CLINICAL EVALUATION OF FLUORESCENT In-Situ HYBRIDIZATION (FISH®) ASSAY FOR MALARIA DIAGNOSIS

By

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156/12778/2009

A thesis submitted in partial fulfillment of the requirement for the award of the degree of Master of Science (Biochemistry) in the School of Pure and Applied Sciences of Kenyatta University.

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April, 2012
DECLARATION
This thesis is my original work and has not been submitted in any other university for an award of a degree.

Signature ___________________________ Date ______________________

We confirm that the work reported here was carried out by the candidate under our supervision.

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Presbyterian University

Signature ___________________________ Date ______________________

Dr. Sabah. A. Omar
Center Director
Kenya Medical Research Institute - Kilifi

Signature ___________________________ Date ______________________
DEDICATION

I dedicate this work to my parents, Moses Nderu Ngugi and Jean Wangari Nderu who are my greatest source of inspiration.
ACKNOWLEDGMENTS

My sincere gratitude goes to my supervisors Prof. Charity Gichuki and Dr. Sabah Omar for guiding me through this research. Your provisions, suggestions, advice, encouragement and patience throughout the course of the work are invaluable and highly appreciated. My successful research at KEMRI, training, working and progress was as a result of tireless efforts and concern of individuals, staff members at various laboratories and sections in the institute, and my fellow students. All malaria laboratory staff, Edwin Too, Francis Kimani, Daniel Mtua, Shirley Undisa, Mrs. Fathia, Rahma Udu and Mr. Kaniaru. My profound gratitude goes to ID-FISH Technology Inc., USA (Dr. Joystna Shah) for financing this research. I am grateful to my brothers, mum and Dad for giving me moral support and encouragement. God bless you all. I can’t forget Evalyne Karanja who literally supported me whenever I was discouraged, thank you so much and am completely indebted to you.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AO</td>
<td>Acridine orange</td>
</tr>
<tr>
<td>DNAs</td>
<td>Deoxyribonucleic Acids</td>
</tr>
<tr>
<td>CMS</td>
<td>Complete Medium with Serum</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent In-Situ Hybridization</td>
</tr>
<tr>
<td>GuHCl</td>
<td>Guanidine Hydrochloride</td>
</tr>
<tr>
<td>GuSCN</td>
<td>Guanidine Thiocyanate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HRP2</td>
<td>Histidine-rich protein 2</td>
</tr>
<tr>
<td>ID-FISH</td>
<td>Infectious Diseases- Fluorescent In-Situ Hybridization</td>
</tr>
<tr>
<td>IFA</td>
<td>Indirect Immunofluorescence Assay</td>
</tr>
<tr>
<td>IPT</td>
<td>Intermittent preventive treatment</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor Residual Spraying</td>
</tr>
<tr>
<td>ITNs</td>
<td>Insecticide Treated Nets</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Potassium dihydrogen phosphate</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>mM</td>
<td>Milli molar</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Disodium hydrogen phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pLDH</td>
<td>Parasite lactate dehydrogenase</td>
</tr>
<tr>
<td>QBC®</td>
<td>Quantitative Buffy Coat®</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red Blood Cells</td>
</tr>
<tr>
<td>RBM</td>
<td>Roll Back Malaria</td>
</tr>
<tr>
<td>RDTs</td>
<td>Rapid diagnostic tests</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNAs</td>
<td>Ribonucleic Acids</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rosewell Park Memorial Institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time PCR</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-Sodium Citrate</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>UNICEF</td>
<td>United Nations Children's Fund</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume by Volume</td>
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<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

Giemsa microscopy has played a major role in malaria control as a standard or reference method in diagnosis. However, this technique has varied performance characteristics while other techniques are more expensive. As a result, there is continued need for new and/or improved techniques that will increase ease of use and lower the cost of diagnosis. Fluorescent In-Situ Hybridization (FISH®) is a technique that uses fluorescent-labeled oligonucleotide probes that are specific to a region in Plasmodium species ribosomal (18S) ribonucleic acid (rRNA) thus aiding detection of malaria parasites present in a blood sample. The aim of this study was to evaluate Fluorescent In-Situ Hybridization technique for diagnosis of uncomplicated malaria. Two hundred and sixteen patients visiting Tiwi Health Center, Coastal Kenya, suspected of having uncomplicated malaria were included in the study. Finger-prick blood samples were collected from the participants to be tested for malaria using Giemsa microscopy and Plasmodium genus ID-FISH® test. The results were then used to determine the diagnostic performance of Plasmodium genus ID-FISH® test against the Giemsa microscopy gold standard. The limit of detection of FISH® test was determined using cultured Plasmodium falciparum strain D6. Sensitivity and specificity were found to be 93% and 84% respectively giving a positive predictive value of 72% and a negative predictive value of 96%. The limit of detection provided additional evidence (higher detection rate of two logs) that Plasmodium genus FISH® test is more sensitive than Giemsa microscopy. Plasmodium genus FISH® was found to be fast and easy to perform as well as more sensitive and specific than Giemsa microscopy. However, it is recommended that Plasmodium genus ID-FISH® is evaluated using a more sensitive and specific gold standard, like PCR, than Giemsa microscopy due to the high discrepancy observed in this study. Evaluation of Plasmodium genus ID-FISH® in malaria non-endemic settings and its effect on the accuracy of anti-malaria drug efficacy studies are vital especially when the rate of low parasitemia cases have increased en route to malaria elimination in Kenya.
CHAPTER ONE
INTRODUCTION

1.1 Background Information

Malaria remains an important parasitic disease worldwide causing an estimated morbidity of approximately 500 million people and 1.5 – 2.7 million mortalities annually (Amexo et al., 2004; Swan et al., 2005; UNICEF, 2008). Ninety percent of these deaths occur in sub-Saharan Africa, mostly in children under five years old (Swan et al., 2005).

In Kenya, malaria accounts for 30 – 50% of all outpatients and 2% of admission in health facilities (Ministry of Health, Kenya, Division of Malaria Control, 2011). The prevalence of malaria burden is varied with some parts having very low cases. In Tiwi, a hyper-endemic area located at the Kenyan south coast, the malaria burden is about 30% of all outpatients (Omar, 2002). Although treatable, malaria poses a huge health burden due to high cost of drugs, drug and insecticide resistance and morbidity (Talisuna et al., 2004).

Routine diagnosis of malaria depends on the microscopic demonstration of parasites in Giemsa stained blood samples as a gold standard. This diagnostic method is limited by its reduced specificity and sensitivity at low parasitemia and its inability to differentiate malaria parasites (Chiodini, 1998). Specific diagnostic methods are becoming more and more important for initial diagnosis to prevent over exposure of patients to anti-malarial drugs for patients with diseases other than malaria and the consequent drug side effects. Specific diagnosis would enhance correct initial treatment, reducing the chances of treatment failure and subsequent drug resistance.
development, improve diagnosis of other febrile illnesses and evaluation of the efficacy of drugs used (Schoone et al., 2000).

A number of new diagnostic methods are now available including Rapid Diagnostic Tests (RDTs) which are cheap, fast and simple to perform. However, at low parasitemia, (less than 100 parasites/µl) sensitivity decreases making them unsuitable (Schoone et al., 2000; Wongsrichanalai et al., 2007). PCR-based assays capable of detecting and differentiating malaria parasites species at low levels have also been developed although their use have been limited by intricate methodologies involved (Swan et al., 2005).

Fluorescent in-Situ Hybridization (FISH®) is a technique that combines molecular techniques and microscopy. FISH® enhances the visibility of parasites during microscopy, differentiates Plasmodium species, is less intricate and has a lower initial capital than PCR. As a result, FISH® has found a wide application in biomedical research (Amann et al., 2001; Levsky and Singer, 2003; Bottarri et al., 2006). The purpose of this study was to clinically evaluate the performance of Plasmodium genus ID-FISH® assay performance for malaria diagnosis compared to the routinely used Giemsa stain microscopy.

