DEVELOPMENT AND USE OF PARASITE LACTATE DEHYDROGENASE ASSAY FOR RESEARCH, DIAGNOSIS AND SCREENING OF ANTIMALARIAL DRUGS IN KENYA

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DECLARATION

I, Rose Aoko Ogwang' Odhiambo, hereby declare that this thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted with our approval as University supervisors.

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DEDICATION

This thesis is dedicated to my husband Wilson Odhiambo Philemon and to my children Franklin Mireri, Denis Young Ogwang', Sam-Martin Cofele and Caleb- Louice Tunu all who have been affected by malaria as a disease and have directly contributed to the success of this study.

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GLOSSARY OF ABBREVIATIONS

ACD:	Acid dextrose
AM:	Amodiaquine
APAD:	3-acetyl pyridine adenine dinucleotide
AT:	Atovaquone
A=0:	Microscopy thick smear negative
A=1:	Microscopy thick smear positive
B=0:	Microscopy thin smear negative
B=1:	Microscopy thin smear positive
CCR:	Centre for Clinical Research
CQ:	Chloroquine
D6:	Chloroquine sensitive malaria reference strain
DAAD:	German Academic Exchange Service for African students
DH:	Dehydroartemisinin
DRIP:	Dissertation Research Internship Programme
F:	Experiments that failed to give reproducible pLDH IC50
Flow Inc:	Flow incorporated Portland Oregon
HF:	Halofantrine
IC50:	Fifty percent inhibitory concentration
IC75:	Seventy five percent inhibitory concentration
IC95:	Ninety five percent inhibitory concentration
ICIPE:	International Centre for Insect Physiology and Ecology

ICT-Pf:	Immunochromatographic malaria *Plasmodium falciparum*
KEMRI:	Kenya Medical Research Institute
LDH:	Lactate dehydrogenase
LSD:	Least significant difference
MF	Mefloquine
NAD:	Nicotinamide adenine dinucleotide
NBT:	Nitroblue tetrazolium
NF54:	International Amsterdam Airport srain of *Plasmodium falciparum* .
OD:	Optical Density
OptiMAL:	Modification of pLDH enzyme assay, a dipstick
Pf HRP2:	*Plasmodium falciparum* histidine-rich protein 2
pLDH:	Parasite lactate dehydrogenase
Parasight-F:	Diagnostic kit for *Plasmodium falciparum* malaria
Parasit:	Parasitaemia
PES:	Phenolmethosulphate
Q:	Quinine
RBC:	Red blood cell
t-statistic:	Statistical method used to derive mean pLDH cut-off
t-test:	Student t-test
W2:	Chloroquine resistant malaria reference strain
WHO:	World Health Organization
WRP:	Walter Reed Project
#:	Number

ABSTRACT

Plasmodium falciparum malaria has routinely been diagnosed using thick smear microscopy, a procedure which has been difficult to standardize because of different levels of technician expertise and quality of reagents in use. More recently, malaria diagnosis has used immuno-diagnostic test for the detection of circulating *Plasmodium falciparum* histidine rich protein 2 (Pf HRP-2) antigen in the parasight-F test. However, this test is limited in use in that the HRP-2 may persist after parasite clearance in some patients, mostly those with high initial parasitaemia and hence give a false positive with the parasight-F test. All the diagnostic tests currently in use to identify malaria parasites in clinical specimens, are restricted to picking circulating parasites. Since mature asexual forms of *P. falciparum* sequester in deep tissue capillaries and may be inaccessible to veni puncture, an alternative diagnostic procedure needs to be developed. The new diagnostic tool should be technically simple to perform, affordable and possess standard sensitivity and specificity for use in *P.falciparum* endemic areas. It will have an added advantage if it could also be used for the evaluation of antimalarial drug susceptibility and sensitivities.

A new diagnostic method namely, Parasite lactate dehydrogenase (pLDH) assay was investigated as a tool for malaria diagnosis and drug studies between 1994-1997 in Kenyan population in laboratory, under field and clinical *in vitro* situations. One hundred and seven (107) healthy Kenyan volunteers from a malaria non- endemic region of Kenya were recruited into the pLDH studies as controls and their plasma and red blood cells measured using the modified pLDH procedure. The results of the controls plasma and red blood cell pLDH indicated significantly lower values compared to those from subjects

from field and clinical studies who were either parasitaemic, symptomatic or asymptomatic from malaria endemic region of Kenya. The results indicated that the controls optical density values could not be used to calculate mean cut-off as well as mean positive values for plasma and red blood cells in field and clinical studies for sensitivity and specificity analysis. The study indicated that individuals living in malaria non- endemic region, who have not suffered malaria attack for three consecutive months have significantly lower red blood cell and plasma pLDH values compared to individuals in endemic regions, who are exposed to frequent infections. Evaluation of the study indicated a new cut-off system for calculating mean plasma and red blood cell pLDH which can be used in any region to calculate sensitivity and specificity of pLDH in a given population for diagnostic purposes, independent of microscopy.

The drug sensitivity profiles for laboratory and field adapted malaria isolates could be comfortably measured using pLDH as opposed to the hypoxanthine assay which requires alot of complicated procedures before IC 50 cut-off could be calculated by probit-analysis. However, in both assays, the results were comparable. pLDH enzyme assay was successfully used to measure the IC 50 of six antimalarial drugs, chloroquine, quinine, mefloquine, dehydroartemisinin, atovaquone and halofantrine but was not successful with the other four antimalarial drugs, doxycycline, azithromycin, pyrimethamine, and sulphadoxine, which are slow acting antimalarials. The latter four drugs did not give consistent results even when the incubation time was raised from 48 hours to 66 hours, by using the reference strains, D6 and W2.

The laboratory isolates indicated a high correlation between pLDH and hypoxanthine assay, which was not the case with field isolates. The Kisumu isolates collected for the study and culture adapted for comparative studies using reference strains were generally chloroquine resistant, nearly mefloquine resistant, quinine sensitive, and sensitive to the remaining new antimalarial drugs, atovaquone, halofantrine and dehydroartemisinin. The results of this study indicate that it is possible to use pLDH for drug sensitivity studies in the field, especially using the six antimalarial drugs, a finding that may improve on malaria chemoprophylaxis without necessarily exerting drug pressure on the few available effective antimalarial drugs.

The study indicated no correlation in pLDH optical density and parasitaemia in culture adapted NF54 gametocytes, strongly suggesting that mature gametocytes (stages three, four and five) may not be producing significant amounts of pLDH in the red blood cell. The study has strongly indicated low parasitaemia as a major limiting factor, a condition limiting its field applicability especially in non-immunes and the high risk groups (children and expectant mothers).

From the study, pLDH compared to ICT and Parasight-F, gives the highest specificity but lowest sensitivity suggesting that it would be a better tool for diagnosis if the sensitivity is improved to enable picking of even low parasitaemias. pLDH was the only procedure among the three that picked pure infection of *P.malariae* as *P. vivax* while the other two missed any pure infection that was not *P. falciparum*. The overall report about pLDH assay is that : it has indicated potential for diagnosis of malaria in endemic areas save for

its low sensitivity, it identifies other species of *Plasmodia* and it is quite fast and safe in drug studies *in vitro*.

CHAPTER ONE

1.0 Introduction and Literature Review

1.1 Incidence, transmission and epidemiology of malaria.

Approximately 270 million people suffer from malaria, and there are between 1 million and 2.5 million deaths every year, most of whom are African children (White, 1996, WHO 1997a). The disease is prevalent throughout the tropics and subtropics (WHO, 1997b). In Africa, *P. falciparum* predominates as it does in Papua New Guinea and Haiti whereas *P. vivax* is more common in central part of South America, North America, the Middle East and the Indian subcontinent (Brabin, 1993). Malaria transmission does not normally occur at temperatures below 16° C or above 33° C and at altitudes greater than 2,000 m, because development in the mosquito (sporogony) cannot take place (Brabin, 1993). Malaria transmission to human depends on several interrelated factors. The most important one pertains to the longevity of the anopheline vector (White, 1996). As sporogony takes over a week, depending on ambient temperatures, the mosquito must survive for longer than this after feeding on human blood carrying gametocyte for malaria to be transmitted. Vectors of malaria parasites differ considerably in their natural abundance, feeding, resting behaviours, breeding sites, flight ranges, choice of blood meal source and vulnerability to environmental conditions (Kamau *et al.,* 1998). Other factors which are not well understood may also influence mosquito populations and lead to fluctuations in the prevalence of malaria (Brabin, 1993; Shililu *et al.,* 1998).

1.2 The human host and parasite burdens

In areas of high transmission, infants and young children are more susceptible to malaria than older children and adults (Marsh and McGregor, 1986). Parasite densities are higher and gametocytaemia is detected more frequently in children (McGregor, 1984). This younger age group probably represents the main reservoir and also the main recipient of infection (Wasonga, 1997; Othoro *et al.*,1999). Those in the older age group also have asymptomatic infections but parasite densities are much lower (McGregor, 1984; Othoro, 1997). In areas that are holoendemic for *P. falciparum,* such as much of tropical Africa or coastal New Guinea, people are infected repeatedly throughout their lives. If the child survives, a state of premunition is achieved where subsequent infections cause little or no problems to the host. Thus, a nonsterile form of immunity develops which is sufficient to control, but not prevent the infection (Riley *et al.,* 1988; Playfair *et al.,* 1990;). The rate at which premunition is acquired may be a function of age of the host and the frequency of subsequent or new infections (Playfair *et al.*, 1990; Othoro, *et al* ., 1999).

1.3 Clinical presentations of malaria

The clinical manifestation of malaria is dependent on the previous immune status of the host (Orago and Facer, 1991). In areas of intense transmission of *P. falciparum* malaria, asymptomatic parasitaemia is usual in adults (premunition). The rate at which specific development of premunition occurs is proportional to the intensity of malaria transmission (Duggan and Hutchson, 1966). The time from sporozoite inoculation until the first positive blood film (prepatent period), and time from sporozoite infection to fever (incubation period), are prolonged by ineffective antimalarial treatment or prophylaxis (Silamut and

White, 1993). The shortest reported incubation period for malaria was on a sailor who docked briefly in West Africa and developed clinical signs of malaria three days later (Bruce-Chwatt, 1985). Primary incubation period may be long, particularly if the infection is suppressed by partially effective chemoprophylaxis. Naturally acquired infections have an incubation period of between ten and thirty days (Bruce-Chwatt, 1985). The parasitaemia level at which fever occurs (the pyrogenic density) varies widely, so that some non-immune patients will become febrile before parasites are visible on blood smears (that is the incubation period is shorter than the prepatent period), whereas semi-immune adults may tolerate up to 100,000 *P. falciparum* parasites per microlitre of blood without fever (Boyd 1949; WHO, 1990), or indefinitely (Makobongo *et al.,* 1997).

