preparation of thin film

Thin films were prepared by placing 2µl of well mixed blood in the slide template. A new slide (spreader) was placed in front of the drop of blood at an angle of 45 degrees. The spreader was then pulled back and held until the blood spread along the spreader

slide but not reaching the edges of the spreader. It was then pushed forward in a smooth continuous motion avoiding jerky motion or hesitation when spreading the blood to produce a feathered edge of the thin films. The thin films were then dried in a horizontal position then fixing done by gently immersing into a coplin jar containing absolute methanol.

2.6.2.4 Thick blood film staining procedure

The films were flooded with F.A.S.T. Malaria stain for 10 minutes. Excess stain tapped off then gently flooded the slides with fresh water for 5 minutes. The slides were then allowed to air dry standing vertically.

2.6.2.4 Thin blood film staining Procedure

The films were flooded with F.A.S.T. Malaria stain for 45 seconds. The stain was then tapped off then gently dipped the slides into fresh water 5 times without agitating. The slides were allowed to air dry standing vertically.

2.6.2.5 Examination and Quantification Procedure

Examination was performed in a dark room with the aid of a ParaLens Advance LED fluorescence attachment at 1000x magnification. A drop of immersion oil was added to the stained films where the monolayer of cells is located. Parasites (viewed as small fluorescent bodies with typical malaria morphological shapes) were counted against 200 leukocytes in the thick blood film and converted to number of parasites per volume assuming 8000 leukocytes/µl of blood. If the examination was done on thin blood films, then the parasitized cells were counted against the 2000 RBCs and converted to number of parasites per volume assuming 450000 RBCs/µl of blood. Slides were considered negative when no parasite was detected after viewing 100 microscopic fields. During the examination species identification on the slides was also done.

2.6.3 SD BiolineTM Malaria Rapid Test

The test devices were placed on a flat dry surface then whole blood drawn and transferred into the round wells. Four drops of assay diluents was added into each square assay well and results read after 15 minutes. A negative result was indicated with the presence of one color band, a positive result with two and an invalid result if the control line failed to appear within the result window and the specimen was retested.

2.6.4 Giemsa Microscopy

2.6.4.1 Preparation of Giemsa buffer

Ten buffer pellets was dissolved into 1000 ml distilled water and mixed thoroughly.

2.6.4.2 Preparation of Giemsa working solution

Ninety ml of buffered water was mixed thoroughly with 10 ml of Giemsa stain in a cyclinder. The cylinder was then labelled with the date of preparation, time prepared, expiration time and the initial of the one who prepared it.

2.6.4.3 Preparation of thick film

Thick films were prepared as in section 2.6.2.1.

2.6.4.4 Preparation of thin film

Thin films were prepared as in 2.6.2.2.

2.6.4.5 Staining

The slides were placed on a staining rack then flooded with Giemsa stain working solution and allowed to stand for 15 minutes. The stain was washed off gently and the slides air dried.

2.6.4.6 Examination of blood films

The examination procedure was performed with the aid of a light microscope at 1000x magnification. A drop of immersion oil was added to the stained films where the monolayer of cells is located. The slides were then viewed using $1000 \times$ magnification. Parasites were counted against 200 leukocytes in the thick blood film and converted to number of parasites per volume assuming 8,000 leukocytes/µl of blood. If the examination was done on thin blood films, then the parasitized cells were counted against the 2000 RBCs and converted to number of parasites per volume assuming 450000 RBCs/µl of blood. Slides were considered negative when no parasites were detected after viewing 100 microscopic fields. During the examination species identification was also done on the slides.

Each slide singly stained by Giemsa stain and the F.A.S.T. malaria stain was read independently by 2 study technicians and a 3rd reader in case of discrepancy at the Malaria Diagnostic Centre, Centre Clinical Research, Kenya Medical Research Institute, Kisumu.

A negative result found by QBC, QBC F.A.S.T.™, SD Bioline™ and Giemsa tests was considered as true-negative because the risk for false positive microscopy results was considered low as the slides were read independently by two experienced technicians.

2.6.5 Sampling and DNA Extraction from Blood Films

Giemsa stained blood films were soaked with 10 μl of phosphate buffered saline (PBS; 0.02 M, pH 7.4) then scraped off from the glass slide by making circular movements

with a sterile scalpel (number 15, Farla Medicals, Antwerp, Belgium). The collected material was transferred in a sterile 1.5 ml tube that contained 90 μl PBS. DNA was extracted with the QIAamp DNA Blood mini kit (Qiagen Benelux, Venlo, The Netherlands) according to the manufacturer's instructions but with reduced buffer volumes: only 100 μl of AL lysis buffer, 50 μl of ethanol and 50 μl of AE elution buffer was used. For each blood film a separate scalpel was used.

2.6.5.1 Plasmodium falciparum Reference Reagent

The WHO international standard for *P. falciparum* DNA nucleic acid amplification technology (NAT) assays, obtained from the National Institute for Biological Standards and Control (NIBSC; Hertfordshire, United Kingdom) was used as the calibration reference reagent for of the *Plasmodium* species assays. The standard consists of a freeze-dried preparation of whole blood collected by exchange transfusion from a patient infected with *P. falciparum*. Following NIBSC recommendations, this lyophilized material was suspended in 500 ml of sterile, nuclease-free water to a final concentration of 16109 IU/ml, which corresponds to a parasitemia of 9.79 parasites/100 red blood cells. The parasite density of the NAT assays after reconstitution was estimated to be 469,920 parasites/ml, based on the average red blood cell count [from uninfected donor] of 4.86106 RBC/ml. Unless otherwise indicated, fresh uninfected whole blood was used as a diluent to prepare serial dilutions. The uninfected whole blood was obtained from donors from Washington DC metropolitan area under WRAIR approved protocol. After reconstitution, genomic DNA was extracted with the EZ1 DNA blood kit on the EZ1 Advanced XL automated sample purification system (Qiagen, Valencia, CA) as recommended by the manufacturer (Kamau *et al*., 2013).

2.6.5.2 Primers and Probes Design

Primers and probes for detection of PLU 3 and RNaseP genes were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA) after the alignment of available GenBank sequences for Plasmodium 18S rRNA gene, accession number

AY579418 and human RNaseP gene, accession number NM_001104546.1. Fluorophores chosen for each assay were carefully selected and each combination extensively tested to allow optimal performance of the multiplex assay. Primer and probe sequences, their lengths and fluorophores used in this study are indicated in Table 2.1.

Table 2.1: Primers and Probes used for qPCR assay

Primers/ Probes	Sequence 5´- 3´	Size (bps)
PLU F	GCTCTTTCTTGATTTCTTGGATG	
PLU R	AGCAGGTTAAGATCTCGTTCG	
PLU P	ATGGCCGTTTTTAGTTCGTG	100
RNaseP F	TGTTTGCAGATTTGGACCTGC	
RNaseP R	AATAGCCAAGGTGAGCGGCT	
RNaseP P	TGCGCGGACTTGTGGA	84

Primers and Probes of *Plasmodium* species with their respective sequences; Forward (F), Reverse (R) and Probe (P).

2.6.5.3 Real-time PCR on Blood Films

The *Plasmodium* PLU/RNaseP real-time PCR (qPCR) assay was performed with the Applied Biosystems 7500 Fast Real-time PCR System v.2.0.5 software.

All standard procedures in the laboratory for molecular analysis including proper decontamination of the work area, proper storage of reagents, ensuring no contamination of reagents and cross contamination were followed. All the required reagents were collected and assembled in the clean area including the primers, probes, master mix

[Quantifast Probe Master Mix by Qiagen] and dH$_2$O. All the primers were at the working concentration of 10 µM and the probes at 5 µM

Each assay was performed by adding 4 µl of reaction master mix which contained all the components required for the qPCR.

The reaction master mix was prepared in multiples of 20 µl. After mixing all the components, a slight pulse vortex [10 sec] was done and then centrifuged to collect all the liquid to the bottom of the micro centrifuge tube. The following components were added to create the reaction

master mix (Table 2.2).

Table 2.2: Components of the reaction master mix used for qPCR assay

Components	*Quantity*
Quantifast Master Mix	11
PLU3F primer [10 µM]	1
PLU3R primer [10 µM]	1
PLU3 probe [5 µM]	1
RNAseP F primer [10 µM]	1
RNAseP R primer [10 µM]	1
RNAseP probe [5 µM]	1
ROX	0.4
dH$_2$0	2.6

Four μl of the reaction master mix was dispensed in each well of the tube following the experiment layout followed by 1μl dH_2O in the NTC wells. One μl DNA was then added to each well then sealed with transparent caps. The reactants were then spun at low speed and loaded into the qPCR system for analysis.

The thermal profile used for the qPCR system was as follows: 5 min at 96°C, 40 cycles of 10s at 96°C; 30 seconds at 60°C, with fluorescence collected at 60°C step. All assays were run with the appropriate controls including Non-Template Control. If the assay did not contain DNA or the DNA was below the detection limit, the assay result was denoted as 'undetermined'. Real-time PCR was performed on 40%(n=52) of the 131 discrepant results at the Malaria Drug Resistance Laboratories, Department of Emerging Infectious Diseases, United States Army Medical Research Unit-Kenya (USAMRU-K), Kenya Medical Research Institute (KEMRI)/Walter Reed Project, Kisumu Kenya. All laboratory technicians were blinded to the results from each of the tests.