1.2 Statement of the Problem

Malaria remains a major health burden in Sub-Saharan Africa, especially amongst children under five years old and expectant women (Swan et al., 2005), despite the enormous investment in its control. In Kenya, malaria accounts for 30 - 50% of all out patient attendance and 2% of all admission in health facilities. Similarly, at Tiwi a hyper-endemic area located at the Kenyan south coast, malaria accounts for 30% of pediatric and adult outpatient attendances (Omar, 2002).
Malaria, like other infectious diseases, results in manifestation of fever which, though non-specific, has been used as basis for diagnosis for a long time. (Amexo et al., 2004; Perkins and Bell, 2008). Poor specificity may lead to over-diagnosis of malaria which in turn leads to over-prescription of anti-malaria drugs thus raising the cost of treatment as well as preventing diagnosis of other ailments (Zurovac et al., 2006). On the other hand, under-diagnosis may subject patients to development of severe malaria that may result in mortality (WHO, 2010). Although Rapid diagnostic tests (RDTs and microscopy have played an important role in improving malaria diagnosis, poor performance (specificity and sensitivity) at low parasitemia has been a major limiting factor. Overcoming this setback is paramount especially when declining malaria prevalence is being reported leading to cases of low parasitemia that may be missed during diagnosis. Therefore it is important to evaluate the performance of novel tests like Plasmodium genus ID-FISH® en route to getting a better diagnostic method than Giemsa microscopy for malaria diagnosis especially at low parasitemia.

1.3 Justification

In situ hybridization tests which are not PCR-based, such as Cyscope® microscopy which detects malaria parasites ribosomal DNA (rDNA) using fluorescent-labelled oligonucleoitides, have improved accuracy of malaria diagnosis, increased ease of use and reduced cost compared to PCR based assays (Hassan et al., 2010). However, detection of malaria parasites using rDNA may also lead to detection of dead parasites thus increasing the false positive rate. Unlike Cyscope® microscopy, Plasmodium genus ID-FISH® uses fluorescent labeled probes to rRNA as a target to detect malaria caused by parasites of the Plasmodium genus eliminating the constraint of detecting dead parasites. The abundance of rRNA in the cytosol and the specificity
of the fluorescence labeled probes make FISH® more accurate and specific, thus improving in
disease diagnosis (Landstrom and Tefferi, 2006; Guy et al., 2007; Gharibi et al., 2010).
Furthermore, the lower initial capital costs and less stringent conditions required for establishing
FISH® compared to PCR would make FISH® more field applicable. Application of *Plasmodium*
genus ID-FISH is expected to bring these advantages to malaria diagnosis. Although FISH® has
been applied in identification and diagnosis of bacteria, cancer cells and protozoa (Babesia), its
application in malaria diagnosis is new (Delong et al., 1989; Jensen et al., 2001). The study
reported here evaluates the sensitivity and specificity of *Plasmodium* genus ID-FISH® using
Giemsa microscopy as the malaria diagnostic gold standard.

1.4 Significance of the Study

Malaria has major negative impacts on children and expectant women. Although there are gains
in reducing the prevalence as a result of concerted efforts in treatment and vector control, much
is needed to maintain the downward trend in malaria incidence. Diagnosis provides an avenue of
preventing empirical use of anti-malaria drugs thus reducing the probability of resistance
development and exposure to drugs side effects. However, current methods face operational and
performance limitations. Development of novel cost effective assays that would improve in the
diagnosis of malaria will significantly reduce deaths due to malaria in Kenya. Therefore
evaluation of *Plasmodium* genus ID-FISH® as a viable alternative would improve detection and
speciation of malaria infections and thus enhance prompt treatment and prevent death due to
malaria.
1.5 Hypothesis

*Plasmodium* genus ID-FISH® test has a higher sensitivity and specificity than Giemsa microscopy.

1.6 Objectives

1.6.1 General Objective

To evaluate *Plasmodium* genus ID-FISH® test for malaria diagnosis in uncomplicated malaria patients.

1.6.2 Specific Objectives

i. To determine the sensitivity and specificity of *Plasmodium* genus ID-FISH® test.

ii. To determine the positive predictive value and negative predictive value of *Plasmodium* genus ID-FISH® test.

iii. To determine the limit of detection of *Plasmodium* genus ID-FISH® test.
2.1 Global Distribution of Malaria

Malaria occurs in 100 countries, but is mainly confined to poor, tropical areas of Africa, Asia and Latin America (Figure 1). Moreover, it is highly endemic in most of sub-Saharan Africa, central and south America, Hispaniola, the Indian subcontinent, the Middle East, Oceania and Southeast Asia (Swan et al., 2005). More than 90% of malaria cases occur in tropical Africa, particularly among young children and pregnant women, with *Plasmodium falciparum* causing almost all malaria deaths. On the other hand, *Plasmodium vivax* is the major cause of malaria morbidity outside Africa, Asia and South America, but rarely fatal.

Figure 1: Global distribution of malaria. Source: Global watch 2004
2.2 Malaria Etiology

Malaria is an insect borne infection caused by a variety of protozoan parasites of the genus *Plasmodium* which are transmitted from one individual to another through the bite of the female *Anopheles* Mosquitoes (Carter and Mendis, 2002). Four *Plasmodium* species infect man namely: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. A fifth species has been included due to reported cases of human infection by a simian malaria parasite known as *P. knowlesi* (WHO, 2006; Cox-Singh *et al.*, 2008).

*P. falciparum* and *P. vivax* are the most encountered malaria parasites with their predominance varying with the region in question. *P. falciparum*, the most virulent, is predominant in Africa while *P. vivax* is predominant outside Africa (Carter and Mendis, 2002). *P. falciparum* malaria is also known as tertian fever because of its characteristic severe and fatal manifestation of the malignant periodic fever. *P. vivax* causes benign tertian malaria and is found sporadically in some temperate regions, where in the past it was prevalent. It is very common throughout much of the tropics and subtropics (Carter and Mendis, 2002). Malaria deaths resulting from *P. vivax* monoinfection are rare. However, this occurs most often due to rupturing of the enlarged spleen.

*P. ovale*, causes quartan malaria and like *P. vivax* has dormant liver stages, hypnozoites, which activate and invade the blood causing a relapse several months or years after a bite by an infected mosquito. On the other hand *P. ovale* can infect individuals who are Duffy antigen negative unlike *P. vivax* giving it a greater prevalence in most of Africa as compared to *P. vivax* which is confined to west and central Africa. Moreover, *P. vivax* has the most limited distribution of all the human malaria parasites (Carter and Mendis, 2002).
*P. malariae* causes an infrequent mild febrile disease. It has a worldwide distribution and the only human malaria parasite that has a four day cycle as compared to the above mentioned that have a three day cycle. *P. malariae* causes a chronic infection that in some instances can last a life time. In some cases *P. malariae* can cause serious complications such as nephritic syndrome which can eventually be fatal (Carter and Mendis, 2002).

*P. knowlesi* unlike the other four human malaria parasites is restricted to vectors belonging to the *Anopheles leucosphyrus* group for their transmission. These mosquitoes are equally attracted to monkeys and humans and feed predominantly in the forest and forest fringe after dusk (Cox-Singh *et al.*, 2008). This has been attributed to the transmission of *P. knowlesi* from its original, monkey host to man (Cox-Singh *et al.*, 2008).