1.4 Pathophysiology of malaria

Pathophysiology of malaria results from destruction of erythrocytes, the liberation of parasite and erythrocyte material into circulation, and the host reaction to these events (White, 1996). *Plasmodium falciparum* infected erythrocytes also sequester in the microcirculation of vital organs, thus, interferring with the microcirculatory flow and the host tissue metabolism (White and Ho, 1992). Cytokines appear to be responsible for many symptoms and signs of the infection, particularly fever and malaise. Plasma concentrations of certain cytokines are elevated in both acute *P. vivax* and *P. falciparum* malaria (Kwiatkowski *et al.*, 1990). Furthermore, there is a positive correlation between cytokine levels and prognosis in severe *P. falciparum* malaria (Kwiatkowski *et al.*, 1990). The process whereby erythrocytes containing mature forms of *P. falciparum* adhere to microvascular endothelium (cytoadherence), and thus disappear from the circulation, is

known as sequestration (White and Ho, 1992) and is thought to be central to the pathophysiology of *P. falciparum* malaria (Oppenheimer *et al.*,1986; White and Ho, 1992).

Cytoadherence begins at the middle of the parasite's 48-hour asexual life cycle. Whereas, in other forms of human malaria, mature parasites are commonly seen on blood smears from peripheral circulation, such are rare in *P. falciparum* malaria and often indicates serious infection. Sequestration occurs predominantly in the venules of vital organs. It is not distributed uniformly throughout the body, being greatest in the brain, particularly the white matter, the heart, liver, kidneys, intestines and adipose tissue, and least in the skin (White, 1996). Cytoadherence and the related phenomena of rosetting lead to microcirculatory obstruction in *P. falciparum* malaria (Howard *et al.,* 1986). The consequences of microcirculatory obstruction are reduced oxygen and substrate supply, leading to anaerobic glycolysis and lactate acidosis (White, 1996). Although death from acute *P. vivax, P. ovale, P. malariae* infections is very rare, *P. falciparum* malaria is a potentially fatal disease. Severe malaria is defined by manifestations of cerebral malaria, severe anaemia (< 5g /dl), parasitaemia >10,000 /μl, renal failure, pulmonary oedema, or adult respiratory distress syndrome (WHO, 1990). The definition also includes hypoglycaemia, circulatory collapse or shock, spontaneous bleeding from gums, nose, gastrointestinal tract, repeated generalized convulsions, acidaemia, microscopic haemoglobinuria and postmortem confirmation of diagnosis. In severe malaria, hypoglycaemia may occur and in the absence of quinine treatment this is accompanied by elevated ketones, raised lactate, alanine and low insulin levels (White *et al.*,1985). The lactate levels in arterial or venous blood or cerebrospinal fluid are elevated in proportion to disease severity (White *et al.*, 1985).

Sequestration of PRBCs in cerebral venules and capillaries is considered to be the

pathologic manifestation of cerebral malaria (Mac Pherson *et al.,* 1985; WHO, 1990b). The preferential location of PRBCs close to the endothelial cells and the packing of the central capillary lumen by both parasitized and non-parasitized RBCs is akin to the phenomenon of *in vitro* rosette formation or binding of these two forms of RBCs. Their role in the pathogenesis of human cerebral malaria is underlined by the protection provided by anti-rosette forming antibodies against cerebral malaria (Handunnetti *et al.*, 1989; Carlson *et al.,* 1989).

1.5 Diagnosis of malaria.

1.5.1 Rationale for diagnosis in malaria

In both developed and developing world, a simple and reasonably sensitive screening test for the detection of the malaria parasite would be of value. More ideal would be a combination screening test for detection and sensitivity for the presently available antimalarial drugs. Since early diagnosis and prompt treatment is the first problem that needs to be addressed in any malaria control programme (WHO, 1988), the association of clinical, epidemiological and laboratory criteria is important to obtain a reliable diagnosis.

1.5.1.1 WHO memorandum for malaria diagnosis

Malaria diagnosis is based on certain findings, including the detection of malaria parasites in humans and the mosquito by microscopy, immunological methods, DNA probes, malaria-related clinical symptoms and an assessment of certain characteristics indicative of current or past malaria infection. The reliable diagnosis of malaria, whether in a hospital or in a rural health clinic or patient's home, is a prerequisite for selecting the correct treatment

and reduction of morbidity and mortality (WHO, 1984, 1986b, 1988).

Treatment at the peripheral level of the health services is generally given following a diagnosis based on clinical symptoms, usually fever. This may be acceptable if the parasite is susceptible to a universally effective, well tolerated and cheap drug such as chloroquine. This procedure however will miss malaria cases not identified as such and also result in the management of fever of non- malarial origin. In addition to being cost effective, it will exert an unduly high drug pressure on the parasite population, which may lead to the development and spread of drug resistant parasites. In fact, chloroquine - resistant and even multi-drug resistant *Plasmodium falciparum* has already spread through many of the malaria endemic areas. Drugs for the treatment of chloroquine - resistant malaria are more expensive and less well tolerated, and resistance against them has been reported elsewhere and will spread unless they are used judiciously. It has therefore become urgent to ensure that specific antimalarial treatment is based on the **rapid** and **reliable** diagnosis of the disease particularly at the peripheral level of the health services delivery systems.

Despite being tedious and labour intensive, light microscopy continues to be the mainstay of diagnosis for epidemiological studies on which current malaria control strategies are based. It must be recognized that the light microscope, although effective, versatile and efficient, has many disadvantages. It is expensive, needs careful maintenance and requires trained and competent operators. Furthermore, continuous use of microscopes induces fatigue which may introduce errors. There is therefore, an urgent need for alternative diagnostic methods which must be cheap, simple, reliable and applicable at the village level by community health workers. The results from these new generation of tests, whether

positive or negative should be clear, unambiguous and available within a few hours so as to be useful when selecting treatment for individual patients. The recent isolation of purified antigens from all stages of the parasite and the development of specific monoclonal antibodies have resulted in the development of the more specific reagents and a new generation of tests. Research can therefore, pursue alternative methods to microscopy for the diagnosis of malaria (Payne, 1988). Precise identification of the four human malaria parasite species in field samples have been carried out by the polymerase chain reaction (Snounou, *et al*, 1993a, b). However, this is largely a research tool unsuited for routine clinical laboratory or field use (Quintana, *et al*, 1998).

1.5.2 Clinical diagnosis of malaria

The classical clinical picture of symptomatic malaria in patients includes malaise, headache, muscular pain, nausea and dizziness. This is followed by a feeling of cold, accompanied by shivering, rigor and raised body temperature, which may then return to normal with a bout of profuse sweating. Fever is often accompanied by nausea, retching and vomiting. In children, the rigor may be replaced by seizures. This classical picture is however, altered by the use of prophylactic drugs or by the development of immunity resulting from a prolonged stay in an endemic area. The fever may be continuous, which is almost a rule in first infections. In complicated cases, there may be marked abdominal pains, drowsiness, a variable reduction of consciousness and even coma as in cerebral malaria. In some cases, fever may be absent, and the only symptoms may be abdominal pain, vomiting, and febrility as in algid malaria. In the absence of classical clinical symptoms, a careful elucidation of the case history, revealing exposure to malaria risk in certain occupations or

while travelling, may assist the diagnosis. The classical picture of paroxysm may be absent in immune or semi- immune persons but variation in this picture is more common in non-immune persons in whom the clinical cause may be severe and even fatal (WHO, 1988). In non-malaria endemic countries, cases of imported malaria may be completely missed, and wrong diagnosis may be made in spite of the best available laboratory facilities often because the clinician does not consider malaria as a possible cause of the illness (Orago A.S.S, personal communication).

1.5.2.1 Complications of clinical diagnosis of malaria

With the increasing frequency of international travel, physicians in malaria free countries are often confronted with febrile travellers returning from areas endemic for the disease. The question that confronts the physician is: what is the likelihood that this febrile episode is due to malaria?. The non- specific nature of the clinical manifestation of malaria alone cannot provide complete evidence for maintaining a high index of suspicion for malaria infection for any febrile patient who has recently travelled to a malaria endemic area. Therefore, diagnosis of malaria ultimately resides with the physician asking the right questions (when and where have you travelled?) and the competent parasitology laboratory technician making a timely and accurate diagnosis (Svenson *et al.*, 1995; Gyorkos *et al.*, 1995).

1.5.2.2 Biochemical tests as indirect indicators of malaria infection

Chemical tests have been used as indirect indicators of many diseases, but their application to the diagnosis of malaria has not been explored. If such tests could be developed for malaria and prove to be cheap and reliable, they would be a valuable diagnostic tool in areas

where the provision of reliable microscopic facilities is impossible. *P. falciparum* malaria is a disease in which biochemical changes are quantitatively produced in the host by parasite induced pathology (Basco *et al.,* 1995). In addition, pathological changes in hepatic functions are usually manifest in acute malaria, even when parasitaemia is low or moderate (Price, *et al.*, 1997). Similarly, major disturbances of renal functions are seen in severe and complicated malaria and malarial nephropathy but rarely in uncomplicated *P. falciparum* malaria (WHO, 1988). However, a mild proteinuria is very common in acute malaria (WHO, 1986a). Tests based on the principle that immune processes in conjunction with hepatic dysfunction often lead to a significant decrease in the albumin and haptoglobin degradation products in urine, as well as for lactate dehydrogenase, glutamate pyruvate transaminase, sorbitol dehydrogenase and sodium haptoglobin levels in blood, may be potential indirect indicators of acute malaria (Basco *et al.,*1995). The potential and the probability of these and other indirect indicators of malaria diagnosis should be evaluated by studies correlating the biochemical changes with both clinical and parasitological parameter.

1.5.3. Light microscopy in the diagnosis of malaria:

Thick blood film has limitations in sensitivity, and needs skilled microscopists to detect and differentiate *Plasmodium* species (WHO, 1988). Despite being tedious and labour intensive, light microscopy continues to be the mainstay of diagnosis for the epidemiological studies on which current malaria control strategies are based. Microscopic examinations of thick blood films technique allows for differentiation of *Plasmodium* species responsible for the actual malaria infection. The examination of thin smears, fixed

and stained in Giemsa, allows for better species differentiation and provides information on haematological parameters, but is of low sensitivity. Low parasitaemia are often missed and species diagnosis may be unreliable (Spielman *et al.*, 1988).

In a review, WHO (1988) observed that new generation alternative diagnostic methods to light microscopy must be cheap, simple, reliable, rapid and applicable at village level by community health workers as a prelude to referrals.

Cerebral malaria can be confirmed from a brain smear after postmortem (WHO, 1990a). A smear of grey matter is examined after staining the slide in the same way as for thin blood film. Capillaries and venules are identified microscopically under low power, and examined under high power. If the patient died in the acute stage of cerebral malaria, the vessels are packed with erythrocytes containing mature parasites and a large amount of pigment (WHO, 1990b).