2.7 Data Analysis

Data analysis was conducted using SPSS software package Version 20.0 (IBM SPSS, Inc, Chicago, IL, USA) and GraphPad Software, Version 5 (GraphPad Software, Inc., La Jolla, CA). Sensitivity (SS), specificity (SP), Positive Predictive Values (PPV) and Negative Predictive Values (NPV) were calculated with 95% Confidence Intervals (CI_{95}). Agreement between tests was determined by calculating Kappa Statistics with 95% confidence intervals and interpreted with the Landis and Koch classification. Relationship in parasite densities between Giemsa microscopy slide reads and QBC F.A.S.T.™ slide reads was determined using Pearson's correlation co-efficient. P values < 0.05 were considered statistically significant.

The sensitivity, specificity, positive and negative predictive values was calculated as follows:

Sensitivity = True Positive/ (True Positive + False Negative) x 100%

Specificity = True Negative/ (True Negative + False Positive) x 100%

Positive Predictive Value = True Positive/ (True Positive + False Positive) x 100%

Negative Predictive Value = True Negative / (True Negative + False Negative) x 100%

2.8 Ethical Approval

Written informed consents were obtained from the parents/guardians of the study participants. The study was approved by National/Kenya Medical Research Institute Ethics Review Committee, Kenya (Scientific Steering Committee Number 2008) (Appendix 1).

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Sensitivity of QBC, QBC F.A.S.T.™, SD Bioline ™ using Giemsa as the gold standard

A total of three hundred and eighty five samples from children under 5 years of age were tested. QBC, QBC F.A.S.T.™, SD Bioline ™ and Giemsa tests were performed in parallel on all samples collected. Positivity rates were 310/385 (81%), 185/385 (48%), 242/385 (63%) and 198/385 (51%) for QBC, QBC F.A.S.T.™, SD Bioline™ and Giemsa tests respectively as shown in Table 3.1. A total of 32 samples were found to be negative by both QBC, QBC F.A.S.T.™, SD Bioline™ and Giemsa tests.

Table 3.1: Total number of positives and negatives for QBC, QBC F.A.S.T.™, SD Bioline ™ using Giemsa as the gold standard

Tests	Positive (%)	Negative (%)	Total
QBC	310 (81%)	75 (19%)	385
QBC F.A.S.T. ™	185 (48%)	200 (52%)	385
SD Bioline™	242 (63%)	143 (37%)	385
Giemsa	198 (51%)	187 (49%)	385

Using Giemsa test as the gold standard, the sensitivities of QBC, QBC F.A.S.T.™ and SD Bioline™ tests were 90% (95% CI: 85-94), 77% (95% CI: 71-83) and 91% (95% CI: 86-94) respectively as shown in Figure 3.1. The Positive Predictive values (PPV) for QBC, QBC F.A.S.T. ™ and SD Bioline ™ tests were 58% (95% CI : 52-63), 83% (95% CI: 77-88) and 74% (95% CI: 63-80) whilst the Negative Predictive values (NPV)

were 74% (95% CI: 63-84), 78% (95% CI: 71-83) and 87% (95% CI : 81-92) (Table 3.2).
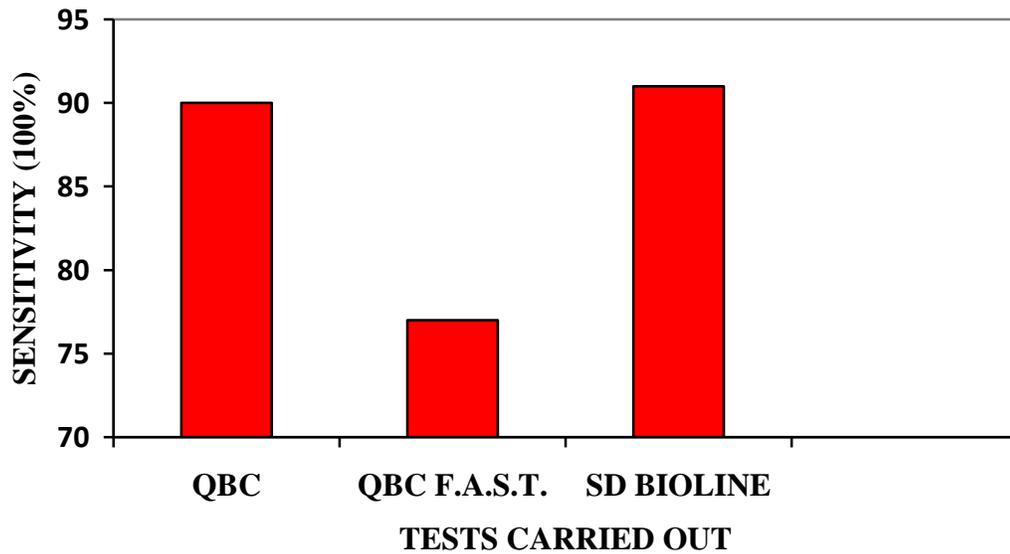
Figure 3.1: Sensitivity of QBC, QBC F.A.S.T.^{TM}, SD Bioline ^{TM} using Giemsa as the gold standard

3.2 Specificity of QBC, QBC F.A.S.T.TM, SD Bioline TM using Giemsa as the gold standard

The Specificity of QBC was 30 % (95% CI: 24-37), QBC F.A.S.T.^{TM} 83 % (95% CI: 77-88) and SD Bioline^{TM} test was 67 % (95% CI: 60-73) as shown in Figure 3.2.
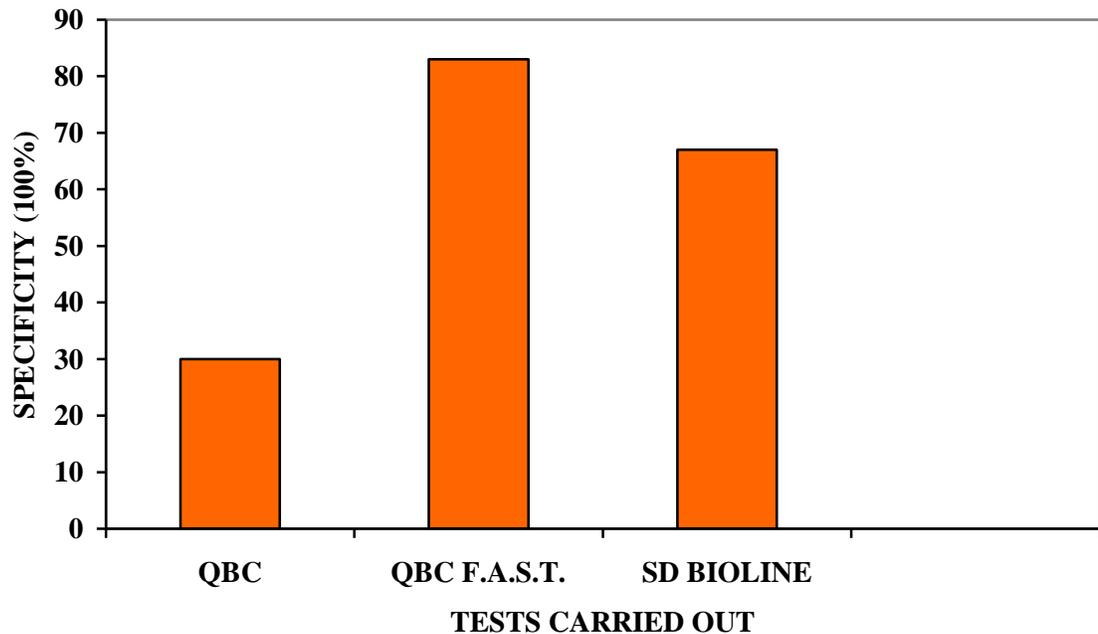
Figure 3.2: Specificity of QBC, QBC F.A.S.T.TM, SD Bioline TM using Giemsa as the gold standard

Three hundred and eighty five samples were tested by all the four tests, of which QBC had the highest number of discrepant results as compared to the other tests using Giemsa as the gold standard. One hundred and thirty one samples were found positive by QBC and not by Giemsa, hence 40% ($n = 52$) of the discrepant results were randomly picked and further analyzed by real-time PCR (qPCR) (Kamau *et al*., 2013) to ascertain the presence of *Plasmodium* DNA. Out of the 52 samples found to be malaria positive by QBC, only 6 samples (12%) had *Plasmodium* DNA by qPCR. This could be attributed to the cell debris in the environment when processing the samples or the presence of bacteria in the sample which fluoresce as malaria parasites.

Concordant rates of the other tests with those of Giemsa were 90.4% (170/198) with QBC, 77.3% (153/198) with QBC F.A.S.T.TM and 90.9% with SD BiolineTM test (Table 3.2).