### 2.3 The Life Cycle of Malaria Parasites

The malaria parasite exhibits a complex life cycle (Figure 2) involving an insect vector (mosquito) and a human host. All four species that infect man exhibits a similar life cycle with only minor variations (Fujioka and Aikawa, 2002). Sporozoites are transmitted to the human host by the bite of infected female *Anopheles* mosquitoes which then invade hepatocytes shortly after inoculation into the blood circulation. The sporozoites develop into pre-erythrocyctic schizonts, through an asexual replication known as exo-erythrocytic schizogony, during the next 5-15 days depending on the *Plasmodium* species. This schizogony culminates in the production of merozoites which are released into the blood circulation and invade the red blood cells (RBCs). However, *P. ovale* and *P. vivax* have a dormant stage known as hypnozoites that may remain in
the liver for weeks to years before development of exo-erythrocytic schizogony (Krotoski et al., 1982; Fujioka and Aikawa, 2002). This results into relapses of malaria infection.

Figure 2: The life cycle of malaria parasites. Source: National Center for Infectious Diseases, Division of Parasitic Diseases (CDC, 2004)

The invasion of RBCs marks the end of exo-erythrocytic cycle and the beginning of erythrocytic cycle. The merozoites invade and develop within the erythrocyte through ring, trophozoite and schizonts stages (erythrocytic schizogony). The erythrocyte containing the segmented schizonts eventually ruptures and releases the newly formed merozoites that invade new erythrocytes (Figure 2, Stage 6). The entire invasion process takes about 30 seconds (Fujioka and Aikawa, 2002). Concomitantly, a small portion of the parasites differentiate, Figure 2, Stage 7, from
newly invaded merozoites into sexual forms, macrogametocyte (female) and microgametocyte (male) which are taken up by a mosquito during a blood meal (Fujioka and Aikawa, 2002).

In the midgut of *Anopheles* mosquitoes, mature macrogametocytes escape from the erythrocytes to form macrogametes. Microgametes exflagellates after a few minutes in the midgut and fertilize the macrogamete leading to the formation of a zygote (Fujioka and Aikawa, 2002). The zygote develops into a motile ookinete which penetrates the gut epithelial cells and develops into an oocyst. The oocyst undergoes multiple rounds of asexual replication resulting in the production of sporozoites. The rupture of the mature oocyst releases the sporozoites into the hemocoel (i.e., body cavity) of the mosquito (Figure 2, Stage 12). The sporozoites migrate to and invade the salivary glands, thus completing the life cycle (Fujioka and Aikawa, 2002).

### 2.4 Clinical Manifestation of Malaria

Malaria presents symptoms that are nonspecific and common with various viral illnesses. These symptoms include: headaches, fever, shivering, joint pains, vomiting, anemia, hemoglobinuria, retinal damage and convulsions (Trampuz *et al.*, 2003). On the other hand malaria presents characteristic symptoms which occur at regular intervals sudden coldness followed by rigor (shaking at high fever) and then fever and sweating lasting for 4–6 hours. The recurrence of these symptoms varies with species with *P. falciparum* having the shortest, 36–43 hours interval (WHO, 2006). At this stage the disease is termed as uncomplicated malaria without mortality and vital organs dysfunction, provided prompt and effective treatment is given (WHO, 2006). As the parasite burden continues to increase severe malaria may develop.
Severe malaria is almost exclusively caused by *P. falciparum* infection and usually arises 6-14 days after infection (Trampuz et al., 2003). Consequences of severe malaria include coma and death if untreated; young children and pregnant women are especially vulnerable. Splenomegaly (enlarged spleen), severe headache, cerebral ischemia, hepatomegaly (enlarged liver), hypoglycemia, and hemoglobinuria with renal failure (causing blackwater fever) may occur (Trampuz et al., 2003). By this stage, mortality in people receiving treatment has increased (more than 20%) and if untreated, severe malaria is almost always fatal (Trampuz et al., 2003; WHO, 2006).

Chronic malaria is seen in both *P. vivax* and *P. ovale*, but not in *P. falciparum*. Here, the disease can relapse months or years after exposure, due to the presence of latent parasites in the liver (Carter and Mendis, 2002). Therefore, describing a case of malaria as cured by observing the disappearance of parasites from the bloodstream can be deceptive. The longest incubation period reported for a *P. vivax* infection is 30 years (Trampuz et al., 2003).

The nature of the clinical disease depends very much on the pattern and intensity of malaria transmission in the area of residence. When intensity is high, children under the age of five and pregnant women are more vulnerable mainly due to lack of immunity against the disease (WHO, 2006; Carter and Mendis, 2002). However, partial immunity acquired from frequent infection is rapidly lost after relocating into an area with low intensity. As a consequence in areas of high transmission, it is children who are at risk of severe malaria and death, whereas in areas of low or unstable transmission, all age groups are at risk.
2.5 Malaria Control Strategies

Malaria control strategies involve blocking of malaria parasite life cycle at a specific point. These strategies vary with local malaria endemicity and generally conform to the key strategies advocated by roll back malaria (RBM) for their epidemiological setting (WHO and UNICEF, 2005). In stable endemic malaria regions, control strategies are classified into two i.e. prevention and treatment (WHO and UNICEF, 2005; Okafor and Amzat, 2007).

The implementation of control strategies have resulted in the reduction of malaria cases and/or transmission (WHO, 2008). However, profound setbacks have emanated from insecticide resistance and anti-malaria drug resistance. Poverty and political instability are some of the factors that have impeded malaria control programs although not directly associated with it (Okafor and Amzat, 2007). Currently, diagnosis and subsequent treatment with effective anti-malaria drugs provide the best control strategy.

2.6 Malaria Diagnostic Methods

Since the discovery of the pathogen that causes malaria, clinical and biological presence of malaria parasite has been the basis of diagnosis. Each method in these categories has its own strengths and weaknesses that help in promoting their use in different parts of the world. Ease of use, sensitivity and cost per test has been the major contributors in selection of a diagnostic method (Peeling et al., 2006).
2.6.1 Clinical (Presumptive) Diagnosis

Clinical diagnosis is the least expensive and is the most commonly used method. It is based on the presentation of malaria-like symptoms (Wongsrichanalai et al., 2007). In settings where access to a diagnostic laboratory service is limited and malaria is endemic, WHO recommends the use of clinical diagnosis especially for treatment of all childhood fever as malaria (Amexo et al., 2004). However, this “fever” is non-specific and overlaps with other tropical diseases.

Populations with different attributable propositions from Philippines, Sri Lanka, Thailand, Mali, Chad, Tanzania, and Kenya have shown a wide range (40 - 80%) of malaria over-diagnosis based on fever (Wongsrichanalai et al., 2007). Moreover, 30% of children reporting to Ugandan health centers had symptoms compatible with both pneumonia and malaria and thus required dual treatment (Amexo et al., 2004). As a result, treating all fever presumptively as malaria masks underlying causes and subjects individuals to unnecessary drug side effects. This leads to a prolonged and worsening illness with loss of income and productivity. In light of this, biological methods of malaria diagnosis have been found to be of utmost importance to ascertain the cause of febrile illness.

2.6.2 Biological Diagnosis

Biological diagnosis is based on the demonstration of the causative agents via a variety of assays and malaria is no exemption (Chan, 1989). Several methods have been developed for malaria diagnosis but differ in the principle. Development of different assays is aimed at improving performance and/or operational characteristics.
2.6.2.1 Microscopy

Discovery of malaria parasites is credited to the examination of Giemsa stained blood films with a light microscopy (Chan, 1989; Wongsrichanalai et al., 2007). Currently, most laboratories depend on this method for malaria diagnosis and is regarded as the most suitable test because it is cheap to perform, differentiates malaria species and quantifies the parasites. However, microscopy requires well-trained, competent personnel and rigorous maintenance of functional infrastructure. Poor reproducibility, parasite sequestration, variable sensitivity and specificity are major limitations of Giemsa microscopy (Kifude et al., 2008).