The definitive diagnosis of malaria has historically been based on the detection of the parasite in the blood. Initially, this was achieved by observing the parasites in the red blood cells in thin blood films, the recognition of the parasites being enhanced by the use of Romanowsky - type differential stains which selectively colour the nuclear and cytoplasmic material (Payne, 1988). The thin blood film still retains its popularity as a diagnostic technique primarily in hospitals and clinics. It has long been recognized (Ross,1897, 1903) that a much more efficient screening technique is that of the thick blood film, a dehaemoglobinized layer of blood cells which is about 20- 30 times denser than the thin film.

Efforts aimed at improving the rate of parasite detection in the thick film by centrifugation have not proved successful, principally owing to staining problems (Bennett, 1962; Worth,

1964; PettyJohn, 1975).

1.5.3.1 Stains used in microscopic diagnosis of malaria

Of the many differential stains that have been developed over the years, the aqueous Romanowsky stains have proved to be the most adaptable and reliable for routine work, while Leishman's stain has proved to be the best all -round stain for the routine diagnosis of malaria. Romanowsky stains have the disadvantage of being relatively expensive, but this is outweighed by Giemsa's stability over time and its consistent staining quality over a wide range of temperatures. It is the stain of choice for peripheral health laboratories. Many alternative microscopical techniques have not been comparable (Shute and Sodeman, 1973; Jamjoom, 1983).

1.5.3.2 Limitations of the microscopical diagnosis

Microscopic diagnosis can be easily influenced by deficiencies in personnel. This includes inadequate training, poor supervision, bad management including the improper utilization of the available expertise and work time (Gevers and Kustner, 1988; Payne, 1988). Substandard or inappropriate equipment is another factor influencing utility and accuracy of microscopy in diagnosis. Conditions of incorrect or poorly coordinated specifications, particularly of microscopes; irregular maintenance and replacement of worn out parts; poor quality control of stains and reagents; inappropriate supply schedules which either cause delays in the supply of materials or accumulate stocks which exceed two shelf - life of the material. Inappropriate technology like the improper use of techniques (thin film for routine malaria diagnosis) may influence diagnosis (Makler and Gibbins, 1991;). Therefore, in the development of new diagnostic techniques for malaria, emphasis should be placed on

simple and standardized technology, as well as low cost, so that they can be used even in the periphery of primary health-care systems, and provide the best possible guidance for the treatment of malaria in endemic areas.

1.5.3.3 Microscopy for drug studies

In recent years, the introduction of various enumerative techniques has enabled the standard thick and thin films to be used to monitor *in vitro* and *in vivo*, the development of resistance to anti malarial drugs and thus serve as an important base in the formulation of appropriate drug policies at the National and International levels. However, this procedure has its limitations (Payne, 1988). Dowling and Shute (1966) concluded that whereas a high proportions of parasites are lost during staining, this loss being significant in scanty infections, parasite count as low as 1 per mm ^{3} could be detected in a thick film examination in ten minutes.

1.5.3 Other diagnostic techniques

1.5.4.1 Antigen detection

Antigen detection has the potential as an indicator of parasitaemia, but may not be suitable for routine diagnosis, mainly due to difficulties in the standardization of the crude reagents used and the necessity of removing all antimalarial antibodies in the test sample to overcome the problem of false positives (Makler and Levine, 1989). An example is the use of two different monoclonal antibodies against the histidine-rich protein (pf HRP-2) *of Plasmodium falciparum* in an antigen capture assay. In the antigen assays, a clear correlation between the amount of antigen and parasitaemia was not observed at low

parasitaemia. One of the reasons is that the antigen persists in the serum for several days after parasite clearance.

All tests for detection of malaria antigens are still at the initial stages of development. It may be possible to adapt a test for diagnosis if the target molecule has the following features: (a) it should not be shed or persist in the circulation independent of the parasite; (b) it should be as abundant as possible to maximize the sensitivity of the test; (c) it should not show great genetic diversity in the parasite population; (d) it should be structurally different from non-malarial antigens likely to be encountered in blood samples such as proteins of the host or other pathogens; (e) it should contain target proteins that are common to all species of human malaria. The test should also be simple, fast and capable of measuring the presence of drug-resistant parasites to have wide spread application .

Detection of malaria antibody can be useful in some circumstances such as confirmation of earlier infection, but has no place in acute diagnosis. Recently, rapid and simple stick test based on a monoclonal antibody against *P. falciparum* histidine rich protein 2 (parasight - F) has performed along with microscopy, in field studies.

1.5.4.1 Quantitative buffy coat (QBC) method

Unlike mature red blood cells, malaria parasites contain DNA and RNA. This can be stained with fluorescent dyes and visualised under ultra violet light microscopy, or with appropriate filters seen under ordinary light. In the QBC technique, blood samples are taken into a specialized capillary tube containing acridine orange stain and a float. Under high centrifugal forces (14,000 g), the infected erythrocytes which have a higher buoyant

density than uninfected cells, become concentrated around the float. Using a modified lens adapter (paralens) with its own light source, the acridine orange fluorescence from malaria parasites can be visualised through an ordinary microscope (Makler and Levine, 1989; Rieckman *et al.*, 1989). In some cases, this system has proved more sensitive than conventional light microscopy, although it does not give parasite counts or speciation with accuracy and it is relatively expensive (Long *et al.*, 1990; Avila *et al.,* 1994; Avila and Ferreira, 1994; Nguyen-Dinh *et al.,* 1994). Kumar *et al*; (1993) tested the QBC in developing countries and reported that although this assay has been reported to be rapid, sensitive and specific for the detection of malaria infection (Anthony *et al.*, 1992; Wongsrichanalai *et al.*, 1992), the technique was only slightly more sensitive than thin blood film and equally sensitive as the thick blood film examination.

Due to its high cost and requirement for a fluorescent microscope, the QBC technique, in spite of its speed and sensitivity, may not have the potential to be a substitute for the conventional blood film examination for the diagnosis of malaria, especially in developing countries (Kumar *et al.,* 1993). The assay has also been used for the detection of chloroquine resistance *in vivo* and it was reported to be an important tool for studying drug resistance (Garin *et al.*, 1992). The QBC was found to be a rapid technique but has a sensitivity of about 56% and a specificity of 85% compared to the thick blood film method in a mesoendemic area in Malaysia (Mak *et al.*, 1992). The technique however, cannot quantify parasitaemia easily and the specimens cannot be stored for future reference and quality control purposes.

Its use may be appropriate in situations like busy blood banks and out patient clinics where rapid screening of malaria infection is needed but where experienced malaria microscopists

may not be available (Mak *et al.*, 1992).

1.5.4.2 Polymerase chain reacton technique (PCR)

PCR has been used to detect the four species of human malaria in field samples, and also in detection of high prevalence of mixed infections (Snounou *et al.*, 1993a,b). Primers exist for the reliable identification of human malarias by PCR analysis. DNA probes have been developed for malaria diagnosis but their utility outside epidemiological surveys is uncertain (Quintana *et al* .,1998).

1.5.4.3 Parasight - F test: History, evaluation and significance of the test

Parasight- F test is a simple rapid manual dipstick test to detect *Plasmodium falciparum* infection (Parra *et al* ., 1991; Taylor and Voller, 1993). This method is based on antigen capture and has been incorporated into a simple, easily interpreted, dipstick by Beckton Dickinson Advanced Diagnostics (Sparks, M.D, USA; Shiff *et al.*,1994). The histidine-rich protein 2 (HRP-2), which is expressed by the blood stages of *Plasmodium falciparum,* has been detected in the plasma of persons infected with *P. falciparum* by dot blot analysis and shown to be detectable in an enzyme linked immunosorbant assay (ELISA) thus forming the basis of this test (Rock *et al.*,1987).

This procedure has been evaluated in various parts of Asia and Africa through clinical trials in malaria endemic countries and in major European referral hospital of parasitologic diseases, as well as trials performed by research division of the manufacturer, Beckton Dickinson Tropical Diseases Diagnostics (Caraballo and Ache, 1996). The Parasight–F

test, by not requiring specimen preparation, equipment, microscopy or electricity, shows promise of being useful in endemic areas as a rapid, specific and sensitive screening test *for P. falciparum* infection in patients reporting to peripheral clinics. The method requires minimal user skill levels and is reproducible when interpreted by inexperienced users. Its requirements fit most of the WHO objectives. HRP-2 may persist after parasite clearance in some patients- mostly those with high initial parasitaemia - and therefore test as positive with the parasight-F tests during that time (Becton Dickinson and Company, 1993).

As an antigen capture test, it only detects active infections with a short-term carry-over following effective treatment. The components of the test are stable and do not require refrigeration hence can be kept at room temperature. Although cost is a factor, use of this test will result in saving of time and effort and will conserve expensive drugs. At present, there is no information about the cost of each test but whatever expense is involved, it must be weighed against the cost of microscopic diagnosis and wastage of drugs from unnecessary treatment.

1.5.4.4 Immunochromatographic (ICT) malaria Plasmodium falciparum evaluation and significance.

The ICT malaria pf is an *in vitro* immuno - diagnostic test for the detection of circulating *Plasmodium falciparum* histidine rich protein 2 (pf HRP -2) in whole blood (Parra *et al.,* 1991; Howard *et al.*,1986). The test uses two antibodies specific for pf. HRP - 2 antigen. One of the antibodies is attached to visible colloidal gold and impregnated into a sample, while the second antibody is immobilised in a line across the test strip. 10μl of whole blood

is added to the sample pad where lysis occurs and any pf. HRP- 2 complexed with the gold labeled antibody is captured by the antibody on the membrane, and a pink line forms. In a negative sample, no pink line forms.

1.5.4.5 Importance of the ICT and Parasight- F in diagnosis

A key feature of the new World Health Organization Global Malaria Control Strategy (WHO, 1992) is the rapid diagnosis of malaria at the village and district level so that effective treatment can be administered quickly to reduce morbidity and mortality. The need for rapid diagnosis of *Plasmodium falciparum* is most acute because of the severe nature of this infection and its non-specific symptomatology. In village-based health clinics, presumptive diagnosis is made and the available recommended drug is dispensed. A common drug, chloroquine, is no longer universally effective due to the widespread chloroquine resistance in *P. falciparum* infections. Policy changes to second line antimalarial drugs (in Kenya, Fansidar) have been considered; however with such compounds, definitive diagnostics at the village level is necessary. There is an urgent need for a simple diagnostic test. Such a test should exclude microscopy, be simple to perform and read and be easily stored. It should also be affordable.

Diagnostic procedures for the detection of malaria differ considerably depending on the aims of evaluation. The current requirement of any laboratory procedure for general application to the detection and diagnosis of malaria include: sensitivity, specificity, simplicity in application, unambiguous interpretation and rapid turn-around time. Presently, the differential stained thick and thin blood smear, examined under the

microscope, remains the most reliable and definitive test for diagnosis of malaria.