Table 3.2: Concordance of QBC, QBC F.A.S.T.™ and SD Bioline ™ tests relative to Giemsa

Tests	Concordance rates
QBC	90.4% (170/198)
QBC F.A.S.T.™	77.3% (153/198)
SD Bioline	90.9% (180/198)

Assessment of agreement between tests using Giemsa as the gold standard showed there was moderate agreement with QBC and SD Bioline ™ (k=0.50 and K=0.58) respectively and a substantial agreement with QBC F.A.S.T. ™ (k=0.61). QBC, QBC F.A.S.T.™ and SD Bioline ™tests had a rather poor agreement when compared to each other: QBC vs. QBC F.A.S.T.™, k = 0.29, QBC vs. SD Bioline™, k = 0.14 and SD Bioline™ vs. QBC F.A.S.T.™, k = 0.46 (Table 3.3).

Table 3.3: Levels of agreement for all the tests QBC, QBC F.A.S.T.™ and SD Bioline™ compared to each other

Tests	QBC VsQBC F.A.S.T.	QBC VsSD Bioline	SD Bioline VsQBC F.A.S.T.
Kappa values	0.29(0.20-0.39)	0.14(0.04-0.23)	0.46(0.37-0.54)

3.3 Assessment of Parasite densities from QBC F.A.S.T.TM Test compared to Giemsa as the gold standard

The continued implementation of different malaria control interventions aim at significantly reducing the morbidity and mortality associated with malaria, and possibly eliminate malaria. The move towards elimination will require more sensitive tests to match the expected reduction in parasitaemia (The malERA Consultative Group, 2011). Consequently, the performance of QBC F.A.S.T.™ and Giemsa tests at different parasite densities was assesed (Figure 3.3). There were 153 positive cases by both QBC F.A.S.T.™ and Giemsa tests. The positive cases were divided into three percentiles representing low parasitaemia (0th and 25th percentiles, $n = 37$), moderate parasitaemia (26th to 75th percentile, $n = 77$), and high parasitaemia (76th to 100th percentile, $n = 39$) so as to determine how the two tests compared at different parasite densities. There was a substantial agreement when all the positive samples ($n = 153$) were compared (r = 0.645, $P < 0.0001$; Figure 3.3- A). As indicated in the figure, at the low parasite densities (0th and 25th percentiles) this relationship was maintained, albeit at a weaker level (r = 0.361, $P = 0.028$; Figure 3.3- B). Similarly, at the moderate parasite density the two tests were significantly correlated at a higher level than for the low parasitaemia(r = 0.478, $P < 0.0001$; Figure 3.3- C). In addition, at high parasite densities (76th to 100th percentile) this relationship was still maintained (r = 0.470, $P = 0.003$; Figure 3.3- D).
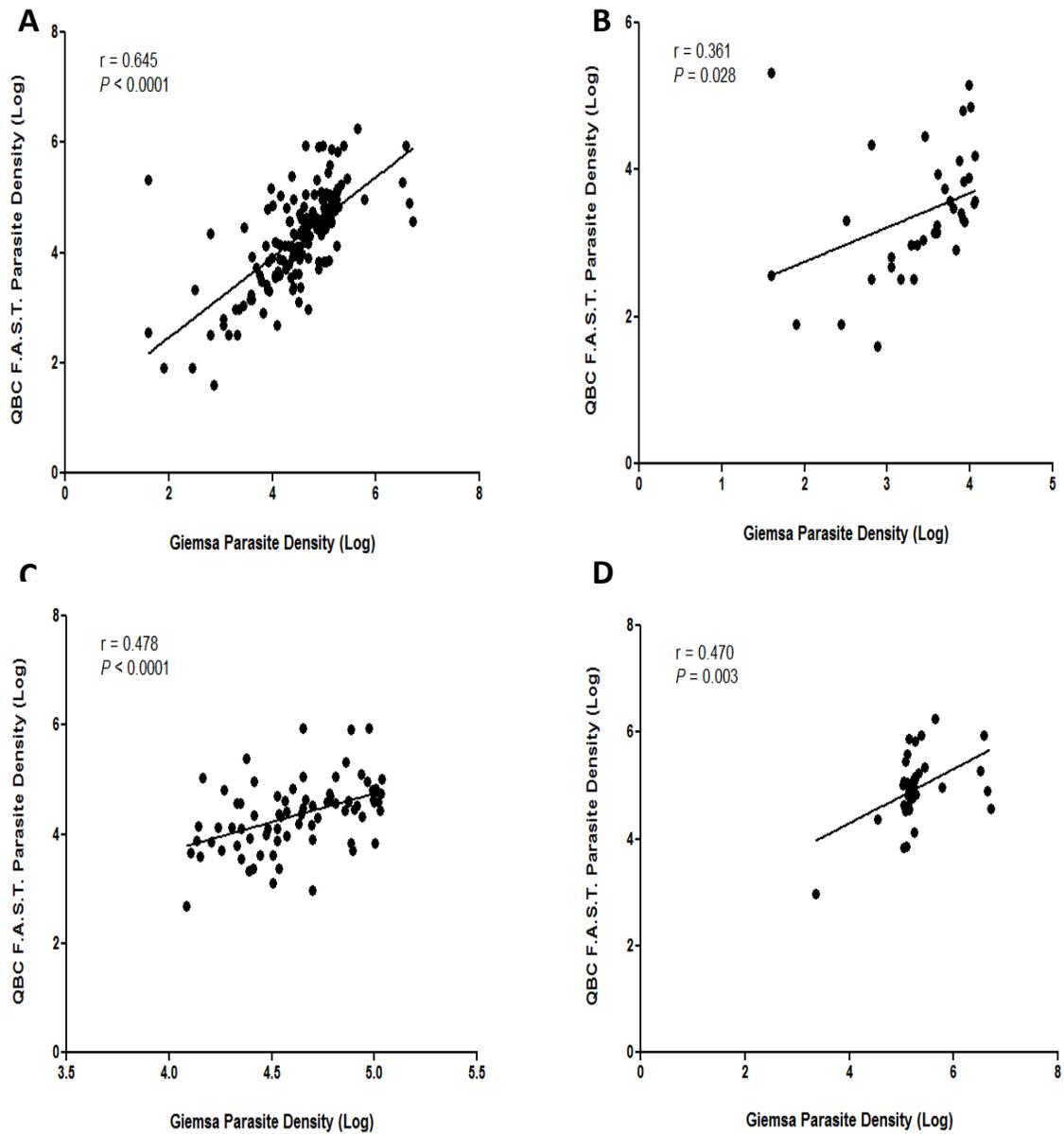
Figure 3.3: Relationship of parasite densities between QBC F.A.S.T. ™ and Giemsa tests

A) All positive samples (n= 153); B) 0th to 25th Percentile (n= 39);C) 26th to 75th Percentile (n=77); D) 76th to 100th Percentile (n=37). Analysis was performed using Pearson's correlation coefficient.

3.4 Species Identification using QBC F.A.S.T TM Test compared to Giemsa as the gold standard

During species identification, out of the 153 positive cases, QBC F.A.S.T.™ detected 150 (98%) *P. falciparum*, 0 (0%) *P.ovale*, 0 (0%) *P. malariae* and 3 (2%) mixed infections. Giemsa test detected 141 (92.2%) *P. falciparum*, 1 (0.65%) *P. malariae*, 1 (0.65%) *P. ovale* and 10 (6.5%) mixed infections. Despite the difference in identification of the mixed infections by both tests, the difference was comparable between the tests.

3.5 Discussion

3.5.1 Sensitivity of QBC, QBC F.A.S.T.TM, SD Bioline TM using Giemsa as the gold standard

The QBC and QBC F.A.S.T.™ tests are fluorescent-assisted microscopy (FAM)-based methods. QBC had 90% sensitivity, a finding that is consistent with previous studies (Schindler et al., 2001; Kuladeepa 2012; Sandhya 2012). The high sensitivity is possibly enhanced through concentration of parasitized erythrocytes and the large volume of blood collected for examination (Kuladeepa 2012). Concerns of leakage and breakage of blood filled QBC tubes in the centrifuge are some of the pitfalls associated with this diagnostic approach as experienced in this study and by other studies (Pinto *et al*., 2001; Salmani 2011). QBC F.A.S.T.™ test was found to be easy to apply as it used the same sample preparation procedure as Giemsa test. Positive cases were easily identified for parasitaemia levels > 500 parasites/µL correlating to evaluation of a test that used the same fluorescence microscopy principle (Sousa-Figueiredo *et al*., 2010). However, the test showed a rather low sensitivity when compared to the other tests. This low sensitivity could be explained by lack of clearly defined ring stages of the parasites.

SD Bioline™ had the highest sensitivity as compared to the other tests. The high sensitivity of SD Bioline™ kits which detect HRP-II antigen gives confidence that most of the malaria cases in the study population were diagnosed in agreement with previous reports (Kosack *et al*., 2013).

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3.5.2 Specificity of QBC, QBC F.A.S.T.TM, SD Bioline TM using Giemsa as the gold standard

QBC test showed a low specificity of 30%, possibly due to high rates of false positives as confirmed by the qPCR results where only 12% of the discrepant results had *Plasmodium* DNA. The low specificity reported in this study is consistent with previous studies (Schindler *et al*., 2001; Morassin *et al*., 2002). This could be explained by the presence of artefacts such as cell debris and bacteria (Bhandari *et al*., 2008; Sandhya 2012). Despite the low specificity, QBC holds promise as a good alternative in malaria diagnosis due to its speed and sensitivity (Datta, 2010).