Fluorescent microscopy increased sensitivity and enhanced scanning through slides for parasites (Hanschheid, 1999). However, skilled microscopy personnel are still needed. Further modification to include centrifugation, before fluorescent microscopy, led to the development of quantitative buffy coat® (QBC®), which improved sensitivity but also introduced new limitations, including increased cost, limited species identification and quantification.

2.6.2.2 Serological Methods

Serological tests include the use of antibodies or antigens produced during the cause of the infection helped to overcome challenges posed by parasite sequestration (Chan, 1989; Kifunde et al., 2008). However, the presence of antibodies is not an indicator of active infection or protective immunity but rather an immunological response (Hanschheid, 1999; Moody, 2002).

Three Plasmodium antigens have been indentified for malaria diagnosis and they include: Histidine-rich protein 2 (HRP2), found only in P. falciparum, parasite lactate dehydrogenase (pLDH) and Plasmodium aldolase, both found in all Plasmodium species (Chan, 1989; Hanschheid, 1999; Moody, 2002; Wongsrichanalai et al., 2007).
Limitations observed while using malaria parasite as antigens led to the development of two techniques: Radioimmunoassay (RIA) and Enzyme-linked immunosorbent assay (ELISA) (Chan, 1989). Of the two, ELISA has found a wider acceptance due to its ease of use and improved clinical reproducibility (Kifunde et al., 2008). Although it overcame the use of “intact” parasites as antigens, difficulties in sourcing standardized antigens limited its use (Chan, 1989).

Rapid diagnostic tests (RDTs) are immunochromatographic assays that detect malaria antigens via monoclonal antibodies impregnated on a strip resulting in the development of a coloured line within 5-20 minutes (Wongsrichanalai et al., 2007). Wide application of RDTs has been prompted by increased ease of use, applicability in the absence of electricity and ease of interpretation. RDTs detect HRP2, pLDH and genus specific aldolase, in blood (Hanscheid 1999; Wongsrichanalai et al., 2007). Some commercial RDTs carry a combination of these antigens and thus achieve detection and speciation of malaria parasites present. For instance, NO~ICT (Binax U. S. A) combines genus specific aldolase enzyme and HRP 2 enabling the test to distinguish infection with non- \textit{P. falciparum} from those due to \textit{P. falciparum}.

Even though the use of RDTs has produced benefits, several limitations have emerged including a drop in sensitivity to values between 11-40% at low parasitemia (< 100/μl). Of particular concern is the occasional failure to detect cases with high parasitemia (>500-1000/μl) attributable to lack of the \textit{HRP-2} gene (Hanscheid, 1999; Koita et al., 2012). Moreover, mutation of this gene may also give false negatives. Similarly, false positive results may be attributed to persistence (7-14 days) of HRP2 and cross-reactivity of antibodies with rheumatoid factor, RF (Wongsrichanalai et al., 2007). The use of pLDH that is only produced by viable parasite and replacement of IgG with IgM improved performance making it possible to follow-up patients after treatment (Wongsrichanalai et al., 2007). Although RDTs have faced several challenges...
they have greatly improved malaria diagnosis with WHO recommending its use, either alone or together with Giemsa microscopy (WHO, 2010).

2.6.2.3 Molecular Diagnosis

Molecular diagnostic tests depend on the detection of the parasite’s nucleic acids via a technique known as Polymerase Chain Reaction, PCR (Hanscheid, 1999). These tests not only have an increased sensitivity and specificity but also enable identification of mixed infection. These tests raised the detection of placental malaria from 42% to 97% and asymptomatic malaria from 17% to 47% (Swan et al., 2005).

However, the lengthy PCR protocol limits its use in routine diagnosis (Hanscheid and Grobusch, 2002). This led to the introduction of an automated real time PCR (RT-PCR) which is less complex and requires less human skill than conventional PCR. This method also allows amplification and distinction of the four *Plasmodium* species using one set of primers which probe the 18S ribosomal RNA (rRNA) gene in a single and shorter run (Swan et al., 2005). Nonetheless, presence of PCR inhibitors, DNA degradation or genotype variation may lead to false negative results, limiting the utilizability of these tests. High cost, danger posed by reagents and inability to distinguish between viable and non-viable parasites further limit adaptation of PCR for diagnosis (Chan, 1989; Swan et al., 2005). To address these drawbacks some modifications have been made with the aim of increasing its utility.

Loop-mediated isothermal amplification (LAMP) is a simple and inexpensive molecular test that detects highly conserved 18S ribosomal RNA gene of the *Plasmodium* genus and species (Erdman and Kain, 2008). Unlike other conventional PCR-based assays, LAMP utilizes a DNA polymerase that amplifies at a single temperature thus improving its efficacy and taking less time.
(Tangpukee et al., 2009). The end point result is seen as a turbid solution or as pellet formation which is detected by eye or measured with a turbidimeter, making it a qualitative and quantitative test. The performance of LAMP has been demonstrated to have a 99% consistency with nested PCR (nPCR) (Poschl et al., 2010). Similar results were obtained using heat treated blood as template without purification reducing cost for parasite DNA preparation process (Poon et al., 2006). Furthermore, use of dried blood on filter paper and incorporation of melt curve analysis has been shown to overcome challenges of blood storage and to improve accuracy (Yamamura et al., 2009). The use of mitochondrial DNA as a target for malaria diagnosis has further increased LAMP sensitivity from a 100 parasites/μl to 5 parasites/μl (Polley et al., 2010). Turbidity as the end point, of LAMP, might lead to a large number of false positives which may result if the heat treated blood is not properly centrifuged (Paris et al., 2007). While this can be overcome by using a real time turbidimeter, its utility is limited by the initial capital needed.

Sensitivity of molecular diagnosis has been improved by use of rRNA as a biomarker compared to the rRNA gene used in the earlier PCR-based tests (Mens et al., 2006). Quantitative nucleic acid sequence based amplification (QT-NASBA) provides a classical example of tests that use 18S rRNA as a template for malaria parasite detection (Schoone et al., 2000; Mens et al., 2006). Schneider et al. (2005) compared the performance of QT-NASBA to RT-PCR and the latter was found to be less sensitive. Use of rRNA as the target has helped improve sensitivity due to its abundance compared to RT-QT PCR that targets rRNA gene (Schneider et al., 2005). Moreover, adaptation of a closed-tube format has helped to reduce chances of contamination thus fewer false positives. If contamination occurs, it is further reduced due to isothermic temperatures that prevent DNA denaturation. Other merits of QT NASBA are: reduced run time, reduce cost per
assay and detect various life cycle stages at a low parasitemia which is important in drug efficacy studies (Schneider et al., 2005; Men et al., 2008).

The integration of PCR-based tests into routine diagnosis of malaria and other diseases has not only been hampered by cost and expertise needed but also by post amplification methods that are used (Mens et al., 2008). This is attributed to the risk posed by ethidium bromide used in gel electrophoresis. A novel technique referred to as nucleic acid lateral flow immunoassay (NALFIA), which eliminates the use of ethidium bromide has been developed (Blazková et al., 2009). A study conducted by Mens et al. (2008) demonstrated that NALFIA is 10-fold more sensitive than gel electrophoresis. In addition, this test detected more clinical samples with pan-Plasmodium (Plasmodium genus) than microscopy and had an excellent agreement with gel electrophoresis. This indicated that NALFIA is more sensitive than Giemsa microscopy and may serve as an alternative of gel electrophoresis. In combination with LAMP, this test provides an opportunity for use of PCR-based test in resource limited areas (Mens et al., 2008). Similarly, Blazková et al. (2009) has demonstrated that NALFIA can also detect different species of the same genus. However, the use of this test has largely been limited by its dependence on PCR based methods (Mens et al., 2008).