1.5.4.6 pLDH diagnosis in malaria

The unique ability of pLDH to utilize 3-acetyl pyridine dinucleotide (APAD) in lieu of NAD as a coenzyme in the conversion of pyruvate to lactate, led to the development of a biochemical assay for the detection of plasmodial parasitaemia (Makler and Hinrichs, 1993). It was further shown that this assay could be used for the determination of plasmodial sensitivity to compounds with known and presumed antimalarial activities (Makler *et al.*, 1993). Drug resistant forms of malaria parasites, particularly *P. falciparum,* are now widespread in many parts of the world. Drug resistance has become a major impediment to effective chemoprophylaxis and chemotherapy of malaria contributing to the world-wide resurgence of the disease (Miller and Warrell, 1990; Wernsdorfter and Payne, 1991). Additionally, the presence of multiple drug resistance parasites in malaria endemic areas has greatly increased the cost of management of malaria as those drugs are either taken or administered empirically. The resulting delay in the institution of effective chemotherapy can lead to higher complication rates and prolonged hospital stay (Rieckman *et al.,*1978).

To date, parasite chemosensitivity to antimalarial compounds is determined by a variation of two basic techniques - the schizont inhibition (Rieckman *et al.*, 1968) and the ^{3}H-hypoxanthine uptake microtest (Desjardin *et al.,* 1979). The former requires microscopy for scoring and is not suitable for bulk processing due to observer bias and fatigue. On the other hand, ^{3}H-hypoxanthine uptake techniques has a requirements for radio labelled materials, is expensive and cumbersome when handling a large number of samples. In

contrast, pLDH assay is non-isotopic biochemical microtest that can be scored objectively with the use of ELISA reader (Makler and Hinrichs, 1993).

The measurement of pLDH has the potential to be developed into a useful format for a diagnostic test for malaria, which is likely to require a low per tests cost with limited or no equipment requirements. The current sensitivity of the pLDH assay offers sufficient promise such that analysis in a field trial appears to be warranted . A final decision as to the eventual usefulness of this assay and appropriate formatting of such an assay could only be developed following such on-site testing (Makler and Hinrichs, 1993).

pLDH assay has also been used for detecting parasite clones in microtiter plates (Gomez *et al.*, 1997; Goodyer and Taraschi,1997). It has been reported to have a number of advantages over the phenol red or Giemsa staining procedures for the identification of malaria clones. It is fast and convenient making it a practical method for processing large number of samples. It does not require a highly skilled technician to grade the wells. It is also likely to be more sensitive than the other method (Kirkman *et al.*,1996).

1.5.4.6.1 History of p(LDH)

Lactate dehydrogenase (L-Lactate NAD^+-oxidoreductase, EC 1.1, 1.27; LDH), the terminal enzyme of glycolysis is present in several species of malarial parasites (Carter, 1978; Sherman, 1979; 1983; Kaushal *et al.*,1985), and plays an important role in regulating the energy metabolism of these parasites (Riandey *et al.*, 1996). Earlier studies on biochemical characterization of LDH from malarial parasites in comparison to the mammalian enzyme, have revealed significant differences between the electrophoretic and kinetic behavior of the parasite and host enzymes (Vandet *et al.*,1981; Kaushal *et al.*, 1985; Goyal *et al.*, 1993)

Immunochemical characterization of *Plasmodium knowlesi* LDH, using hyperimmune monkey anti *P. knowlesi* serum in an immunodot enzyme staining method, have also shown that *Plasmodium* LDH is immunochemically different from the host enzyme (Watts *et al.*, 1987; Kaushal *et al.*, 1988, 1993; Lang-Unnasch, 1992)

1.5.4.6.2 Field study using pLDH dipstick

A study carried out by Jelinek *et al.*, (1996a) in 429 Ugandan patients for pLDH diagnosis of *Plasmodium falciparum* infection, indicated low (58.8 and 62.2 %) sensitivity and specificity respectively. The positive and negative predictive values failed to meet necessary standards in the study above. Hence, the authors concluded that the methods for measurement of pLDH activity of malaria infection in endemic areas required improvement although it was potentially useful for the fast diagnosis of malaria.

1.5.4.6.3 Development of pLDH as a dipstick

1.5.4.6.3.1 The optiMAL® assay

Most of the diagnostic tests currently in use to identify malaria parasites in clinical specimens depend upon the presence of circulating parasites. However, mature asexual forms of *P. falciparum* sequester in deep tissue capillaries and do not readily appear in peripheral blood. As a consequence, accurate determination of parasitaemia in cases of *Plasmodium falciparum* malaria can be a problem. The pLDH test can measure parasite enzyme in infected erythrocytes or released into serum following schizont rapture *in vitro*. Thus a potential advantage of the pLDH test, unlike other more routine tests, is that it is not completely dependent on circulating infected erythrocytes.

Flow inc.(Portland, Oregon, U.S.A) developed a rapid malaria assay: the OptiMAL® dipstick assay. This assay is a sensitive, simple method which permits the diagnosis and the specification of the major forms of human malaria, *P. falciparum* and *P. vivax*. OptiMAL® dipstick assay permits health personnel to monitor patient therapy.

The dipstick is a preparation of nitrocellulose and glass fibre pre- treated with a mouse monoclonal antibody (mAb) against HRP-2 and applied in a line across about 1 cm from the base of the dipstick (Becton Dickinson Tropical Disease Diagnostics, 250 Schilling Circle Cockeysville, MD 21030, U.S.A). A second dotted line of HRP2 antigen is incorporated in the dipstick about 2-3 mm above the line of mAb as a reagent control.

The OptiMAL® assay uses a series of monoclonal antibodies to detect a unique parasite antigen, parasite lactate dehydrogenase (pLDH). The OptiMAL® method is simple to operate.

1.5.4.6.3.2 Optimal® - assay procedure

A small sample of blood from a finger prick is mixed with OptiMAL® reagent A. Next, this mixture migrates up the OptiMAL® strip dipstick. After 8 minutes, the OptiMAL® strip is cleared with reagent B and the strip is cleared with reagent and the strip is read to determine the presence and the species of malaria parasite. The OptiMAL® assay is based on the detection of an abundant intracellular metabolic enzyme (pLDH) produced by viable malarial parasites. pLDH is present in, and released from, parasite infected red blood cells.

The OptiMAL® assay detects the pLDH enzyme with a series of monoclonal proteins. Differentiation of malarial species is based on antigenic differences between the pLDH

isoforms. Because the presence of pLDH in the blood reflects the presence of viable malarial parasites, the OptiMAL® assay can be used to evaluate the effectiveness of antimalarial chemotherapy. It is a simple way for health care personnel to check for anti-malaria susceptibility or resistance (Flow Inc. Portland, Oregon, USA).

1.6 Sources and action of some antimalarial drugs:

Extracts of the plant qinghaosu (*Artemesia annua*), known as qinghaosu, have been used in traditional medical practice in China for over two millenia. The antimalarial properties of qinghaosu were rediscovered in 1971 though the low temperature ethylether extracts of the compound was discovered in 1945 by the U.K. research group. Antimalarial biguanides-proguanil and subsequently chlorproguanil compounds were later shown to inhibit the plasmodial enzyme dihydrofolate reductase (DHFR). In early 1950s, the 4-aminoquinolines, chloroquine, pyrimethamine and proguanil were used for malaria prophylaxis (Pampana, 1969; Hagos *et al.,* 1993). Antimalarial drug resistance was not taken seriously until chloroquine resistance in *P. falciparum* malaria developed almost simultaneously in South East Asia and South America in the early 1960s. Mefloquine and Halofantrine are the result of the effort in addressing the expanding tide of antimalarial drug resistance, together with the looming conflict of the Vietnam and the manifest failure of eradication programme which prompted the U. S. army- led research effort to screen and test new antimalarial compounds (WHO, 1990a). A naphthoquinone compound (Atovaquone, a modification of a compound discovered over 40 years ago), appeared to be a safe and effective antimalarial although resistance developed rapidly thereafter

(Pongponratn *et al.*, 1991).

The most important development in the recent years has been the rediscovery and development of drugs related to artemisinin (qinghaosu) in China (Klayman, 1985). These drugs are structurally unrelated to existing antimalarials. They are rapidly effective, appear to be safe and may be the only drugs available for treatment of malaria (Li and Jiang', 1992). By the early 1980s, chloroquine was no longer effective in many countries and the first ominous reports of resistance in the East coast of Africa appeared (Watkins *et al.*, 1988).

The available antimalarials fall into three broad groups:

(1.) (quinine, quinidine, chloroquine, amodiaquine, mefloquine, halofantrine, primaquine)

(2)Antifols

(Pyrimethamine, proguanil, chlorproguanil, trimethoprim)

(3) Artemisinin compounds: artemisinin, artemether, artesunate. (ter Kuile *et al.*, 1993). Of these artemisinin drugs have the broadest time window of action on the asexual malaria parasites from medium sized rings to early schizonts, and produce the most rapid therapeutic responses (Wyler 1990; ter Kuile *et al*; 1993). Several antimalarial drugs also have antiplasmodial activity, although, in general, their activity is slow and they are used in combination with the antimicrobial drugs e.g. sulphonamides, sulphones, tetracyclines and the macrolides. Azythromycin has been evaluated for prophylaxis in Africa and South America (Milhous *et al.*, 1985).

In vitro studies of antimalarial drug sensitivity and resistance to mefloquine, quinine and halofantrine is linked. As suggested by drugs susceptibility studies, chloroquine and mefloquine resistance are not linked (Brasseur *et al.*, 1988). Indeed there is some evidence

that increasing mefloquine resistance is associated with increasing susceptibility to chloroquine (Brasseur *et al.*,1988; Warsame *et al.*, 1991) . *Plasmodium falciparum* has developed resistance to all available antimalarial drugs with such alarming speed that there is possibility of new strains of *P. falciparum* appearing before the beginning of the next millennium (White, 1992).

1.6.1 Quinine:

Quinine is a bitter powder obtained from the back of the cinchona tree. It is widely used as a flavouring (tonic water, bitter lemon) and as treatment of night cramps, as well as for malaria (Taylor, 1945). Quinine acts principally on the mature trophozoite stages of parasite development. It does not prevent sequestration or further development of formed meronts, and does not kill the pre-erythrocytic or sexual stages of *P. falciparum* (Warsame *et al*; 1991). Investigations demonstrate that it is possible to produce aminoquinolines active against chloroquine, mefloquine, and multiple - resistant *P. falciparum* parasites by modifying the diaminoalkane side chain. These investigations may help resolve the current questions about the mechanism of aminoquinoline resistance, and may lead to the production of compounds effective for the treatment of multi-drug resistant *P. falciparum* infections (Dibyendu *et al*,. 1996).