SD Bioline ™ showed a relatively low specificity of 67%. This may have been due to the persistency of HRP-II antigen in the blood for up to 56 days after treatment (Nyunt *et al*., 2013) or ability of the RDT to detect low parasite densities (Bell, 2002) or possible deletions of HRP- II (Gamboa *et al*., 2010) although this is rare in Africa (Baker, 2005). These pose serious diagnostic challenge in malaria endemic regions resulting in over diagnosis with overtreatment. However, this is considered to have less consequence as compared to low sensitivity in the context of presumptive diagnosis. Despite the test being unable to detect non-falciparum malaria it is likely to be sensitive for the clinically most significant falciparum malaria cases as *P. falciparum* dominates the study area and it is the most lethal malaria causing *Plasmodium* species (Strom *et al*., 2013).

3.5.3 Assessment of Parasite densities from QBC F.A.S.T.TM Test compared to Giemsa as the gold standard

The parasite densities of all positive cases by both Giemsa and QBC F.A.S.T. ™ tests strongly correlated (r = 0.645, *P* < 0.0001) indicating a good performance of QBC F.A.S.T.™ on quantitative diagnosis. The weak correlation (r = 0.361, *P* = 0.028) at low parasite densities could be attributed to the field of view from Giemsa test which is visually clear enhancing distinct parasite morphology making parasite counting easier as

compared to the challenges obtained from QBC F.A.S.T.™ test where the morphology of the parasite is not clearly visible.

3.5.4 Species Identification using QBC F.A.S.T TM Test compared to Giemsa as the gold standard

Accurate identification of *Plasmodium* species is very critical because the results employed assist in correct deployment of specific control intervention strategies (Obare *et al*., 2013). Therefore, prompt and correct case diagnosis leading to accurate epidemiological assessments and optimal case management remains a critical research agenda especially in malaria endemic areas (Sousa-Figueiredo *et al*., 2010). Reliable differentiation of malaria infections is imperative since *Plasmodium* species differ in their biology, clinical symptoms and treatment regimens (Barber *et al*., 2013). Importantly, QBC F.A.S.T.™ was found to differentiate between *Plasmodium* species making it applicable to regions where falciparum and non-falciparum malaria cases are common. This was possible through the fluorescing of the parasites with typical malaria morphological stages. The test however is not very satisfactory in the diagnoses of *P. malariae* due to the lack of a very clear visual distinction of the morphology of the parasite. With the increased implementation of various control measures, significant reductions in malaria transmission intensities have been reported in some regions (O'Meara WP, 2010). The reduction in transmission intensities is expected to be accompanied by reduction in malaria parasite densities, which will require more sensitive diagnostics for better case management and possible elimination (The malERA Consultative Group, 2011). It is therefore desirable that new sensitive diagnostic tools are developed or the available tools be improved to make them more sensitive and robust. Despite malaria being a major cause of paediatric morbidity and mortality in most sub-Saharan African countries (Schumacher and Spinelli, 2012), the diagnostic tools development has remained slow and there is need for redoubled effort in the development of highly sensitive and robust point of care malaria diagnostics.

This study had limitations. Real-time PCR was performed only on the highest number of discrepant results obtained between the tests. It might be considered incorrect to use Giemsa blood stained microscopy as the gold standard for the entire study samples as compared to using real-time PCR which has a high performance. However, this was done because the risk of getting false-positives was considered low as the slides were read by trained technicians and supervised by the Malaria Diagnostics Centre, Kisumu, Kenya. Real-time PCR is a very useful gold standard in that it is highly sensitive, easily reproducible and can detect cases with low parasitemia missed by other tests. Its limitation is that the test is expensive and time and labour-consuming hence it was therefore used only to confirm the accuracy of microscopy (Johnston *et al*., 2006b).

CHAPTER FOUR

CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

All the study objectives were met and from the results it can be concluded that:

1. SD Bioline™ test had higher sensitivity relative to QBC and QBC F.A.S.T^{TM} tests with Giemsa as the gold standard.

2. QBC F.A.S.T^{TM} test had higher specifity relative to SD Bioline™ and QBC tests with Giemsa as the gold standard.

3. The parasite densities of all positive cases by both Giemsa and QBC F.A.S.T^{TM}tests strongly correlated indicating a good performance of QBC F.A.S.T^{TM}on quantitative diagnosis.

4. QBC F.A.S.T^{TM} was found to differentiate between the *Plasmodium* species.

4.2 Recommendations

From the results and conclusions of the current study the following recommendations can be made:

1. QBC can be used as a diagnostic test due to its high sensitivity.

2. SD Bioline™ may be useful to identify pockets of infected people due to its high sensitivity.

3. QBC F.A.S.T. [TM] test can be used as a point-of-care malaria device because it showed a good performance on qualitative and quantitative diagnosis.

4. Further studies should be done on the efficacy of QBC F.A.S.T. [TM] test in malaria diagnoses especially quantitative diagnoses on low parasitaemia and a distinct visual morphology of the *Plasmodium malariae*.

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APPENDICES

Appendix 1: Ethical Approval

KENYA MEDICAL RESEARCH INSTITUTE

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

ESACIPAC/SSC/101403   4th February, 2013

Bernhards Ogutu

Thro'
    Director, CCR
    NAIROBI

Forwarded 7/2/2013

REF: SSC No. 2008 (Amendment) – Human blood collection for the maintenance of quality malaria diagnostics in clinical and research settings

I am pleased to inform you that the above mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its 198th meeting held on 15th January, 2013 and has since been approved for implementation by the SSC.

Kindly submit 4 copies of the revised protocol to SSC within 2 weeks from the date of this letter i.e. 18th February, 2013 for onward transmission to the ERC office.

We advise that work on this project can only start when ERC approval is received.
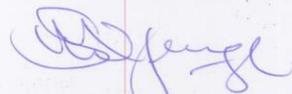
Sammy Njenga, PhD
SECRETARY, SSC

Appendix 2: Consent Form

QUANTITATIVE BUFFY COAT MALARIA TEST, QBC F.A.S.T.™ TEST AND SD BIOLINE™ MALARIA RAPID TEST IN MALARIA DIAGNOSIS AT AHERO SUB-COUNTY HOSPITAL, KISUMU COUNTY

Information to parents and guardians of children's participating in the Study.

Name of the Researcher: Anastasia Wanda Adera

Name of the institution: Kenya Medical Research Institute

Name of sponsor: Drugs for Neglected Diseases Initiatives-DNDi

My name is _____and I am working for the Kenya Medical Research Institute (KEMRI).

Purpose

Kenya Medical Research Institute (KEMRI) and Drugs for Neglected Diseases Initiative (DNDi) are conducting a study of evaluating different malaria diagnostic tests (Quantitative Buffy Coat Malaria Test, QBC F.A.S.T.™ Test and SD Bioline™ relative to Giemsa microscopy the gold standard).Malaria is one of the leading causes of morbidity and mortality in Sub Saharan Africa. Effective management of malaria requires rapid and accurate diagnosis and prompt treatment with efficacious anti-malarial drugs. Accurate diagnosis of malaria requires laboratory confirmation of the presence of malaria parasites in the blood of a febrile patient. Conventional Giemsa stained peripheral blood smear examination remains the gold standard for diagnosis of malaria in developing endemic countries. However, this technique is time-consuming, requires specialized training and may give poor results in cases with low parasitaemia hence this cross-sectional prospective cohort study will evaluate the performance of QBC, QBC F.A.S.T.™ - improved QBC system and SD Bioline^{TM} malaria rapid tests for the detection of *Plasmodium* species in human blood from children < 5 yrs in a malaria endemic area relative to Giemsa microscopy as the gold standard. We would

like to invite your child to participate in this study. For complete elimination of malaria then there is need to evaluate other techniques, compare and assess their effectiveness in malaria diagnosis. It is for this reason that this study is conducted.

Procedures

In this study, we are planning to involve 385 children between 6 to 59 months of age. Thick and thin blood smears (two replicates) will be prepared then stained independently with Giemsa stain and F.A.S.T.Malaria stain for viewing of any malaria parasite present. QBC capillary tubes will be prepared for malaria parasite examination and SD Bioline rapid test will also be done on the blood samples.

If you agree for your child to be involved in this study, your child will be seen by a doctor and a small amount of blood (5mls) will be drawn from her/his hand to verify if the child has malaria parasites.

Risks and discomforts

When participating in this study, there is a possibility of discomfort to your child during blood sampling.

Benefits

Participating in this study could help us compare and assess the effectiveness of QBC, QBC F.A.S.T.™ - improved QBC system and SD Bioline^{TM} relative to Giemsa microscopy in malaria diagnosis so as to aid in complete elimination of malaria which is still a major cause of morbidity and mortality in sub- Saharan Africa.

Confidentiality

The information that we collect from this research project will be kept confidential. Blood slides and blood samples will be stored as recommended by the responsible authorities for future reference and not for new studies.