2.6.2.4 Fluorescent In-Situ Hybridization (FISH)

Despite the improvement in malaria diagnosis, the Giemsa test is still considered the gold standard and thus the diagnosis of malaria therefore remains pegged on a poor gold standard and there is need to investigate other, more reliable diagnostic tests. Fluorescent In-Situ Hybridization (FISH) is an in vitro method which effectively detects specific nucleic acid sequences of intact cells and tissues. This involves two partially or completely complimentary strands of DNA, RNA or combinations (of the two) which act as probes (Levsky and Singer,
Initially radio-labeled probes were used for detection of specific DNA/RNA sequences in a heterogeneous cell population (Levsky and Singer, 2003). This also allows simultaneous determination of biochemical and morphological characteristics of the examined cells and pathogens thus assisting in their identification (Bottari et al., 2006). Although sensitive, radiolabels limited its use. The replacement of radiolabels with fluorescent labeled probes removed the limitations instability, low resolution, cost, duration of test and irradiation hazards (Levsky and Singer, 2003). In addition, FISH allows easier differentiation of *Plasmodium* species; requires less time for scanning through a slide; removes the need for a thermocycler and post-amplification detection techniques, such as electrophoresis and the risk associated with it.

In carrying out FISH, samples deposited on slides are fixed as described for both thick and thin smears in Giemsa microscopy (Endeshaw et al., 2008). The fixative helps in preserving the morphology of cells or tissues such that nucleic acids are retained within the cell matrix. This also cross-links and/or precipitates cell membrane proteins, enabling penetration and subsequent hybridization of probes (Shah and Harris, 1999). Fluorescent moieties are used for labeling but when testing for multiple targets different moieties are used (Oda et al., 2000; Bottari et al., 2006). The size of a probe complex molecule preferably ranges between about 10-30 nucleotides (Amann et al., 2001; Bottari et al., 2001). It is noteworthy that excess probe (100:1) is needed for efficient hybridization (Shah and Harris, 1999).

Hybridization is achieved in stringent conditions provided by a hybridization buffer (Shah and Harris, 1999). The exact concentration of buffer is dependent on the melting temperature (Tm) of the probe, probe sequence, probe length, and experimentation. Presence of some reagents in the buffer enable hybridization to occur at a low temperature for example, presence formamide enables hybridization to occur at 42°C as opposed to 60-65°C of sodium chloride (Shah and
Harris, 1999). Similarly, hybridization performed in the GuSCN or GuHCl hybridization buffers take place at a lower temperature of 37°C.

The non-hybridized probes are rinsed from the sample, generally by applying a series of washes with a wash buffer e.g. 0.3 M sodium chloride, 0.03 M sodium citrate, 0.5% NP40 and phosphate buffered saline, PBS (Shah and Harris, 1999; Bottari et al., 2006). Evans Blue is used to counter stain the host cells. Those that hybridized are then visually detected by fluorescent microscopy (DeLong et al., 1998; Shah and Harris, 1999).

Current diagnosis of many cellular pathologies depends on microscopic evaluations, cellular morphological parameters, staining characteristics, and the presence (or absence) of certain antigens. Many of these methods of diagnosis are not entirely accurate or sufficiently sensitive. Fluorescent in situ hybridization having improved these parameters has therefore been applied in various fields of biomedical research. This test provides an alternative for RT-PCR in diagnosis of infectious diseases because it requires less initial capital. Recently this technique was applied for diagnosis of Babesiosis (IGeneX.inc). Application of FISH® for diagnosis of malaria is new and therefore its evaluation is critical.
CHAPTER THREE

METHODOLOGY

3.1 Area of Study

This study was approved by the KEMRI Ethical Scientific steering committee. The area of study was the catchment sites for the clinic (Tiwi health center) within Tiwi town located in Kwale district about 30 Km south of Mombasa, coastal Kenya (Figure 3). Tiwi is an urban area with a population of 0.75 to 1.05 million people of which 20-27% are under five years (Omar, 2002). The study site falls in a malaria hyper endemic region. Moreover, there are two rainy season i.e. long rains (February to June) and short rains (October- December) with an annual rainfall of 1000mm. Malaria transmission occurs all year round with peaks in the months of May, June and July (Omar, 2002).

Figure 3: Map showing the study site. Source: www.Googlemaps.com
3.2 Inclusion and Exclusion Criteria

Outpatients visiting Tiwi health center were included in the study if they met the following criteria: provided informed consent (for patients below the age of 18 years consent was provided by a parent or guardian), presenting malaria symptoms and no history of anti-malarial drug administration. However, patients were excluded from the study due to personal withdrawal and presence of other health complication.

3.3 Sample Collection and Preparation

Peripheral blood was collected by lancet prick from a fingertip for Giemsa microscopy and *Plasmodium* genus ID-FISH® Kit test. For each patient, two slides were made containing a thick and thin smear. Thin smears were fixed with 100% methanol, air-dried and stored at room temperature.

3.4 Malaria Diagnosis

3.4.1 Giemsa Microscopy

Thick and thin (fixed with 100% methanol) blood smears was flooded with Giemsa stain for 15 minutes and rinsed carefully. Air-dried Giemsa-stained slides were observed under a light microscope at 100X magnification.

3.4.2 ID-FISH *Plasmodium* Genus FISH® Kit Assay

*Plasmodium* genus ID-FISH® test is a diagnostic kit developed by ID-FISH technology Inc. (San Antonio, California) for detection of all *Plasmodium* species. This kit was composed of the following reagents: Tube A (contained the fluorescent labeled probe), Tube B (contained the
hybridization buffer), 3 Buffers (Pretreatment buffer, Wash Buffer I and Wash Buffer II) and Evans Blue Counter Stain. These reagents were used as described in the sections below.

3.4.2.1 Preparation of Plasmodium Genus FISH® Assay probe mix

The probe mix was prepared by using 12μl solution A (contains genus probe) and solution B (contains hybridization buffer). The solution was vortexed gently for proper mixing.

3.4.2.2 Hybridization

Thin smears were prepared as described for Giemsa microscopy (only fixed with 100% methanol) (Endeshaw et al., 2008). However, thick smears were first pretreated with a pretreatment buffer in coplin jars until it became clear and then air dried. The thick smear was then fixed with 100% methanol and aired dried completely. The probe mix, 10μl, was applied onto the corresponding smear (that is 20 μl per slide) and cover slip placed thereafter. Plaster slide holders were used to incubate the slides at 37°C in a humid incubator for 30 minutes in the dark.

3.4.2.3 Washing

Cover slips were removed gently from each slide. In order to remove excess probe, the slides were washed twice with Wash Buffer I and once with Wash Buffer II, successively, at 15-30°C with 2 minutes intervals. The wash buffers were removed by tilting the slides. Slides then flooded with 1ml of Evan’s Blue for 15 minutes and air-dried completely. Fluorescent microscope (100X) was used to view the slides. The presence of malaria parasites was indicated by only apple green fluorescence.
3.5 Plasmodium Genus ID-FISH® Test Clinical Evaluation protocol

Clinical evaluation of the ID-FISH® Kit assay was conducted using samples collected from the study site. One set of the sample slides was processed according to the Giemsa microscopy (at the site of study) protocol while the other set was done according to the FISH® Kit protocol. Giemsa microscopy was used as the gold standard.