1.6.2 Chloroquine

This is a 4-aminoquinoline. It acts mainly on the large ring forms and mature trophozoite

stages of the parasite. It acts more rapidly than quinine. It is therefore used widely for treatment of *P. vivax*, *P. malariae* and *P. ovale* infections but is being replaced for the treatment of *P. falciparum* infection (White, 1992). *In vitro* studies in Kenya revealed that significantly more isolates were sensitive to amodiaquine compared to chloroquine (Nevill *et al.*, 1994). In contrast, the response to chloroquine was very poor with one third of patients failing to respond clinically, and 59% overall parasitological resistance, of which 17% was at the R3 level (Nevill *et al.*, 1994). Chloroquine is recommended as the drug of first choice for the treatment of malaria throughout eastern Africa although this has changed due to resistance(Republic of Kenya, Ministry of Health, 1990; 1997; WHO,1994). The users are therefore likely to suffer the severe consequence of treatment failure which may include progressive anaemia, long term debility and ultimate death (Nevill *et al.*, 1994). Fansidar remains very effective against 4-aminoquinoline resistant cases. However, the useful therapeutic lifespan of this drug is likely to be short as has been well documented in South East Asia, probably due to the rapid selection of resistance catalysed by the long half-lives of its two components (Hurwitz *et al.*,1981; Boudreau *et al.*, 1982). As a result, it is imperative that the poor developing countries obtain the very best use of the few remaining cost-effective antimalarials before moving into expensive short life-span alternatives (Nevill *et al.*, 1994). Amodiaquine was recommended to replace chloroquine in a Kenyan study because of its demonstrated efficiency, lack of proven therapeutic toxicity and low cost in the face of chloroquine's marked clinical failure (Nevill *et al.*, 1994). Fansidar was recommended to remain a reasonable choice for treatment of blood- slide- confirmed 4-aminoquinoline resistant malaria, and quinine to be retained for severe or complicated malaria before new drugs are made affordable and available (Royer *et al.*, 1986; OMS,

1990). Artemether should be used for the treatment of severe *falciparum* malaria where there is evidence that the antimalarial efficacy of quinine is declining (Kager,1993; WHO,1994). The other common antimalarial drugs should be used for management of uncomplicated malaria and protection of travellers (Weiss, *et al*,1995).

1.7 An overview of antimalarial drug therapy in Kenya

1.7.1 Chloroquine

In Kenya, treatment of malaria with chloroquine was associated with 33% case fatality rate compared with 11% for children treated with more effective regimens (pyrimethamine /sulfa, quinine, or trimethoprim/sulfamethoxazole for five days) (Zucker *et al.,* 1996). Children with primary diagnosis of malaria who received Fansidar or Quinine for five days had a significantly lower case fatality rate compared to those children who received only chloroquine (Zucker *et al.,* 1996). The proportions of deaths attributable to the ineffectiveness of chloroquine treatment was 60% (Bloland *et al.*, 1993).

Reports of studies done in the southern Rift Valley area of Kenya (Nevill *et al.,* 1990) also showed 91.3 % amodiaquine sensitivity and only 40.6 % sensitivity to chloroquine. In view of the above findings it is advisable to reassess the role of chloroquine in the management of *P. falciparum* malaria in areas of documented high chloroquine resistance (Republic of Kenya, Ministry of Health,1997).

1.7.2 Mefloquine

This is a fluorinated four quinoline methanol compound. The parasiticidal action is similar to that of quinine. It is very insoluble in water and is used for single dose oral treatment of

uncomplicated multidrug resistant *Plasmodium falciparum* malaria (Harinasuta and Bunnag, 1988). *Plasmodium falciparum*, the most lethal human malaria parasite, has become increasingly difficult to control. Strains have emerged that are resistant to established antimalarials such as chloroquine and quinine, and also to newer therapeutic agents such as mefloquine, halofantrine and artemisinin (Peters, 1985).

1.7.3 Pyrimethamine

This is a dehydrofolate reductase (DHFR) inhibitor. It is used commonly in combination with long-acting sulfanomides such as sulfadoxine and sulfalene. The DHFR inhibitors inhibit development of malaria trophozoite stage of the asexual parasite, in addition to having pre-erythrocytic and sporontocidal activities (Taylor, 1945; Pampana, 1969)

1.7.4 Qinghaosu

This is also known as artemisinin. It is a sesquiterpene lactone peroxide extracted from the leaves of the shrub *Artemesia annua*. The parent compound, artemisinin, and the three derivatives, the oil soluble ethers, artemether and arteether, in addition to the water soluble artesunate, are all converted *in vivo* to a common biologically active metabolite dihydroartemisinin (Lee and Hufford; 1990; Fisher, *et al.*, 1994)). Comparative studies have shown that they predictably give faster parasite and fever clearance than the other antimalarial drugs. There is some concern that if the oral artemisinin derivatives are used alone, then resistance of these valuable compounds may develop rapidly. (Boudreau *et al.*, 1982; Schapira *et al.,* 1993).

1.7.5 Atovaquone

Also coded 566C80, atovaquone is a hydroxynaphthoquinone, antiparasitic drug. Trials have been carried out for the treatment of malaria, *toxoplasmosis, Pneumocystis carinii* pneumonia using this drug (Hughes *et al.*, 1990; Fisher *et al.,* 1994). In animal studies, it has shown usefulness in treating leshmaniasis (Fry and Pudney, 1992). Its chemical structure differs from that of the other antimalarial drugs. The site of action is the ubiquinone - cytochrome C-reductase known as complex III of the mitochondrial respiratory chain, which is a new target molecule (Fry and Pudney, 1992; Hudson, 1993) . The structural difference, the unique target site and good tolerance of atovaquone in humans, are important features that hold promise for the development of this drug (Brasco *et al.,* 1995). In rodents, atovaquone inhibits the hepatic stages in the rodent malaria at lower concentrations than pyrimethamine, suggesting its potential use for casual prophylaxis. Atovaquone phase I studies have reported the drug as safe and well tolerated (Hughes *et al.*, 1991) and phase II have reported its usefulness for treatment of acute, uncomplicated malaria (Hudson, 1993).

1.7.5.1 Why is atovaquone considered a special drug?

A study carried out by Brasco *et al.,* (1995), showed that there was no evidence for *in vitro* cross resistance between atovaquone and any of the antimalarial drugs tested. In another study, atovaquone was shown to be extremely effective against *Babesia divergens* both *in vitro* and in the gerbil model. *In vitro,* it was the most effective agent against the parasite in human erythrocytes more effective than standard antimalarials, the most effective of which was quinine (Pudney and Gray, 1997). It is already apparent that atovaquone is the only drug registered for use in humans with a high degree of activity against *B. divergens* , and could be used both for the treatment of clinical disease and also prevention in asplenic individuals exposed to infection (Pudney and Gray, 1997). A drug that is both effective against *babesia* infections and is also registered for use against other human pathogens is required, and atovaquone seems to fit this zoonotic description (Hudson *et al*; 1991; Gray, 1983; Pudney and Gray, 1997). Atovaquone has no cross resistance with other antimalarial drugs due to the difference in target sites. The four amino quinolines and aminoalcohols accumulate in the food vacuole of the parasites, where they are thought to inhibit the heme polymerase enzyme. The target molecules of the antifols is dehydrofolate reductase. Artemisinin and its derivatives generate free radicals through the mediation of iron and /or heme (Brasco *et al*; 1995). The site of action of atovaquone is complex III of the mitochondrial respiratory chain, the inhibition of which results in the disruption of pyridine biosynthesis (Fry and Pudney; 1992).

1.7.6 Malaria situation and drug response

Plasmodium falciparum infections are widespread and contributes to more than one million paedriatic deaths annually (WHO, 1994). The spread of chloroquine resistance across the African continent has diminished the effectiveness of treatment with chloroquine, the most widely used antimalarial drug, leading to the risk of persistent parasitaemia and anaemia in young children (Pettersen *et al.,* 1981; WHO, 1990b). The impact of malarial treatment on malarial illness has been investigated by various researchers because malaria accounts for the largest amount of admissions, diagnoses and deaths, and effective treatment could be readily defined. Even though chloroquine resistant *P. falciparum* malaria was first reported in East Africa in 1978 in a tourist (Petterson *et al.*, 1981), currently, chloroquine resistance can be found at different levels in all the endemic areas of the country. In the wake of widespread drug resistance, new antimalarial drugs need attention (Vennerstrom *et al.*, 1995).

1.8 Rationale for this study

In 1990, 41% of all outpatient visits in Kenya were malaria related and 4.5 million Kenya shillings were spent for antimalarial drug purchases in South Nyanza district alone (Republic of Kenya, Ministry of Health, 1994). Therefore, antimalarial drug purchases are the single most expensive item in the health budget of not only Kenya but of several poor countries with endemic malaria. Accurate malaria diagnosis is one factor that complicates the treatment of the disease. In the absence of an effective malaria vaccine, chemoprophylaxis remains the primary tool for malaria control for children, non-immunes such as tourists and all the people visiting and living in malaria infested environments.

Proper diagnosis and rational use of drugs could save money that can then be used to provide other essential health care needs. The rational use of antimalarial compounds could shorten hospital stay for admitted patients and reduce complication, mortality and morbidity rates from malaria. Reduction in the indiscriminate use of antimalarial drugs could also reduce the rapid development of parasite resistance to the few effective antimalarial drugs. Determination of antimalarial drug sensitivity profile of patients presenting to a hospital in an endemic area can provide the needed database on drug resistance patterns in a population. This database can then be accessed by visitors to the endemic area for rational choice of a chemoprophylactic regimen. Currently, there is no single technique that can diagnose, speciate and give drug sensitivity pattern of malaria. This study investigated the usefulness of modified parasite lactate dehydrogenase (pLDH) assay for laboratory, field and clinical diagnosis of malaria and for monitoring the drug sensitivity patterns of antimalarial drugs in a malaria endemic region of Kenya.

CHAPTER 2

2.0 OBJECTIVES OF THIS STUDY

2.1 General objective

The main aim of the study was to determine the usefulness of parasite lactate dehydrogenase (pLDH) assay as a new method for diagnosis of malaria and to assess its potential usefulness for drug sensitivity studies *in vitro* and in clinical set- ups in Kenya.

2.2 Specific objectives

2.2.1 To determine the kinetics of pLDH blood clearance during treatment of *P. falciparum* infection.

2.2.2 To compare pLDH and light microscopy as diagnostic tools for malaria in school going children and in clinical (hospital) samples from western Kenya.

2.2.3 To measure drug sensitivity profiles for both used and candidate antimalarial drugs through pLDH and tritiated hypoxanthine uptake assays.

2.2.4 To determine and compare sensitivity and specificity of Immunochromatographic (ICT) test, Parasight- F and pLDH optiMAL® dipstick using light microscopy as gold standard.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1The study subjects.