Communication

This study has been reviewed and approved by the ethical clearance committee of the Kenya Medical Research Institute (KEMRI) which has the mandate of ensuring that studies meets the required standards and that patients are protected from harm. If you wish to find out more about the Kenya Medical Research Institute Ethical Review Committee (IRB) and ethical issues related to this study, please contact _____

Dr. Bernhards Ogutu   0733812613

Dr. John Michael Ong'echa   0733447920

Miss Grace Owino   0723260445

Miss Anastasia Adera 0724519354

Consent form

I have been invited for my child to take part in this study. I have received information about the reasons for the study which is to compare and assess the effectiveness of QBC, QBC F.A.S.T.™ - improved QBC system and SD Bioline™ relative to Giemsa microscopy in malaria diagnosis so as to aid in complete elimination of malaria in children aged 6 months to 59 months.

I have read the information above/ the information above has been read to me. I have had an opportunity to ask questions and got satisfactory answer for each question. I consent voluntarily for my child to participate in this study.

By signing this form I agree my child to participate in the study

_____

 Full name of the patient (write)

_____

Name of the Parent/Guardian (write and indicate which one)

_____

Signature of the parent/guardian (or thumb print)

Date :

If the consent is verbal: By signing this form: a witness not benefiting from the study or the patient confirms that parents of the above named patient, voluntarily agreed to participate in this study.

I have witnessed the parent of this child correctly reading or the consent form of this study correctly read to him/her and had the opportunity to ask questions.

I confirm the parent/guardian to have agreed voluntarily to participate in the study

_____

Name of the Witness (capital letters)

_____

Signature of the Witness (thumb print)

Date:

I have read or I have witnessed the parent/guardian of this child correctly reading or the consent form of this study correctly read to him/her and had the opportunity to ask questions.

I confirm the parent/guardian to have agreed voluntarily to participate in the study

Name of the researcher (write)

Signature of the researcher:

Date_____

Date: /_____/_____/_____/

Copy of this consent form has been given to the parent/guardian of the participant's child.

_____ (Initial of the researchers)

Consent Form (Luo Version)

OBOKE MAR NONRO MAR POGO PIM MAR QUANTITATIVE BUFFY COAT MALARIA TEST, QBC F.A.S.T.™ TEST AND SD BIOLINE™ KUOM GIEMSA E PIMO MALARIA EI AHERO SUB-COUNTY HOSPITAL, KISUMU COUNTY

Ler ne jonyuol gi jorit mag nyithindo mantie e nonro.
Oboke mar nonro mar pogo pim mar quantitative buffy coat malaria test, qbc f.a.s.t.™ test and sd bioline™ kuom giemsa e pimo malaria ei Ahero sub-County hospital, Kisumu County

Nying Jatend nonro: Anastasia Adera
Nying Migawo: Kenya Medical Research Institute
Jomochung ne nonro: Migawo mar yedhe mag touché ma ok odew (DNDI)

Nyinga en _____ Kendo atiyo gi jo Kenya Medical Research Institute (KEMRI).

Gima omiyo
Jo Kenya medical research institute(KEMRI) kachiel gi migawo mar yedhe mag touché ma ok odewo(DNDI)timo nonro kuom pim mar yudo Malaria ee del miluongoni Quantitative Buffy Coat Malaria, QBC F.A.S.T.™ gi SD Bioline™ mopogre gi Giemsa

49

microscopy mar kawaida ee ospital. Pim gi ibiro tiyogo kuom nyithindo matuo.Wach mongere en ni Malaria osebedo ka kelo thoo mang'eny kuom nyithindo matindo manpiny mar higni abich. E thiedho malaria yedhe gi pim malong'o dwarore mondo watiek tuoni edala wa kaa. Pim malongo mar malaria dwarore ni kute mag malaria onenre eremb jatuo.Pim mosebedo kitiyogo eosiptande okgol duoko maler ka kute mag malaria ni piny kuom jatuo kendo dwaro ngat molony e weche mag pim emomiyo watimo nonro manyien mondo wane ka pimgi mokongere eosptande nyalo konyo ee pim mar kutegi mayot. Mano emomiyo dwaher mar gwelo nyathini mondo obed jachiwre enonroni niketch tieko tuoni dwaro mondo watim nonro kaka magi.

Gik ma itimo

E nonoroni,wachano mar rwako nyithindo 385 nyiri gi yawuoyi mantie e kind dweche 6-59.

Remo mapek gi mochot idhi mien erameny asto ikete e liquid miluongo ni Giemsa gi F.A.S.T mabiro konyo wa eneno kute gi mag malaria. Remo be ibiro ket e tube moro asto ipimo kute mag malaria. Pim moro miluongoni SD Bioline be ibirotim.

Kiyie mondo warwak nyathini enonroni, laktar bironene asto ibirogol remo matin elwete (5ml) mibiropimgo kute mag malaria.

Rach:

Ka nyathini obedo jachiwre e nonroni, nitie thuolo ni onyalo winjo malit e kinde mi golo remo.

Ber:

Ka iyie ni nyathini odonj e nonroni, inyalo konyo wa pogo pim mar QBC, QBC F.A.S.T.™ - improved QBC system and SD Bioline™ gi Giemsa mosedo kitimo eosiptande. Mae biro konyo wa e tieko tuoni mar malaria ee dala wa kaa.

Maling ling

Weche ma wachoko kuom nonroni ibiro kan e yo momungore.Weche mag nyathini ma ibiro choki koa e nonro ibiro kano e file ma ok ondikie nying nyathini to mak mana namba mare. Remo ma omien e glas kod mamoko mokaw ibiro kano kaka chik migao dwaro mondo otigo e kinde mabiro to ok kuom nonro manyien.

Tudruok

Nonroni osepuodhi gi bath migao mochung ne puodho nonro e bwo migawo maduong mar Kenya Medical Research Institute(KEMRI) man gi thuolo mar chiwo chik ni nonro otim kaka chik dwaro kendo ni jochiwre ogengne gi hinyruok.

Kadiher ngeyo kuom migawo matin ma e bwo KEMRI Ethical Review Committee (ERC) (Bura mangi'yo ratiro mar timo Nonro) kod chike moluwore gi nonro ni yie itudri gi Jagoro mar KEMRI ERC, c/o Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya Tel. (020) 2722541.

Kapo in kod penjo moro amora inyalo penjo sani kata bange.ka dibed gi penjo bange to tudrigi jogi:

Dr. Bernhards Ogutu 0733812613

Dr. John Michael Ong'echa   0733447920

Miss Anastasia Adera 0724519354
Miss Grace Owino 0723260445

Otas Yieruok

Osegwela e chiwo nyathi e nonroni.

Asewinjo gima omiyo idwa tim nonro mar pogo pim QBC, QBC F.A.S.T.™ - improved QBC system and SD Bioline™ gi Giemsa mosedo kotim eosiptande mar nyiyo kute mag malaria mondo watiek tuoni kuom nyithindo ma e kind dweche 6-59 man kod tuo mar malaria.

Ase somo weche mamalogo/weche mamalogo osesomna.Aseyudo thuolo mar penjo kendo aseyudo douko mar penjo duto,ayie kuom herona mondo nyathina ochiwre e nonro.

Keto seyi e oboke ni nyiso ni ayie mondo nyathina obed e nonro

_____

Nying jatuo duto(ndiki)

_____

Nying janyuol/jarit(ler ni en mane)

_____

Lwet janyuol/jarit kaka(lith lwete mathuon)

Tarik: _____

Ka oyie gi dhok:ka ogo seyi janeno ma ok otudore gi nonro kata jatuo onego bedgi gi
adiera ni janyuol mar nyathi manyinge ni malono noyie chiwruok e nonroni.

Aseneno janyuol mar nyathini ka osom obokeno gadiera/obokeno osomne gadiera
kendo oyudo thuolo mar penjo.

Aseneno gadiera janyuol/jarit koyie kuom herone bet e nonro

_____

Nying janeno(nyukta madongo)

 _____

Seyi mar janeno(lith lwete mathuon)

Tarik:

Asesomo kata aseneno ka janyuol/jarit mar nyathini kasomo oboke mar nonroni abidha
kata otas mar yieruokno osesomne kendo obedo gi thuolo mar penjo.

Aneno gadiera ka janyuol/jarit oyie gi chiwruok e nonroni.