3.6 Plasmodium Genus ID-FISH® Limit of Detection Assay

To determine the limit of detection of FISH® Kit assay 10 log dilutions (1:10) of cultured *P. falciparum* strain D6 (provided by Malaria Culture Laboratory, Malaria Unit, Center for Biotechnology Research and Development, KEMRI.) parasites, were prepared using a complete culture medium (CMS) with serum and RBCs. CMS was prepared by mixing 86.22% (v/v) of RPMI/HEPES, 3.7% (v/v) of 5% NaHCO₃, 15% (v/v) human serum and 5% non-parasitised RBCs. From each log 10 slides, each with a thick and thin smear, were prepared. Only the thin smears were fixed with absolute methanol. Diagnosis of each log was done as described previously (Giemsa microscopy and FISH® kit assay). Negative and positive samples were used as negative and positive controls respectively.

3.6 Data Recording and Analysis

Results, for both assays, were recorded as either Positive (Pos) or Negative (Neg) to indicate positive or negative respectively. However, for any Giemsa positive test the parasitemia was determined and appropriate prescription given (Artemisin lumefantrine) as described by the national guidelines for diagnosis, treatment and prevention of malaria for health workers in Kenya (MoH, 2006). Sensitivity was determined as the percentage proportion of true positives
identified by FISH® test while specificity was determined as the percentage proportion of true negatives identified by FISH® test. Negative and positive predictive values were determined as described by Altman and Bland (1994). The clinical utility (using likelihood ratios, LR) of FISH was determined and the following criterion was used: LR less than 2 = not useful, LR between 5 and 10 = moderate and LR greater than 10 = conclusive.

Accuracy of this test was calculated using a Receiver Operating Characteristics (ROC) curve of SPSS version 12.0 (plotting sensitivity against 1-specificity or the false positive rate). The ROC curve was analyzed using the area under the curve, AUC. This was compared to AUC of 0.5, if less than 0.5 then the assay cannot distinguish between a malaria positive and negative sample but if greater than 0.5 then the assay is able to distinguish between malaria positive and negative samples. The kappa value was used to determine the degree of agreement between the two tests with a kappa value of 0 indicating no agreement and that of 1 indicating perfect agreement.
CHAPTER FOUR
RESULTS

4.1 Demographic Profile

The number of patients included in the study was 216 with a mean age of 20.7 years (minimum age: 3 years, maximum age: 56 years and standard deviation: 11.4). Females and males constituted 44% (n = 95) and 56% (n = 126) of the samples that were collected respectively. When the patients were categorized based on age 41.7% and 58.3% represented children (less than 18 years) and adults respectively (Table 1).

Table 1: Summary of clinical samples collected for the study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (n =)</td>
<td>216</td>
</tr>
<tr>
<td>Male</td>
<td>95 (44%)</td>
</tr>
<tr>
<td>Female</td>
<td>121 (56%)</td>
</tr>
<tr>
<td>Children (less than 18 years)</td>
<td>90 (41.7%)</td>
</tr>
<tr>
<td>Adults (above 18 years)</td>
<td>126 (58.3%)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>3 – 56 years</td>
</tr>
<tr>
<td>Mean</td>
<td>20.7 years (SD: 11.4)</td>
</tr>
<tr>
<td>Median</td>
<td>20.5 years</td>
</tr>
</tbody>
</table>

SD, Standard Deviation
4.2 Malaria Diagnosis by Giemsa Microscopy and *Plasmodium* Genus ID-FISH®

Diagnosis of malaria using Giemsa microscopy gave a prevalence of 31.5% (n= 68), average parasitemia of 20844 parasites/μl with a minimum parasitemia of 120 parasites/μl, while *Plasmodium* genus ID-FISH® test gave a prevalence of 40.3% (n = 87). This represented a 28% increase in detection rate according to fluorescent microscopy diagnosis (Table 2). Moreover, this demonstrated a significant detection difference of 8.80% (P = 0.0008) between the two tests.

Table 2: Summary of clinical diagnosis based on assay.

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>PR 95%CI</th>
<th>Negative</th>
<th>NR 95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giemsa Microscopy</td>
<td>68 (31.5%)</td>
<td>25.3-38.1</td>
<td>148 (68.5%)</td>
<td>61.9-74.7</td>
</tr>
<tr>
<td>FISH® Test</td>
<td>87 (40.3%)</td>
<td>33.7-47.1</td>
<td>129 (59.7%)</td>
<td>52.9-66.3</td>
</tr>
</tbody>
</table>

CI, confidence interval; PR, positive result; NR, negative result

While examining FISH® processed slides, it was observed that there was a variation in the intensity of the apple green fluorescent signal from the *Plasmodium* parasites as shown in Figure 4.
Figure 4: *Plasmodium* Genus ID-FISH® assay thin blood smear. This illustrates a sample positive for *Plasmodium* species. Infection. Arrow A shows a strong fluorescent (apple green) signal while Arrow B shows a weak fluorescent signal of parasites.

Based on age, *Plasmodium*- Genus FISH® test and Giemsa microscopy gave a detection rate of 51% and 44%, respectively, in children (less than 18 years of age) while in adults it was 33% and 24% respectively (Figure 5). Malaria prevalence was observed to reduce with increasing age.
4.3 Evaluation of Diagnostic Performance of *Plasmodium* Genus ID-FISH®

When the results for *Plasmodium* genus ID-FISH® test were compared to the gold standard, Giemsa microscopy 63 (29.2%) tested positive and 124 (57.4%) tested negative for both tests. This represented an agreement of 87% (187 true positive and true negative results) between the two tests. However, a total of 29 samples gave discrepant results with 11.1% (24) and 2.3% (5) being false positive and negative respectively (Table 3).
Table 3: Comparison of malaria diagnosis results based on the test used.

<table>
<thead>
<tr>
<th></th>
<th>Giemsa Microscopy</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmodium Genus FISH®</strong></td>
<td>Positive</td>
<td>63 (29.2%)</td>
<td>24 (11.1%)</td>
<td>87</td>
</tr>
<tr>
<td>Kit Test</td>
<td>Negative</td>
<td>5 (2.3%)</td>
<td>124 (57.4%)</td>
<td>129</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>68</td>
<td>148</td>
<td>216</td>
</tr>
</tbody>
</table>

Table 4 shows the diagnostic performance of *Plasmodium* genus ID-FISH® assay with reference to Giemsa Microscopy. *Plasmodium* genus ID-FISH® assay had a sensitivity of 92.65% (95% CI 83.67% - 97.57%) and a specificity of 83.78% (76.84% - 89.33%). Moreover the test had positive and negative predictive values of 72.41% and 96.12% respectively. Since sensitivity, specificity and predictive values vary with disease prevalence, likelihood ratios were used to analyze the clinical usefulness of the test. *Plasmodium* genus ID-FISH® test gave a positive likelihood ratio (PLR) of 5.71 (95% CI 3.94 - 8.29) and a negative predictive value of 0.09 (95% CI 0.04 to 0.20). This indicated that a negative result is highly reliable compared to positive results.
Table 4: Diagnostic Performance of FISH® test compared to Giemsa microscopy.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>92.65%</td>
<td>83.67% to 97.57%</td>
</tr>
<tr>
<td>Specificity</td>
<td>83.78%</td>
<td>76.84% to 89.33%</td>
</tr>
<tr>
<td>PLR</td>
<td>5.71</td>
<td>3.94 to 8.29</td>
</tr>
<tr>
<td>NLR</td>
<td>0.09</td>
<td>0.04 to 0.20</td>
</tr>
<tr>
<td>PPV</td>
<td>72.41%</td>
<td>61.79% to 81.46%</td>
</tr>
<tr>
<td>NPV</td>
<td>96.12%</td>
<td>91.19% to 98.73%</td>
</tr>
</tbody>
</table>

CI, confidence interval; PLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value

Accruacy of this test was analyzed using a receiver operating characteristic (ROC) curve and it gave an area under the curve (AUC) value of 0.886 (95% CI 0.832 – 0.932; p < 0.0001). This indicated that Plasmodium genus ID-FISH® test was significantly able to distinguish between malaria positive and negative samples since AUC was above 0.5 (Figure 6). Moreover, the agreement between the gold standard, Giemsa microscopy, and the Plasmodium genus ID-FISH® test was analyzed using the kappa value. It was observed that this test had a moderate agreement, kappa value of 0.711 (95% CI 0.58 – 0.84), with that of Giemsa microscopy.
Figure 6: ROC curve analysis of the accuracy of FISH assay. The area under the curve is 0.886 (95% CI 0.832 – 0.932) with a p < 0.0001.