3.1.1 Patient recruitment

In Kenya, malaria accounts for about 30% of all illnesses reported (WHO, 1994). Based on this prevalence rate, the minimum number of subjects required to obtain this estimate with 95% confidence and 5% absolute precision in this study approximately 96 patients with clinical signs and symptoms of the disease (Greenberg *et al.*, 1993). Patients with slide positive and negative malaria were enrolled for this study from Nairobi, (a non-malaria endemic region) for pLDH negative control studies, Kisumu (a malaria endemic region) for field and clinical studies and Rift Valley Nakuru (a non- malaria endemic region) for pLDH blood clearance and rational use of antimalarials follow- up studies.

The inclusion criteria were:

(a) Age greater than one year and not breast feeding

(b) Thick film peripheral blood positive for pure *P. falciparum* malaria parasites.

(c) No antimalarial drug use within the past two months

(d) Informed consent from patient (if older than 18 years) or from parents/guardian (see appendix).

(e) Informed consent from local hospital physician stating that there is no medical contra indication to obtaining 3.0 ml of pre- treatment blood sample from each patient for culture and drug sensitivity studies (see appendix).

(f) Ethical clearance from a competent body.

The exclusion criteria were:

(a) Child over one year but on regular breast feeding

(b) Responsible local hospital physician could exclude any patient from participation or remove a participating patient from protocol at any time for medical reasons.

(c) Patients with severe anaemia (Hb < 6gm/dl) were excluded from pLDH kinetics study.

(d) Pre-treatment urine test positive for chloroquine drug screening, (Lelijveld and Kortman, 1970).

(e) Any recruited patient opting to withdraw from the study.

3.1.2 Study subjects

Healthy Kenya residents of Nairobi (a city in a non-endemic malaria region in Kenya), who had not travelled to a malaria endemic area within the past three months, had slide negative malaria diagnosis and with no history of chemoprophylaxis served as negative controls. Test subjects included healthy school children, n=130, (field study subjects) who had not taken antimalarial drugs within two months prior to the study (confirmed by interview) and were residents of Kisumu (an area holoendemic for malaria). Clinical samples were obtained from patients attending Kisumu District Hospital (n=156) who qualified after rigorous examination using exclusion and inclusion criteria (Appendix 1 for consent and appendix 2- Malaria investigation form). Adult volunteers for pLDH, ICT and Parasight-F comparative studies were subjects (n=308) from Siaya District, Ndori station, (an area endemic for malaria), which had been previously marked for drug studies. For pLDH blood clearance treatment the subjects were students and workers (n=65) staying in the Rift

Valley, who had history of travel to malaria endemic regions of Kenya and who were blood slide positive for malaria. Informed consent was obtained from adults, teachers and children before blood samples were collected and all individuals with a blood smear positive for malaria received appropriate antimalarial drug treatment. Three millilitres of blood were collected from the subjects by venipuncture prior to treatment for culture, setting of drug sensitivity studies in pLDH and tritiated hypoxanthine assays. Fingerpricks for determination of parasitaemia were done daily for seven days for both ambulatory and in- patients on pLDH kinetics study. Clinical and parasitological data for each patient were entered daily into charts (Svenson *et al.*, 1995).

3.2 Blood collection procedures

One ml of venous blood was obtained from all patients prior to treatment for setting up of antimalaria drug sensitivity using pLDH and tritiated hypoxanthine assays. Fingerprick for determination of parasitaemia was done daily in the patients on the follow-up protocol (Zucker *et al.*, 1996). An additional 0.5 ml of blood was obtained daily from a selected number of hospitalised patients with HB>6g/dl for the pLDH kinetics study. All patients admitted into the study gave 3ml of blood, a urine sample and a complete record of physical examination data from one of the participating physicians. Urine was screened for antimalarial drug use by Dill Glazko and Sulphur tests (Lelijveld and Kortman, 1970). The admission blood was used for the preparation of a thick and thin smear and for tritiated hypoxanthine and pLDH assays against the antimalarial drugs. Local hospital admitting physician prescribed therapy according to standard practices at the hospital. Daily blood smears were obtained from hospitalised patients for a maximmum of seven days and in

cases of discharge before the period, then the patients were followed until the seven days were over. An additional 0.5ml of blood was obtained daily from hospitalised and ambulatory patients for the determination of pLDH blood clearance. For the latter study, time for the clearance of parasitaemia was used to assess clinical outcome and treatment failure due to drug resistant parasites (WHO, 1988).

3.3 Cultivation of parasites

Parasite isolates identified as *P. falciparum* by microscopy were cultivated using standard techniques (Trager and Jensen, 1976). *Plasmodium falciparum* reference strains D6 and W2 were kind donations from Dr. Milhous of the Walter Reed Army Institute of Research, Washington DC, through Dr. Klotz of the Walter Reed project, Kenya Medical Research Institute). The international Amsterdam airport strain (NF54) was donated by Dr. Sam Martin (Walter Reed army Institute of Research, Washington DC). The other *P. falciparum* isolates used in the study included, KA95, KC95, K155, K044, KS140, KS041, KS021, KS608, KS155, KS157, KS193, KS211, KS012, C1589, KS031, KS168, KS047, KS063 and KS192 which were collected from the field each from a single patient or study volunteer and transported to the laboratory using the appropriate transport media (Ofulla *et al.,* 1994). The other isolates Som A6 (Somali strain), M24 (Mombasa strain), FCB (kind donation from Wellcome Trust Research Laboratories, Kenya), K39 and S104 were culture adapted *P. falciparum* isolates stored in liquid nitrogen at Kemri malaria laboratories. All parasites were maintained *in vitro* in flasks in RPMI 1640 medium to which was added human type A, O, B red blood cells (depending on the blood group typing of the isolate to be set) and 10% heat inactivated human serum blood group matched (Udomsangpetch,

1993). All cultures were placed in a humidified incubator at 37°C with a gas at controlled environment of 5% O_2, 3% CO_2 and 92% N_2 from BOC gases, Nairobi, Kenya, and maintained according to established standards. Parasitaemias were assessed using both thin and thick smear procedures as well as pLDH and tritiated hypoxanthine uptake. When parasitaemias were above 1%, drug tests were set up after diluting the cultures to initial parasitaemia of 0.2 % or 0.4% depending on the drug to be tested and the incubation period for the drug (Appendix 3).

3.4 Sources of reagents and drugs

The reagents used in this study for pLDH analysis were obtained from Sigma (St. Louis, Mo) and included Lactic acid, Lithium salt, trizma buffer, triton X-100, nitroblue tetrazolium (NBT) , phenazine ethosulphate (PES), APAD and NAD. The malstat® reagent, which is a formulation for parasite LDH detection, was obtained from Flow, Inc. (Portland, OR.) as outlined by Makler and Hinrichs (1993). The RPMI 1640 medium was obtained from Gibco Company, UK. The pre-dosed microtitre drug test plates were ordered from WHO Regional Office, Manila (The Philippines). The antimalarial drugs used for the study included, chloroquine, amodiaquine, quinine, pyrimethamine and sulfadoxine as separate drugs, which were predosed plates. Later, drug plates were prepared in KEMRI laboratories using standard procedures for the drugs, chloroquine, mefloquine, quinine, dehydroartemisinin, atovaquone, halofantrine, doxycycline, azythromycin, pyrimethamine and sulfadoxine (which were kindly donated by United States Army Medical Research, Unit. through, Dr. F. Klotz). Drug plates were prepared at KEMRI.

3.5 Transportation and processing of malaria samples

The isolates were collected from malaria patients in Kisumu or Rift- Valley, filtered through cotton plugged needles and added under sterile conditions into transport medium, bovine albumin (BAM). These were transported in cool boxes with ice packs through Securicor courier services to Nairobi. After spinning the samples at 3,000 rpm and 4° C, for 5 minutes, the supernatant was removed and 4 mls of BAM with glucose without para-amino benzoic acid (PABA) and folic acid were either added for direct testing, or the isolates were culture adapted immediately for further testing (Ofulla *et al.*, 1994). The samples transported from the field site to Nairobi were only tested for the red blood cell pLDH as the serum had already been diluted with the transport medium.

3.6 Determination of parasitaemia

3.6.1 Determination of malaria parasitaemia by light microscopy

Thick and thin blood films were examined under an oil immersion objective (1,000 x) by two experienced microscopists. The parasites were examined against 300 leucocytes, and the microscopists also counted parasites per 5,000 erythrocytes to determine percentage parasitaemia (Orago and Solomon, 1986) for laboratory cultivated *P. falciparum* isolates.

3.6.2 Synchronization of *Plasmodium falciparum* erythrocytic stages in culture

Five ml of asynchronized culture were centrifuged at 200 g for 5 minutes, the supernatant discarded and the pellet, approximately 0.5 ml, resuspended in 2.5 ml of aqueous 5% D-sorbitol (0.274 M) for 5 min. at room temperature (Lambros and Vanderberg, 1979). After two additional centrifugation, an equal volume of RPMI 1640 containing 10%

human serum was added to the pellet. Cultures were re-established by adding uninfected erythrocytes and culture medium to give 12.5 % haematocrit with an appropriate starting parasitaemia (0.1%). The culture medium was then changed daily.

3.6.3. Quantification of parasitaemia

The number of parasitized red blood cells per 5,000 erythrocytes was counted on giemsa-stained smears for culture adapted parasites, whereas, in field samples from volunteers, the number of parasites per millimetre cubed (mm^3) was calculated as follows:

Number of *Plasmodium falciparum* counted (#pf)/200 white blood cells (WBC); number of *Plasmodium malariae* counted (#pm)/200 WBC; number of *Plasmodium ovale* counted (#PO)/200 WBC= number of Plasmodia species in 200 white blood cells of thick smear counted.

Therefore, number of parasites per mm^3 of blood

= (number of parasites (#) x WBC x 1000) / (200 x WBC)

= 5 multiply by the number (#)of parasites

where # parasites is the number of parasites counted per 200 white blood cells (Greenberg, *et al.*, 1993)

The number of species of *Plasmodium* parasites were read per 200 white blood cells (WBC). Parasite density was calculated based on the number of WBC /mm^3 determined by the total blood white cell count. Total blood white cell counts were obtained using a coulter counter (Miami, FL, Counter, Model M 350).

3.6.4. Determination of parasitaemia by pLDH assay

Plasma and red blood cell pLDH was assessed only in samples processed in the field site . Those transported to the Nairobi laboratory in serum free transport media and cultured in the laboratory were only assessed for RBC pLDH. One ml blood was mixed with 150 microlitres of acid dextrose (ACD) anticoagulant in a 1.5 ml eppendorf centrifuge. Cells and plasma were separated by centrifugation (one minute, at 3,000 rpm, at 4°C). Cells were washed 3 times with serum free culture medium and resuspended to 1% hematocrit. Approximately 50 μl of infected RBCs suspension or plasma was added to duplicate wells of a 96 well microtitre plate after the resuspension. The samples were then tested for the presence of parasite LDH by using malstat® reagent which is directly related to microscopy parasitaemia (Makler and Hinrichs, 1993).