Nying jatij nonro(ndiki)

Seyi mar jatij nonro:_____

Tarik:/_____/_____/___/

Oboke machal gima osechiw ne janyuol/jarit mar nyathi_____

(nying jatij nonro e yo machuok)

Appendix 3: Publication

Performance of Quantitative Buffy Coat, QBC Fluorescence and Staining Technologies™ Test, and SD Bioline™ Malaria Rapid Test in Malaria Diagnosis in Western Kenya

Anastasia Adera[1*]   Peter Obare[2,3]   Andrew Nyerere[1]   Hellen Kutima[1]   Angela Achieng[3]
Hoseah Akala[3]   John Ong'echa[4]   Bernhards Ogutu[2,3]

1. School of Biological Sciences, Jomo Kenyatta University of Agriculture and Technology, PO box 62000-00200, Nairobi, Kenya
2. Malaria Diagnostics Centre, Centre for Clinical Research, Kenya Medical Research Institute, PO box 54-40100, Kisumu, Kenya
3. Department of Emerging Infectious Disease, United States Army Medical Research Unit-Kenya (USAMRU-K), Kenya Medical Research Institute (KEMRI)/Walter Reed Project, PO box 54-40100, Kisumu, Kenya
4. Centre for Global Health Research, Kenya Medical Research Institute, PO box 1578-40100, Kisumu Kenya

Abstract

Malaria remains the most important parasitic disease in sub-Saharan Africa as a cause of morbidity and mortality. Effective management of malaria relies on prompt and accurate diagnosis to guide treatment. The World Health Organization (WHO) recommends that all suspected malaria cases be tested before initiation of treatment, thus diagnosis of malaria requires parasitological confirmation of malaria parasites in the blood of suspected patients. This cross-sectional study conducted at the Ahero County Hospital, Kisumu, Kenya, evaluated the performance of quantitative buffy coat (QBC) (QBC Fluorescence and Staining Technologies™(QBC F.A.S.T.™)-improved QBC system and SD Bioline™ malaria rapid tests) against 'gold standard' (Giemsa blood stained slides microscopy) for the detection of Plasmodium species in children<five years old (n=385)in a malaria holo-endemic area of western Kenya. Real-time PCR was performed on discrepant samples across the tests and the gold standard (microscopy).Sensitivity of QBC, QBC F.A.S.T.™ and SD Bioline™ malaria rapid tests were 90% (95% CI: 85-94), 77% (95% CI: 71-83) and 91% (95% CI: 86-94), respectively, while specificity was 30% (95% CI: 24-37), 83% (95% CI: 77-88) and 67% (95% CI: 60-73), respectively. The positive predictive values (PPV) were 58% (95% CI: 52-63), 83% (95% CI: 77-88) and 74% (95% CI: 63-80), respectively, while the negative predictive values (NPV) were 74% (95% CI: 63-84), 78% (95% CI: 71-83) and 87% (95% CI: 81-92), respectively.Although the standard QBC malaria test and the SD Bioline™ malaria rapid diagnostic test (RDT) showed better sensitivity relative to the improved QBC F.A.S.T.™ test, the latter had a better specificity. The performance of these tests remains modest against microscopy.

Keywords: Malaria, Quantitative buffy coat, QBC F.A.S.T.™, SD Bioline™

1. Introduction

People living in more than 97 countries around the world remain at risk of malaria, with 198 million cases and about584,000 of deaths reported in 2013 (WHO 2014). Over 80% of these deaths occur in sub-Saharan Africa (WHO 2011). Malaria diagnosis and prompt treatment with artemisinin combination therapy (ACT) remains the mainstay of malaria control in Africa (Gosling 2008). Parasitological diagnosis is the diagnostic cornerstone of choice since malaria symptoms and signs overlap with other febrile diseases (White 2005). The World Health Organization (WHO)currently recommends that all suspected malaria cases be parasitologically-confirmed before initiating treatment (WHO 2010) and this has been adopted by many countries, including Kenya. By 2013, the WHO African Region reported the largest increase in the number of suspected cases being tested (62%) compared to 47% in 2010 when this was initiated (WHO 2014).

Confirmatory malaria infection requires the availability of affordable, rapid, sensitive, and specific tests. Currently, the malaria diagnosis 'gold standard' method remains light microscopy with a variable limit of detection of 20-40 parasites/μL in good hands (Schindler *et al.* 2001). Microscopy also allows estimation of parasite density. However, the technique is human dependant, labour intensive requiring well-trained microscopists for reliable accurate results. Microscopy is not readily deployable in remote areas that lack equipped laboratories (Pinto *et al.* 2001). Due to these limitations there has been an increase in development of alternative diagnostic tools. A number tools, such as quantitative buffy coat (standard QBC) test and immunochromatographic tests such as rapid diagnostic tests (RDTs) have been developed and deployed for routine to complement microscopy. These methods have been found to be easier to use, sensitive and accurate (Bhandari *et al.* 2008)(RDT evaluation programme) but these methods detect malaria antigens in blood. Most RDTs available on the market target *Plasmodium falciparum*-specific,histidine-rich protein II (HRP-2) and *Plasmodium*lactate dehydrogenase enzyme (p-LDH). Some tests detect pan-specific pLDH or adolase from the parasite glycolytic pathway found in all *Plasmodium* species (Wongsrichanalai *et al.* 2007). A well-known

limitation of the RDT is the occurrence of false positive results caused by persistent antigenaemia even after effective anti-malarial treatment. This is peculiar to those tests that detect the parasite antigen HRP-2 specific to *P.falciparum* (Gitonga W. Caroline and Snow W. Robert 2012). More sensitive molecular techniques, such as polymerase chain reaction (PCR) and flow cytometry, have also been explored in laboratories to enhance detection of malaria parasites at very low parasite densities. These molecular methods remain costly, limiting their use to reference laboratories (Tangpukdee *et al.* 2009). All these techniques have their own limitations with respect to sensitivity, specificity, turnaround time, cost effectiveness, and ease of performance of procedures.

Even though there has been a rapid increase the use of RDTs globally, like other biological tests, malaria RDTs are temperature sensitive (Gitonga W C and Snow W R 2012) and performance alters in case of exposure to extremes of weather. There is a need to develop and deploy more robust methods. This study evaluated the performance of the QBC malaria test, QBC F.A.S.T.™test (improved QBC)and the SD Bioline™ malaria RDT against microscopy using Giemsa-stained blood slides for malaria diagnosis in young children aged <five years residing in a malaria holo-endemic area of western Kenya.

2. Methods
2.1 Study area
The study was conducted from May to September, 2013 at the Ahero County Hospital in Kisumu County, Kenya. The study area is situated in a rice irrigation scheme with high malaria transmission (Bukhari 2011). The hospital has antenatal and child health clinics from Mondays to Fridays from where potential study participants were screened and enrolled.

2.2 Study design
A hospital-based, cross-sectional study was conducted in children <five years of age at the Ahero County Hospital, Kisumu County, Kenya after fulfilling the inclusion criteria (children aged six-59 months presenting with fever and who had not taken any anti-malarial drugs within 14 days of reporting to the hospital,fever of $\geq 37.5°C$). Individuals who had taken anti-malarial drugs within 14 days of reporting to the hospital were excluded from the study. The children were enrolled after parents/guardians provided a written informed consent obtained by research nurses and clinicians.

2.3 Ethical approval
The study was approved by National/Kenya Medical Research Institute Ethics Review Committee, Kenya (SSC# 2008).

2.4 Sample collection
Consecutive parents/guardians of patients aged six-59 months presenting at the study site were approached by study nurse/clinicians for recruitment. After consenting, capillary blood samples by finger-prick were collected into 0.5 mL microtainers (K_2EDTA-BD, USA) and processed within 2 hours of collection. The sample processing and conduct of the malaria diagnosis tests were performed at the study site by trained laboratory technicians. Prior to study initiation the technicians had a refresher training on malaria diagnosis methods by the Malaria Diagnostic Centre team, Centre Clinical Research, Kenya Medical Research Institute, Kisumu.

2.4.1 Quantitative buffy coat test
Approximately (55-65μL) of blood samples were filled into QBC capillary tubes by tilting the well-mixed blood tubes and placing the capillary tubes nearest to the blue lines in contact with the blood, keeping the tubes slightly above horizontal. The tubes were then rolled several times so as to mix the blood with the white anticoagulant coating, then tilted to allow blood to flow to the opposite end of the tubes into the orange reagent coating to mix with the acridine orange coating. The tubes were then sealed and a float inserted into each tube at the unsealed end and centrifuged at 12,000 rpm for 5 min. The centrifuged QBC tubes were then inserted into a paraviewer, two to three drops of fluorescence optical oil added for examination of the buffy coat area at 1,000× magnification using ParaLens Advance LED fluorescence attachment. The samples were processed and read at the study site by one of the study technicians.

2.4.1.1 Preparation of thick and thin blood films
Two thick and thin films per sample were prepared from each sample using6μL of blood to prepare thick film and 2μLfor thin film. The slides were air-dried and thin films were then fixed in methanol before the slides were singly stained with QBC F.A.S.T.™ or 10% Giemsa (Obare *et al.* 2013).

2.4.2 QBC F.A.S.T.™test
Thick films were individually flooded with F.A.S.T. Malaria stain for 10 min followed by fresh water for 5 min. Thin films were flooded with the stain for 45 sec and then rinsed by dipping in fresh water five times singly. Films were allowed to air dry vertically before examination. Examination of films was performed in a dark room with the aid of a ParaLens Advance LED fluorescence attachment at 1,000× magnification. A slide was

only considered negative if no parasites were detected after 100 fields were examined. Parasites were viewed as small fluorescent bodies with typical malaria morphological shapes.

2.4.3 Giemsa microscopy

Buffered water was used to prepare a 10% working Giemsa solution before staining. Blood films were individually flooded with the stain for 15 min followed by rinsing with water. Films were allowed to air dry vertically before examination. Examination of the films was performed with the aid of a light microscope at 1,000× magnification. A slide was considered negative if no parasites were detected on examining 100 microscopic fields.

All slides were read independently by two study technicians and a third reader (tie breaker) in case of discrepancy at the Malaria Diagnostic Centre, Centre Clinical Research, Kenya Medical Research Institute, Kisumu.