4.4 Limit of Detection

The growth of malaria parasites used for limit of detection study was not synchronized. The parasitemia varied (2%, 4% and 8% for trial A, B and C respectively) based on Giemsa microscopy. *Plasmodium* genus FISH® test could detect malaria parasites 2 logs higher than Giemsa microscopy (Table 5).
Table 5: Limit of detection: Comparison of the lowest number of parasites that can be detected by Giemsa microscopy and *Plasmodium* genus ID – FISH® kit test

<table>
<thead>
<tr>
<th>Slide ID</th>
<th>Trial A</th>
<th>Trial B</th>
<th>Trial C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Giemsa (n/n)</td>
<td>FISH (n/n)</td>
<td>Giemsa(n/n)</td>
</tr>
<tr>
<td>c0</td>
<td>10/1</td>
<td>7/1</td>
<td>8/1</td>
</tr>
<tr>
<td>c1</td>
<td>3/1</td>
<td>2/1</td>
<td>2/1</td>
</tr>
<tr>
<td>c2</td>
<td>-</td>
<td>4/20</td>
<td>1/20</td>
</tr>
<tr>
<td>c3</td>
<td>-</td>
<td>3/50</td>
<td>2/50</td>
</tr>
<tr>
<td>c4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>c5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>c6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cn</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Slide ID, slide Identification; n/n, average number of parasites counted/average number of microscopic fields observed; -, negative results
5.1 Discussion

Malaria is an important parasitic disease with its burden being enormous in sub-Saharan Africa. Recently the prevalence has decreased due to the concerted efforts employed with the aim to eradicating it (Okiro et al., 2007; D’Acremont et al., 2010). Continued decrease in incidence is threatened by development of drug and/or insecticide resistance, making early diagnosis and prompt treatment critical in sustaining the gains achieved (Okafor and Amzat, 2007; WHO, 2010).

Lack of a good diagnostic gold standard for malaria poses a challenge in its control (Peeling et al., 2006). Development of malaria diagnostic tests started from a clinical approach which has been observed to be equivocal in distinguishing malaria from other febrile illnesses (Amexo et al., 2004; Perkins and Bell, 2008). Consequently, tests that can demonstrate the presence of parasites, either directly or indirectly, leading to improved management of malaria have been developed (Chan, 1989; Kong and Chung, 1995).

5.1.1 Malaria diagnosis using Plasmodium Genus ID – FISH® and Giemsa microscopy

In this study, the prevalence of malaria was observed to be 31.5% and 40% by Giemsa microscopy and Plasmodium genus ID – FISH®, respectively. This was a high incidence rate which could be attributed to the high transmission at the time (May and June) of sample collection. In Kwale district malaria transmission is at its peak in the month of May, June and July (Omar, 2002). The malaria incidence rate observed in the study is similar to that reported by
Okiro et al. (2007) in Kwale district. These results are in agreement with the estimated malaria burden of about 30% (based on Giemsa microscopy) of all outpatients in Kwale district (Omar, 2002).

Malaria prevalence was observed to be highest amongst children below 5 years compared to those between the age of 5 to 18 years and adults. Even though the majority of patients included in the study were adults (58.3%), malaria prevalence was found to be highest in children (below 18 years). This might be attributed to acquired immunity against malaria parasites which decreases the rate of patients presenting malaria symptoms with ageing (Baird, 1998; Ladeia-Andrade et al., 2009).

The lowest parasitemia observed while using Giemsa microscopy for diagnosis was 120 parasites/μl. Although not determined for Plasmodium genus ID – FISH®, it was expected that a lower parasitemia could be detected due to increased sensitivity and therefore making it possible to detect malaria parasites missed by Giemsa microscopy. This was demonstrated by a significant detection difference (P = 0.0008) between Giemsa microscopy and FISH® suggesting that some infections may have been missed. A study conducted in Kwale district revealed a high rate of self medication of fever in both children and adults (Abuya et al., 2007). This may affect the health of the parasites reducing the test’s target (rRNA) concentration leading to weak fluorescent signals from parasites which can be easily missed. In light of this, laboratory staff using Plasmodium genus ID-FISH® should not rule out the possibility that patients have self mediated. Moreover, it should be emphasized to the public that seeking treatment after diagnosis improves management of diseases. Similarly, immunity development might also lead to under diagnosis (Iqbal et al., 2002; Harris et al., 2010).
During diagnosis by *Plasmodium* genus ID-FISH®, it was observed that the intensity of the fluorescent signal of some of the detected parasites was weak and thus they may be missed. Such instances are common while observing a thick smear, where a parasite(s) may be in an erythrocyte that is under layers of other non-infected RBCs. On the other hand, it was observed that some parasites had a weak signal in the thin smear. This may be attributed to variation of the concentration of the target rRNA resulting to fewer fluorescent probes hybridizing. A similar observation was reported in studies conducted by Poulsen *et al.* (1993) and De Vries *et al.* (2004). Antibiotics impeding growth rate of *Plasmodium* parasites may as well have a similar effect on the signal intensity.

*Plasmodium* genus ID – FISH® demonstrated a higher detection of malaria resulting to an increase of 28%. This finding suggests that FISH would detect 28% more cases than Giemsa microscopy. The increment in detection observed using the FISH appears to indicate that lower parasitaemias, not detected by Giemsa, became detectable. This is because fluorescent labeled probes enhance the visibility and thus detection of parasites even those lying below layers of red cells. An increased malaria detection rate, based on tests, has also been reported in studies evaluating the performance of a similar test referred to as cyscope® fluorescent microscopy (Hassan *et al.*, 2010; Sousa-Figueiredo *et al.*, 2010). Similarly, a study conducted by Mens *et al.* (2007) reported that molecular tests lead to identification of more patients (especially children) with low parasitemia thus increasing the detection rate. The use of fluorescent labeled probes specific to parasites made it unlikely that fluorescence was from debris or artifacts thus the increment in detection was a true reflection of increase in detection (of parasitemia) below the detection limit of Giemsa microscopy. Moreover, *Plasmodium* genus ID-FISH® test reduced the
time taken to look through the slide because of use of fluorescence. This is corroborated by observations made by Kong and Chung (1995) and Guy et al. (2007).

5.1.2 Diagnostic performance of Plasmodium Genus ID-FISH® test

The specificity calculated in this study was (83.78%) below the acceptable lower limit of 90% for RDTs (WHO, 2006). This emanated from a high discordance rate between the two diagnostic methods for positive results. It is noteworthy that the use of an imperfect gold standard may lead to an overestimation of the false negatives rate which may be the case in this study because of lack detection at low parasitemia (Ohrt et al., 2002).

Although Plasmodium genus ID-FISH® demonstrated a lower specificity (83.78%) than 100%, it was an improvement from that reported for Giemsa microscopy (71%) by Ohrt et al. (2002). This is attributable to improved detection ability which exposed malaria parasites missed by Giemsa microscopy (Guy et al., 2007). However, poorly prepared slides for Plasmodium genus ID-FISH® test might lower specificity due to auto-fluorescence. Diagnosis using Plasmodium genus ID-FISH® depends on the observation of a fluorescent signal making it easier to spot parasites than Giemsa stained parasites (Ohrt et al., 2002; Bronner et al., 2009; Cnops et al., 2010). This as well may have contributed to the improved specificity.