3.6.5 Determination of RBC pLDH

A standard curve was generated for each plate by making double dilutions of an infected sample of known parasitaemia from a non-synchronous culture of a laboratory reared isolate. About 100 μl of malstat reagent (Flow Inc. Portland, OR. USA) was added to each well and incubated for 20 minutes at room temperature. Approximately 10μl of freshly prepared nitroblue tetrazolium (NBT) and phenazine ethane sulfate (PES) (Flow Labs) were then added to each well and incubated for a further 20 minutes in darkness at room temperature. The reaction was stopped by adding 20 μl of 5% acetic acid and mixed on a microplate shaker to eliminate bubbles. The colour changes were measured in an ELISA plate reader at 650 nm (Molecular Devices, Monlo Park CA). Plasma pLDH was read as optical density, whereas RBC pLDH was read from optical density and extrapolated as % parasitaemia from a standard curve using a software programme (Soft Max 3.2, Menlo Park , CA) for Macintosh computers.

3.6.6 The pLDH Enzyme assay and OptiMAL ® assay

When the pLDH enzyme assay indicated limitations, a decision was made to use OptiMAL ® assay which is a dipstick, modification of the enzyme assay (Flow, Inc. 6127 SW Corbett, Portland, OR 79201 USA). This assay has been reported to be intended for use in Research and Development only.

About 10μl of whole blood was obtained from 308 volunteers from the study area and tested for malaria infection using standard procedures indicated by the manufacturers (Flow, Inc. 6127 SW Corbett, Portland, OR 79201 USA) and the results recorded as positive or negative.

3.7. Diagnosis of malaria using Parasight-F and immunochromatographic test (ICT) .

3.7.1. The Parasight - F test

Patients enrolled in the study were volunteers (n=308) in Ndori, Siaya district who were recruited after meeting the standard criteria. A drop of finger prick blood was placed in a well on a plastic plate and haemolysed with a drop of the detergent. The dipstick was placed in the haemolysed blood which is rapidly absorbed. Subsequently, a drop of developing reagent was applied to the base of the dipstick. This consisted of a suspension of micelles (phospholipid vesicles) containing sulpho- rhodamine B as a marker and coupled to rapid antibody raised against HRP2. When the reagent had been absorbed, two drops of clearing reagent was applied. This cleared the haemolysed blood and in *P. falciparum* malaria positive cases would leave a thin red line across the wick with a broken line above it as reagent control. In negative cases, only the reagent control broken line could be seen. The whole test took 10 minutes (Shiff *et al.*, 1993, 1994). The tests were carried out simultaneously with ICT, pLDH and light microscopy, hence, sensitivity, specificity, negative and positive predictive values were evaluated using light microscopy as gold standard, and in comparison with OptiMAL® which was the new diagnostic test under evaluation .

The study was conducted from May to July 1997 in Ndori station of Siaya District, where a special drug study was operational. Finger prick samples of blood was obtained from each volunteer who was recruited. A trained assistant made thin and thick blood films which

were later stained with 10% Giemsa stain and examined separately without reference to the field test. Simultaneously, three other assistants also collected blood and performed the rapid manual Parasight-F test. The principle used was as previously described (Shiff *et al.*, 1993). The results were recorded and confirmed by all the three trained assistants and the investigator.

3.7.2.The immunochromatographic (ICT) test.

The same samples collected for the 308 volunteers in Ndori were used in this procedure. The test was carried out using standard procedures and kits (ICT Diagnostics, 3/14 Roseberry Street Balgowlah NSW 2093, Sydney, Australia, -patents pending). Venous blood was collected by the standard venipuncture procedure into EDTA or Heparin tube. The kit was stored at 2-8°C when not in use. The test card was removed from the pouch just prior to use. The card was opened and laid flat on the work surface. The adhesive liner was removed and discarded and adhesive on the right hand side of the test card was exposed. The capillary tube was filled 3/4 full by capillary action using venous blood. The blood from the capillary tube was added to just cover the entire purple area of the sample pad. This was done by holding the capillary tube vertically and gently pressing the end across the purple pad in several places. Once the purple pad was saturated, the capillary tube was discarded. The tip of the dropper bottle was dried with tissue paper before dispensing drops to ensure that an accurate drop size is added to the pads. The bottle was held vertically and one drop of reagent A was gently added below the area where the blood sample had been dispensed. Four drops of reagent A were then added to the clearing pad on the top of the left hand side of the test card. The sample was then allowed to run up the membrane until

the red lysed blood front reached the limit line, before the card was closed. The results were read through the viewing window after the blood had cleared. The test procedure took 3 - 5 minutes and results were recorded and confirmed by all the three trained assistants and the investigator. The test was positive if two lines (test and control) were seen through the window. Any trace of a line in the test line area indicated a positive test result. The test was negative if only the control line was seen. The test was invalid if the control line did not appear. Whenever this occurred the test was repeated.

3.7.3 Calculation of mean positive cut - off on plasma and RBC pLDH

This was determined using the mean plasma and red blood cell pLDH value from one hundred and seven (107) healthy Kenyan volunteers who had no travel history to an endemic area, were not on any malaria chemoprophylaxis and thick and thin smear negative for malaria. Calculation of the mean positive cut off using this population indicated that the same control cut-off could not be used for field and clinical samples hence a new procedure was sought for field and clinical pLDH cut off. Field samples included 130 healthy volunteers from the study village who had no clinical symptoms of the disease but some were parasitaemic and others, non- parasitaemic. Clinical subjects were 156 study patients derived from the outpatient clinic within Kisumu District Hospital. The cut-off values for plasma and red blood cell was calculated by using the range of the values obtained by measuring pLDH optical density. Different cut offs were used and for each cut off the mean plasma and red blood cell for all subjects below and above this cut off was calculated.

T-statistics value for the difference between the two means was then calculated as:

$$T = \{M_1 - M_2\}/\{S\sqrt{(1/n_1 + 1/n_2)}\}$$

Where M_1= mean of subjects above

M_2= mean of subjects below

n_1 = number of subjects above

n_2= number of subjects below

S is the pooled standard deviation for the two groups (above and below)

$S^2= \{(n_1-1)\ S_1^2 +(n_2-1)\ S_2^2\}/\ (n_1+n_2-2)$

Where S_1^2=Varience of subjects above

S_2^2= varience of subjects below

The cut off with maximum T-value was selected as the best. Using this best cut off, the sensitivity and specificity of the assay were obtained.

3.8 Sensitivity and specificity analysis

This was done using standard procedures previously reported (Galen and Gambino, 1975; Greenberg *et al.,* 1993). The sensitivity and specificity of pLDH was calculated using both thick and thin smear microscopy as gold standards. The reliability of the pLDH was calculated from the method (Greenberg *et al.,* 1993) derived from the table below.

The reliability of a diagnostic test.

Microscopy		New test	pLDH	
		+	-	total
Standard test	+	a	b	a+b
	_	c	d	c +d
Total		a+c	b+d	a+b+c+d=n

From the derivation of reliability of a diagnostic test the other parameters were calculated as follows:

Probability of false negative= β = b/(a +b)

Probability of false positive = α= c/(c+d)

Sensitivity =(1-β) $\times$ 100%

Specificity = (1-α) $\times$ 100%

Predictive value of a positive test = a/(a+b)

Predictive value of a negative test = d/ (c+d)

After calculation of the cut-off values then these were used to calculate the sensitivity and specificity of the new diagnostic assay, pLDH when thick and thin smear microscopy were used as the standard and by using individual readings independent of microscopy. For Parasight-F and ICT, sensitivity and specificity were calculated using thick smear microscopy as the standard.

3.9 Assessment of anti-malarial drugs

3.9.1 Preparations of drug test plates:

Drug plates were made using folate free RPMI 1640 with hepes and with neither sodium bicarbonate nor serum. The serum type used, A, O or B, depended on the blood grouping of the field isolate to be tested. The red blood cell counts were done using haemocytometer according to basic haematologic techniques (1081 - 1083).

3.10 Drugs used for assays

The antimalarial drugs used for the various assays in this study included chloroquine diphosphate (Sigma Chemicals St. Louis, Mo), mefloquine hydrochloride, (Walter Reed Army Institute Research (WRAIR), Washington DC), quinine sulfate (Sigma St. Louis, Mo) , halofantrine hydrochloride (WRAIR). The remaining drugs were all from Sigma, St. Louis) through WRAIR (dehydroartemisinin, azithromycin, doxycycline, sulfadoxine, pyrimethamine and atovaquone). The drug test plates were categorised into two groups as follows: Plates labelled A were the fast acting drugs which were set at 0.2 % initial parasitaemia for 48 hours. These included: chloroquine, {starting concentration (sc) 100 ng/ml}, mefloquine 100 ng/ml, halofantrine 10 ng/ml, quinine 500 ng/ml, dehydroartemisinin 10 ng/ml, and atovaquone 10 ng/ml}. B-plates were the slow acting drugs which were set at 0.4% initial parasitaemia, for 66 hours. These included: doxycycline {starting concentration (sc) 1000 ng/ml}, azithromycin {starting concentration (sc) 10,000 ng/ml}, pyrimethamine{starting concentration (sc) 250 ng/ml}, and sulfadoxine {starting concentration (sc) 10,000 ng/ml}.

3.11 Drug sensitivity assays

All assays were performed in triplicates. Eight-fold 1 in 2 serial dilutions of drugs were distributed into 96 well microtitre plates in which fresh and infected erythrocytes were added to give a total volume of 200 μl well and initial parasitaemia of 0.2 % or 0.4 % depending on the time of incubation (48 or 66 hours) and the drugs for testing. Control wells with uninfected erythrocytes and infected erythrocytes in medium without drugs were always included in each plate to check on parasite growth. The 96- well drug plates were prepared in a horizontal manner such that the first ten wells contained diluted drug, well number 11 had no drug while well number 12 had no drug, no parasitized red blood cells but only uninfected red blood cells. 100µl of prepared culture medium was mixed with equal amount of the starting concentration of the drug, mixed and finally a two fold dilution was carried out upto well number 10, then the remaining medium discarded. The 96- well drug plates were divided into three chambers to allow for multiple comparative studies, that is: chamber1 (A,B,C) for hypoxanthine studies; chamber2 (D,E,F) for p(LDH) studies while the last chamber(G,H) was for microscopy. Each parasite isolate was tested using one plate for comparison purposes.

Plates were incubated at 37° C for 24 hours in a humidified chamber in the gas mixture, following which 10 µl per well 0.5 µci of ^{3}H hypoxanthine was added to the appropriate wells (Ofulla *et al.,* 1994) and the trays were incubated for a further 24 hours for the hypoxanthine assay.