2.4.4 Quantification of parasite densities by QBC F.A.S.T.™ and Giemsa methods

Thick blood films were examined against 200 leukocytes. Parasite densities were estimated as parasites per volume assuming 8,000 leukocytes/µL of blood. If the parasites counted per microscopic field were 100 or more, then thin blood film examination was recorded by counting the number of parasitized cells against 2,000 red blood cells (RBCs) and converted to number of parasites per volume assuming 450,000 RBCs/µL of blood.

2.5 SD Bioline™malaria rapid test

Blood samples were added to the round wells followed by four drops of assay diluents into the square assay well. Results were read after 15 min. A negative result was indicated with the presence of one colour band, a positive result with two colour bands and an invalid result if the control line failed to appear and the test repeated with a new device.

A negative result by QBC, QBC F.A.S.T.™, SD Bioline™, and Giemsa microscopy was considered as true-negative because the risk for false positive microscopy results was considered low since the slides were all read independently by two experienced technicians and a third tie breaker.

2.6 Malaria parasite identification by PCR

Giemsa-stained blood films were soaked with 10 µL of phosphate buffered saline (PBS) 0.02 M, pH 7.4) then scraped off the glass slide by making circular movements with a sterile scalpel (Farla Medicals, Antwerp, Belgium). For each blood film, a separate scalpel was used. The collected material was transferred in a sterile 1.5-mL tube which contained 90 µL PBS. DNA was extracted with the QIAamp DNA Blood mini kit (Qiagen Benelux, Venlo, TheNetherlands) according to manufacturer's instructions. The standard used in the assay was the WHO International Standard for *P. falciparum* DNA Nucleic Acid Tests (NAT) obtained from National Institute for Biological Standards and Control (NIBSC; Hertfordshire, UK) as described by (Kamau *et al.* 2013).

2.6.1 Real-time PCR on blood films

Primers and probes for the amplification of the *Plasmodium* species were used to target PLU3 gene of all *Plasmodium* species and RNAse P, a human housekeeping gene, as described previously (Kamau *et al.* 2013). The assay was performed with the Applied Biosystems 7500 Fast Real-time PCR System, v.2.0.5 software. The thermal profile used for the qPCR was as follows: 5 min at 96°C, 40 cycles of 10secat 96°C; 30 sec at 60°C, with fluorescence collected at 60°C step. Each reaction contained 1 µL of template DNA and a reaction master mix containing Quantifast Probe Master Mix with Rox dye (QIAGEN,USA),10µMof each primer,5 µM of each probe, and dH$_2$O. All assays were run with the appropriate controls including non-template control.

Real-time PCR was performed on 40% of the discrepant results at the Malaria Drug Resistance Laboratories, Kenya Medical Research Institute, Kisumu Kenya. All laboratory personnel were blinded to the results from each of the tests.

2.7 Data Analysis

Data analysis was conducted using SPSS software package Version 20.0 (IBM SPSS Inc, Chicago, IL, USA) and GraphPadSoftware, Version 5 (GraphPad Software, Inc, La Jolla, CA, USA). The performance of the tests (QBC, QBC F.A.S.T.™ and SD Bioline™) against Giemsa microscopy was expressed as true-positive (TP), true-negative (TN), false-positive (FP), or false-negative (FN). The formulae used to calculate performance were TP/TP+FN for sensitivity (SS), TN/TN+FP for specificity (SP), TP/TP+FP for positive predictive values (PPV) and TN/TN+FN for negative predictive values(NPV). The results were interpreted with 95% confidence intervals (CI$_{95}$). Agreement between tests was determined by calculating Kappa statistics with 95% CIs and interpreted with the Landis and Koch classification. Relationship in parasite densities between Giemsa microscopy slide reads and QBC F.A.S.T.™ slide reads was determined using Pearson's correlation co-efficient. P values <0.05 were considered statistically significant.

3. Results

3.1 Performances of the tests

A total of 385 samples were tested. The total number of positive cases was found to be high with QBC test (310/385) as compared to the other tests (Table 1). Overal, SD Bioline™ had the highest sensitivity (91%) with QBC F.A.S.T.™ demonstrating a lower sensitivity (67%) when compared to the other tests. On the other hand, QBC test reported the lowest specificity of 30% as compared to 83% and 67% of QBC F.A.S.T.™ and SD Bioline™ tests respectively(Table 2). A total of 32 samples were found to be negative by both QBC, QBC F.A.S.T.™, SD Bioline™, and Giemsa microscopy.

Table 1. The total number of positives and negatives for each test in the study

Tests	Positive (%)	Negative (%)	Total
QBC	310 (81%)	75 (19%)	385
QBC F.A.S.T.™	185 (48%)	200 (52%)	385
SD Bioline™	242 (63%)	143 (37%)	385
Giemsa	198 (51%)	187 (49%)	385

QBC test gave the highest number of positive cases 310 (81%) while QBC F.A.S.T.™ 185 (48%) test gave the lowest number of positive cases as compared to the other tests.

Table 2. Diagnostic Performance of QBC, QBC F.A.S.T.™ and SD Bioline™ using Giemsa as the gold standard and pair-wise comparison of concordant tests.

Tests	Sensitivity	Specificity	Positive Predictive Value(PPV)	Negative Predictive Value(NPV)	Kappa (95%CI)
QBC	90 (85-94)	30 (24-37)	58 (52-63)	74 (63-84)	0.45 (0.38- 0.52)
QBC F.A.S.T.™	77 (71-83)	83 (77-88)	74 (63-80)	78 (71-83)	0.61 (0.53-0.69)
SD Bioline™	91 (86-94)	67 (60-73)	83 (77-88)	87 (81-92)	0.58 (0.50-0.663)

SD Bioline™ had the highest sensitivity 91% followed by QBC and lastly QBC F.A.S.T.™ while QBC had the lowest specificity 30% as compared to the other tests

   Three-hundred and eighty-five samples were tested by all the four tests, of which QBC and Giemsa microscopy had the highest number of discrepant results. One-hundred and thirty-one samples were found positive by QBC and not by Giemsa, hence 40% (n=52) of the discrepant results were randomly picked and further analysed by qPCR (Kamau *et al.* 2013) to ascertain the presence of *Plasmodium* DNA. Out of the 52 samples found to be malaria positive by QBC, only six samples (12%) had *Plasmodium* DNA by qPCR.

   Assessment of agreement between tests using Giemsa as the gold standard showed a moderate agreement with QBC test (k=0.45) and SD Bioline™ (k=0.58) and a substantial agreement with QBC F.A.S.T.™ (k=0.61) (Table 2). The other tests had a rather poor agreement when compared to each other: QBC *vs* QBC F.A.S.T.™ (k=0.29), QBC *vs* SD Bioline™ (k=0.14) and SD Bioline™ *vs* QBC F.A.S.T.™(k=0.46) as shown in Table 3. The concordant rates of the tests relative to Giemsa microscopy were 90.4% (170/198; QBC), 77.3% (153/198; QBC F.A.S.T.™) and 90.9% with SD Bioline™ test.

Table 3. Levels of agreement for all the tests QBC, QBC F.A.S.T.™ and SD Bioline™ compared to each other

Tests	QBC VsQBC F.A.S.T.	QBC VsSD Bioline	SD Bioline VsQBC F.A.S.T.
Kappa values	0.29(0.20-0.39)	0.14(0.04-0.23)	0.46(0.37-0.54)

When the test agreement was compared against each they performed poorly with Kappa values of as low as 0.14.

3.2 Assessment of parasite densities from QBC F.A.S.T.™test compared to Giemsa

The continued implementation of different malaria control interventions aim at significantly reducing the morbidity and mortality associated with malaria, and possibly eliminate malaria. The move towards elimination will require more sensitive tests to match the expected reduction in parasitaemia (The malERA Consultative Group 2011). Consequently, we assessed the performance of the QBC F.A.S.T.™ test and Giemsa microscopy at different parasite densities (Fig.1). There were 153positive cases by both QBC F.A.S.T.™test and Giemsa microscopy. The positive cases were divided into three percentiles representing low parasitaemia (0th and 25th percentiles, n=37), moderate parasitaemia (26th to 75^{th}percentile, n=77), and high parasitaemia (76th to 100th percentile, n=39) so as to determine how the two tests compared at different parasite densities. Overall, the two tests correlated relatively well when all the samples (n=153) were compared (r=0.645, P<0.0001; Fig.1A). At low parasite densities (0th and 25th percentiles) this relationship was maintained, albeit at a weaker level (r=0.361, P=0.028; Fig.1B). Similarly, at the moderate parasite density the two tests were significantly