A similar study conducted by Sousa-Figueiredo et al. (2010) evaluating the performance of cyoscope fluorescent microscopy recorded a lower specificity of 38.8% compared to Giemsa microscopy. However, an earlier study reported that cyoscope® was very specific (98%), performing better than Plasmodium genus ID-FISH® test (Hassan et al., 2010). Lower specificity was attributed to a change in the study population; high number of samples with low parasitemia leads to a low parasite detection ability (Dijk et al., 2009; Hassan et al., 2010; Sousa-Figueiredo
et al., 2010). This illustrates that *Plasmodium* genus ID-FISH® test may record a varied performance depending on the study site or population.

A total of 5 (2.3%) samples diagnosed to be negative for malaria infection by *Plasmodium* genus ID-FISH® test were positive by Giemsa microscopy thus false negative results. This included samples with a high and low parasitemia. Weak signals from parasites affected by drugs other than anti-malaria drug might have lowered the detection rate at high parasitemia. On the other hand low parasitemia increases the chances of there being no parasite in a given smear and therefore higher chances of false negative results. Several studies have demonstrated that a decrease in parasitemia decreases the sensitivity of many tests including fluorescent microscopy (Keiser et al., 2002); RDTs and PCR-based methods (Coleman et al., 2006; Erdman and Kain, 2008; Kim et al., 2008; Rakotonirina et al., 2008; Bendezu et al., 2010).

The accepted level of sensitivity of rapid diagnostic tests for malaria is 95% at a parasite density of 100 parasites/μl (Hassan et al., 2010). In this study, the calculated sensitivity of FISH® assay was 93% (negative predictive value of 96%). Although not above the 95% threshold, it was above the sensitivity recorded for basic microscopy 91% (Ohrt et al., 2010). This sensitivity is also similar to that of cystscope® microscopy for diagnosis of malaria in children (Sousa-Figueiredo et al., 2010). Moreover, improved sensitivity further explained the significant increased malaria detection rate observed in this study.

Sensitivity of FISH® assay might be reduced if samples used are collected from patients who are on prescription drugs which reduce the concentration of the test target (rRNA) unlike Giemsa microscopy which depends on the affinity of the dye (Giemsa stain) to the nucleic acid (DNA) which is not affected by such drugs (Poulsen, et al., 1993; De Vries et al., 2004). This results in fewer *Plasmodium* genus ID-FISH® test probes binding to the target (rRNA) and reduced
intensity of the fluorescent signal, thus leaving parasites undetected giving false negative results. Consequently, patients who may need malaria treatment may remain untreated.

Finding an accurate and precise diagnostic test is important for malaria to be controlled. It is now recognized that although Giemsa microscopy is recommended for malaria diagnosis it is an imperfect diagnostic tool (Ohrt et al., 2002; Amexo et al., 2004). In this study Plasmodium genus ID-FISH® showed an accuracy (or agreement) of 88.6% (95% CI 83.2 – 93.2). Nicatri et al. (2009) reported a higher accuracy (93.5%) due to use of a better reference test (PCR) and therefore accuracy may improve on re-evaluation with such a gold standard. This demonstrates that this test may be used as a guide for prudent prescription of anti-malaria drugs in order to prevent malaria parasites from developing drug resistance. Modifications of Plasmodium genus FISH test will promote the possibility of replacing Giemsa microscopy as a gold standard. Furthermore, this shows that Plasmodium genus ID-FISH® test may help in ruling out malaria infection and as a result improving the management of other febrile diseases that might be present especially in children below 5 years.

Positive and negative likelihood ratios are independent of the prevalence of the disease in the population and can be used to determine the accuracy of positive and negative results (Bendezu et al., 2010). In this study, a low likelihood ratio for the positive test, below 10, was found for Plasmodium infection indicating that a Plasmodium genus ID-FISH® positive result had a moderate clinical value. This shows that patients with Plasmodium genus ID-FISH® positive results need to be diagnosed with another test to confirm the positive test. A poor gold standard like Giemsa microscopy might lower the clinical value of positive results due to high discordance. This validates why it is important to use a better gold standard than Giemsa microscopy like PCR for evaluation of Plasmodium genus ID-FISH® although this was beyond
the scope of this study. On the other hand, the clinical value of a *Plasmodium* genus ID-FISH® negative result was observed to be high, NLR = 0.09, indicating that this test can be used to rule out malaria infection in febrile patients. This is further collaborated by a high negative predictive and therefore a negative *Plasmodium* genus ID-FISH® test result can be used with confidence. This demonstrates that use of this test will help save resources that could have been used for purchase of anti-malaria drugs by governments which in turn will be used establishing these tests in the long term.

5.1.3 Limit of Detection for *Plasmodium* Genus ID-FISH® test

In this study, *Plasmodium* genus ID-FISH® illustrated the capacity to detect malaria parasites two logs higher than Giemsa microscopy. This indicates that *Plasmodium* genus ID-FISH® is more sensitive, detecting parasites at lower levels, as was observed when calculating sensitivity and specificity. Moreover, this demonstrates that the false positives that were detected may have been true positive missed by Giemsa microscopy. Coleman *et al.* (2006) supported this by regarding false positives of nested PCR (nPCR) to be true positives since it was more sensitive than Giemsa microscopy.

During the limit of detection study, it was observed that in one trial *Plasmodium* genus ID-FISH® detected one log higher than Giemsa microscopy. This variation may have been due to use of *Plasmodium falciparum* strain D6 culture whose growth was not synchronized. Consequently, the proportion of malaria parasites at a particular growth stage might have varied and possibly was not detected. This might have also influenced the limit of detection. A study conducted by Khairnar *et al.* (2010) supported this by revealing that the limit of detection was
significantly affected by the stage of malaria parasites; a tenfold difference although samples had the same parasitemia.

5.2 Conclusions

- *Plasmodium* genus FISH\textsuperscript{®} assay is more sensitive and more specific than Giemsa microscopy for malaria diagnosis.
- The negative predictive value indicates that *Plasmodium* genus FISH\textsuperscript{®} assay can be used to rule out malaria in febrile patients.
- The limit of detection of *Plasmodium* genus FISH\textsuperscript{®} assay is two folds higher than that of Giemsa microscopy.

5.3 Recommendations

- Since *Plasmodium* genus FISH\textsuperscript{®} assay is more sensitive and more specific than Giemsa microscopy for malaria diagnosis and has a limit of detection two folds higher than of Giemsa microscopy, it is recommended that *Plasmodium* genus FISH\textsuperscript{®} be evaluated against more sensitive assays like PCR to rule out the false positives observed in this study.
- Kenya has put stringent malaria control strategies which have resulted in lower parasitemias within populations at risks. Considering that Giemsa microscopy fails to detect low parasitemias in the populations at risk, and that Kenya in intent on eliminating malaria, it is recommended that the *Plasmodium* genus FISH\textsuperscript{®} be used to detect the low parasitemias that Giemsa microscopy is unable to detect.
Since *Plasmodium* genus FISH® is two folds lower parasitemia than Giemsa microscopy, it is recommended that *Plasmodium* genus FISH® replaces Giemsa in ruling out malaria in febrile patients and also in ascertaining cures following malaria chemotherapy.
REFERENCES


large proportion of asymptomatic *Plasmodium* infections with low and sub-microscopic parasite densities in the low transmission setting of Temotu Province, Solomon Islands: Challenges for malaria diagnostics in an elimination setting. *Malaria Journal* 9:254.