correlated at a higher level than for the low parasitaemia (r=0.478, $P<0.0001$; Fig.1C). In addition, at high parasite densities (76th to 100th percentile) this relationship was still maintained (r=0.470, $P=0.003$; Fig.1D).
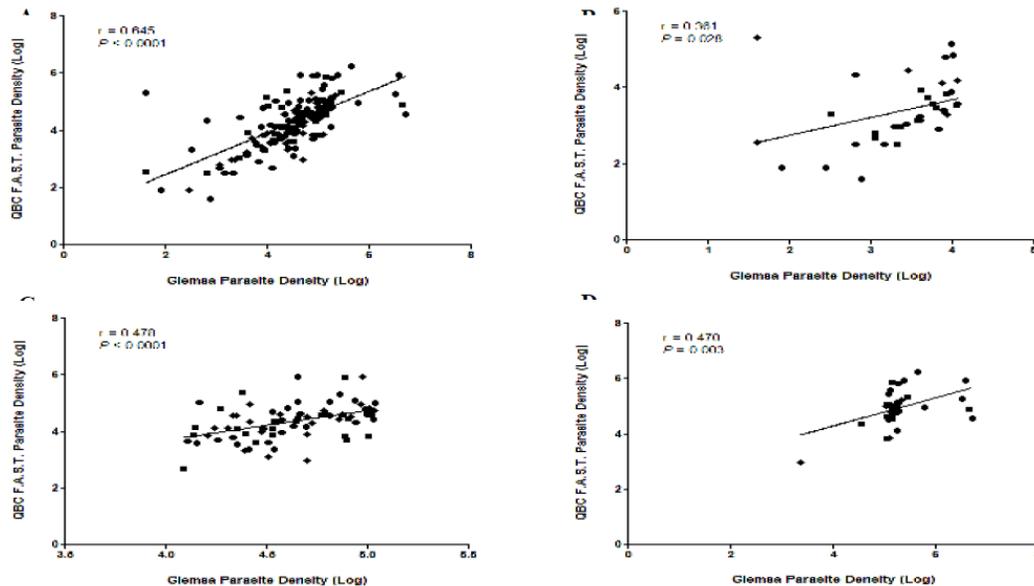
Fig.1 Relationship of parasite densities between QBC F.A.S.T. ™ and Giemsa tests

A) All positive samples (n=153); **B)** 0th to 25th percentile (n=39); **C)** 26th to 75th percentile (n=77); **D)** 76th to 100th percentile (n=37). Analysis was performed using Pearson's correlation coefficient.

QBC F.A.S.T. ™ and Giemsa tests correlated relatively well (r=0.645, $P<0.0001$) on all positive cases (n=153) albeit they had a weaker correlation (r=0.361, $P=0.028$) at low parasitaemia (n=39).

3.3 Species identification by QBC F.A.S.T™ test compared to Giemsa

Of the 153 positive cases, QBC F.A.S.T.™ test detected 150 (98%)*P.falciparum*, zero (0%)*Plasmodium ovale*, zero (0%)*Plasmodium malariae,* and three(2%) mixed infections. Giemsa microscopy detected 141 (92.2%)*P.falciparum*, one(0.65%)*P.malariae*, one(0.65%)*P.ovale*, and ten (6.5%) mixed infections. Despite the difference in identification of the mixed infections by both tests, the difference was comparable between the two.

4. Discussion

The QBC and the QBC F.A.S.T.™ tests are fluorescent-assisted microscopy (FAM)-based methods. QBC had 90% sensitivity, a finding that is consistent with previous studies (Schindler *et al.* 2001 Kuladeepa 2012 Sandhya 2012). The high sensitivity is possibly enhanced through concentration of parasitized erythrocytes and the large volume of blood collected for examination (Kuladeepa 2012). However, the test showed a low specificity of 30%, possibly due to high rates of false positives as confirmed by the qPCR results, whereas only 12% of the discrepant results had *Plasmodium* DNA. The low specificity reported in this study is consistent with previous studies (Schindler *et al.* 2001, Morassin *et al.* 2002). This could be explained by the presence of Howell-jolly bodies and artefacts (Bhandari *et al.* 2008 Sandhya 2012). Concerns of leakage and breakage of blood-filled QBC tubes in the centrifuge are some of the pitfalls associated with this diagnostic approach as experienced in this study and by other studies (Pinto *et al.* 2001, Salmani 2011). Despite the low specificity, QBC holds promise as a good alternative in malaria diagnosis due to its speed and sensitivity as reported by Datta and his team (Datta 2010).

SD Bioline™ had the highest sensitivity compared to the other tests. The high sensitivity of SD Bioline™ kits, which detect HRP-II antigen, gives confidence that most of the malaria cases in the study population were diagnosed in agreement with previous reports (Kosack *et al.* 2013). Sensitivity improves with parasitaemia, however two cases with parasitaemia>10,000parasites/µL were diagnosed as negative. The false negative results could possibly be explained by the pro-zone effect (Kosack *et al.* 2013). A relatively low specificity of 67% obtained may have been due to the persistency of HRP-II antigen in the blood for up to 56 days after treatment (Nyunt *et al.* 2013), or the ability of the RDT to detect low parasite densities (Bell, 2002)or possible deletions of HRP-II (Gamboa *et al.* 2010), although this is rare in Africa (Baker and Kyle DE, 2005). These pose serious diagnostic challenge in malaria-endemic regions resulting in misdiagnosis with poor treatment outcome. Despite

the test being unable to detect non-falciparum malaria, it targets the most lethal *Plasmodium* species (Strom *et al.* 2013), hence greater impact. QBC F.A.S.T.™ test was found to be easy to apply since it uses the same sample preparation procedure as Giemsa microscopy. Positive cases were easily identified for parasitaemia levels >500 parasites/µL correlating to evaluation of a test that used the same fluorescence microscopy principle (Sousa-Figueiredo *et al.* 2010). However, the test demonstrated low sensitivity when compared to the other tests and this could be due to lack of clearly defined ring stages of the parasites.

The parasite densities in all positive cases by both Giemsa and QBC F.A.S.T.™ strongly correlated, indicating a good performance of QBC F.A.S.T.™ on quantitative diagnosis. The weak correlation at low parasite densities could be attributed to the field of view by Giemsa microscopy, which is visually clear, enhancing distinct parasite morphology, making parasite counting easier compared to the challenges obtained from QBC F.A.S.T.™ test where the morphology of the parasite is not clearly visible.

Accurate identification of *Plasmodium* species is critical because the results employed assist in correct deployment of specific control intervention strategies (Obare *et al.* 2013). Prompt and correct case diagnosis leading to accurate epidemiological assessments and optimal case management remains a critical research agenda, especially in malaria-endemic areas (Sousa-Figueiredo *et al.* 2010). Reliable differentiation of malaria infections is imperative since *Plasmodium* species differ in their biology, clinical symptoms and treatment regimens (Barber *et al.* 2013). Importantly, QBC F.A.S.T.™ was found to differentiate between *Plasmodium* species making it applicable to regions where falciparum and non-falciparum malaria cases are common. This was possible through the fluorescing of the parasites with typical malaria morphological stages. The test however is not very satisfactory in the diagnoses of *P. malariae* due to the lack of a very clear visual distinction of the morphology of the parasite. With the increased implementation of various control measures, significant reductions in malaria transmission intensities have been reported in some regions (O'Meara WP 2010). The reduction in transmission intensities is expected to be accompanied by reduction in malaria parasite densities, which will require more sensitive diagnostics for better case management and possible elimination. Despite malaria being a major cause of paediatric morbidity and mortality in most sub-Saharan African countries (Schumacher and Spinelli 2012), diagnostic tools development has remained slow and there is need for redoubled effort in the development of highly sensitive and robust point of care malaria diagnostics.

Study limitations were performance of real-time PCR on only the discrepant results obtained between the tests, and the use of Giemsa blood-stained microscopy as the gold standard for the entire study samples compared to using real-time PCR which has a better parasite detection limit. However, this was done because the risk of getting false positives was considered low as the slides were read by trained technicians and supervised by the Malaria Diagnostics Centre, Kisumu, Kenya. Real-time PCR is a very useful gold standard in that it is highly sensitive, easily reproducible and can detect cases with low parasitaemia missed by other tests. Its limitation is that the test is very expensive and time- and labour-consuming, hence it is used only to confirm the accuracy of microscopy (Johnston *et al.* 2006).

5. Conclusions

QBC test still remains a sensitive, rapid and accurate optical test although it should be supplemented with Giemsa due to limitations in species determination and parasite quantification. However, QBC is less sensitive compared to RDTs but the QBC F.A.S.T.™ test holds promise in rapid malaria diagnosis as differentiation of ring stage morphology is addressed.

Abbreviations

CI_{95}:Confidence intervals; CCR:Centre for Clinical Research; DNA:Deoxyribonucleic acid; GM:Giemsa microscopy; HRP-II:Histidine Rich Protein II; IMCI:Integrated Management of Childhood Illness; NAT:Nucleic acid tests; NPV:Negative predictive value; PBS:Phosphate buffered saline; PCR:Polymerase chain reaction; p-LDH: *Plasmodium*lactate dehydrogenase enzyme; PPV:Positive predictive value; QBC:Quantitative buffy coat; QBC F.A.S.T. ™:QBC (Fluorescence and Staining Technologies) Test; qPCR:Real-time polymerase chain reaction; RBC:Red blood cell; RDT: Rapid diagnostictests;SD BiolineTM:Standard Diagnostics Bioline™ Malaria Antigen *Pf* Test; SSC:Scientific Steering Committee; SS:Sensitivity; SP:Specificity; WBC:White blood cell; WHO:World Health Organization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AAW, OP, NAK, KHL, OJM, and OB participated in the design of the study. AAW, AOA and AMH participated in data collection. AAW, OP, NAK, KHL, OJM, and OB participated in data analysis. All the authors made input in the writing of the manuscript. All authors read and approved the final manuscript.

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