A standard parasite dilution sheet (Appendix3) was prepared for purposes of parasite dilution for drug testing for pLDH, ^{3}H- hypoxanthine assays and microscopy. The 48- hour

isotopic microtest was performed according to the modified method of Desjardins *et al.*, (1979).

3.12 Testing drug plates by microscopy, pLDH and ^{3}H- hypoxanthine techniques.

The drug plates which were set at 1% haematocrit with the same starting parasitaemia were serially diluted upto well 10. The plate was divided into 3 rows diluted in triplicate for microscopy, 3 for ^{3} H- hypoxanthine and 3 for pLDH assays. The wells A - C were harvested, D- F preserved for pLDH and wells G-F, the contents put in eppendorf tube, centrifuged, suspension removed, 100 µl of foetal calf serum (FCS) added. These were centrifuged again, then 2.5 µl of foetal calf serum added to the pellet, mixed well and thick and thin smears made which were stained using 2.5 % Giemsa solution for 30 minutes.

Cells were harvested using a cell harvester that lyses the cells and deposits washed particulate material onto filter papers (skatron). These filter papers discs were individually placed into scintillation vials containing 1ml scintillation fluid and radioactivity counted in a liquid scintillation counter to determine the ^{3}H-hypoxanthine incorporation. Percentage inhibition of the growth compared to the control was calculated from the 3H- hypoxanthine incorporation and IC50 values determined from these points by means of a computer fitted curve. Each assay was carried out on at least 2 occasions.

The Giemsa stained slides were read by microscopy and isotopic uptake was calculated by probit analysis to determine 50 % inhibitory concentration values for drug concentrations. Log_{10} dose concentration probit regressions were fitted to the reduction in the 3-hypoxanthine data. The lethal concentration at 50%, 75%, and 95% reduction (IC50, IC75 and IC95) were then determined using the probit regression model (Mead and Curnow 1986).

3.13 Malaria diagnosis in school children resident in an endemic area by microscopy, red blood cell (RBC) and plasma pLDH

Randomly selected children between ages of 9 and 17 years who participated in the survey were recruited from Nyalunya village school in Kisumu. A total of 130 children were selected and teachers were informed about the nature of the investigation before they gave their consent. The study sample comprised 73 males and 57 females with mean (± SE) age of 13 ± 1.6. Blood was drawn from the study group and both thick and thin smears prepared. The same venous blood was processed, separating the plasma from cells and storing the samples immediately in the fridge at 4°C. These samples were then run within three hours after collection for plasma and red cell pLDH optical density (OD) using the original pLDH enzyme assay (modified method of Makler and Hinrichs, 1993). Sensitivity and specificity of pLDH assay were calculated using standard thick and thin film microscopy (Knobloch and Henk, 1995) and also by using the highest t-statistic value (independent of microscopy) for determination of mean cut-offs.

3.14 Comparison of parasitaemia of *P. falciparum* isolates from clinical samples by microscopy and red cell pLDH.

The isolates were collected from symptomatic outpatients (n=156) who were screened for malaria with a thick blood film on reporting to Kisumu District Hospital. Asexual parasite numbers were counted for five thousand red blood cells on the thin film with species confirmation on the thin film. Thirty minutes after thick smear positive samples

were detected, 1ml of venous blood was drawn from the volunteers who had signed the consent form and filled in the information on the clinical investigation form (Appendix 1 and 2). The clinical symptoms of the ambulatory patients were recorded on the first day of attendance of the outpatient clinic at the Kisumu District Hospital. The clinical signs were then coded as follows:

1- Headache
2- Headache + weakness or aches (pains)
3- Headache + weakness or aches (pains) + fever
4- Headache + weakness or aches (pains) + fever + vomiting or nausea
5- Headache + weakness or aches (pains) +fever + vomiting or nausea + diarrhea
6- Headache + weakness or aches (pains) + fever + vomiting or nausea + diarrhea + restlessness or convulsions
0- Other non-specified symptoms suggestive of cerebral malaria.

The blood was immediately transferred into two tubes, one with 50 ul of ACD anticoagulant and the other tube containing transport media (Ofulla *et al.*, 1994). The samples in transport media were transferred to Nairobi by courier services and set into culture one day (24 hours) after withdrawal. The same samples in the eppendorf tube with anticoagulant were processed in the field; washed twice with the transport media (using Dade serofuge, at 500g for 30 seconds), diluted to 1 % haematocrit and tested for RBC pLDH using a modified standard techniques described previously by Makler and Hinrichs (1993). For samples transported to Nairobi, plasma components were not assayed as the samples arrived already in transport medium. The predicted parasitaemia was determined using the standard curve generated by the laboratory cultivated isolates and extrapolated using an ELISA reader (Makler and Hinrichs, 1993) while microscopy readings were determined using standard techniques.

The field studies were carried out the same way as for the samples transported to Nairobi but in this case they were processed at the field site immediately. The samples were processed both for plasma and red blood cells in order to allow for comparison. The plasma samples were run using ELISA reader but only optical densities were read and not predicted parasitaemia as in red blood cell assays. The pLDH assay was run using standard curves for D6 and W2 clones with known chloroquine sensitivities. The basis of the assay was a calorimetric technique, where the darker the colour, the more the parasites, hence the enzyme pLDH.

3.14.1 Comparison of the three diagnostic tests: pLDH, Parasight-F and immunochromatographic test (ICT) using microscopy as gold standard.

The study patients were 308 healthy volunteers from Kisumu, Siaya District, Ndori station, who were parasitaemic or aparasitaemic but with no clinical symptoms of the disease. Blood was withdrawn and the samples were tested using the three techniques by independent individuals recording the results without knowledge of microscopic results. The microscopist read the results independently and recorded the results on a different sheet. Any discordant result were read again by the investigator and the results recorded. Whole blood was tested and improvement on parasite quantification was done by counting the number of parasites on thick smear for every 200 white blood cells. Species identification was carried out using thin smears. In this study, the pLDH used was OptiMAL ® dipstick and not an ELISA plate as previously done. Measurement of

association of the techniques was done at 95 % confidence interval and calculation of sensitivity and specificity carried out as previously reported (Galen and Gambino 1975; Greenberg *et al.,* 1993).

3.14.2 Determination of correlation between pLDH optical density (OD) of red blood cell (RBC) and parasitaemia in laboratory, field and clinical culture adapted malaria parasites.

Isolates with consistent growth rates and from various locations, namely, Somalia (Som A6), Mombasa (M24) FCB from wellcome, Kenya, Amsterdam strain NF54; Kisumu (K39) and Siaya (S104) were cultivated *in vitro*. The NF54 gametocytes were also cultivated according to standard techniques (Ogwang *et al.*, 1993) for the same. When good exflagellation was noted, spent medium was removed as per routine medium change, and warm suspended animation buffer immediately added so as to achieve at least a 1:100 times dilution. Gametocytes were spun at at 37°C and the pellet resuspended in warm suspended animation buffer. The gametocytes were then diluted out using 1% heamatocrit control red blood cells in warm suspended animation and then tested for pLDH. When parasitaemias for the other isolates were stable, the isolates were serially diluted by red blood cell control (at 1% haematocrit) which also served as control at the 12^{th} well and then tested for pLDH, using optical density reading by ELISA plate reader while thin smear parasitaemias were read. Correlation analysis was carried out to determine any linear relationship between optical density (OD) of red blood cells and parasitaemia. Linear regression analysis was also carried out to obtain equation for predicting O.D using parasitaemia or predicting parasitaemia using OD for each isolate.

3.15 Determination of correlation between percentage plasma pLDH and parasitaemia in laboratory, using sampled clinical isolates from the study site.

Malaria positive samples were collected from the District Hospitals study site (ambulatory patients) and the samples transported immediately to the laboratory in ACD anticoagulant using cool box. Immediately the samples were span using Dade serofuge, at 500g for 30 seconds, plasma was then separated from the red blood cells and serial dilution of plasma carried out in 96 well plate using culture medium (Appendix 3). Each isolate was then tested separately from the original (neat) to the lowest dilution using negative plasma sample, serially diluted, as control. The plates were immediately tested for presence of p(LDH) using malstat® reagent at the same time thick and thin smear slides for microscopy were prepared. These samples were initially selected as thick smear positive, but on reaching the laboratory (4 hours later), some tested negative. The thick and thin smear microscopy readings of the samples were then correlated with pLDH using t- test procedure.

3.16 Relationship between clinical presentation and p(LDH) red blood cell and plasma levels in symptomatic ambulatory patients in a malaria endemic zone.

The 156 volunteers in the study were ambulatory patients from Kisumu District Hospital. These were symptomatic outpatients who were screened for malaria with a thick blood film and the results recorded as low (+, representing one or occasional parasite per 10 microscopic fields scanned, moderate (++, two or less than ten parasites per 10 microscopic field scanned), or high (+++, over 10 parasites per 10 microscopic fields scanned) using

thick blood film (Kitchen, 1949). Both serum and red blood cell pLDH was run for comparison purposes with microscopy and clinical symptoms. The cut off plasma and red blood cells negative and positive cut-offs were calculated and analysis of variance (ANOVA) was used to compare the mean RBC and plasma for the different clinical signs groups.

3.17 Progression of plasma pLDH post treatment using -single dose fansidar in malaria endemic clinical trial (retrospective study).

Blood from 30 malaria patients who had been registered into malaria drug study at Kisumu District Hospital were stored frozen at - 80°C in freezer for three years and later on tested for pLDH activity. The samples were collected on day 0, that was the first day of treatment with Fansidar® (pyrimethamine\sulphadoxine) with follow up collections on days 7, 14 and 28. These samples for pLDH analysis levels were in storage at -80°C for three years after treatment. Data was generated for plasma pLDH and analysis of variance was used to compare the plasma levels.

3.18 Measurement of drug sensitivity profiles for used and candidate antimalarial drugs using pLDH assay.

The 96-well flat-bottomed antimalarial drug sensitivity test plates predosed with chloroquine, amodiaquine, quinine and sulfadoxine /pyrimethamine were purchased from WHO Regional Office of the Western Pacific (Manila, Philippines). The isolates, D6, W2, and NF54, were cultivated up to parasitaemia of > 1% using standard methods (Trager and Jensen, 1976). The fourteen field isolates, KS021, KS140, KS012, KS030, KS044, KS063,

KS155, KS001, KS168, KS157, KS211, KS031, KS193, and KS047 were the only field parasites that adapted (using modified method of Trager and Jensen, 1976) to a level that could be tested for pLDH. The other field parasites either contaminated or did not reach the desired parasitaemia (1%) by the third week for drug sensitivity testing. The parasites could not be kept in culture for a longer period for fear of variation in IC50 results (Ofulla, unpublished observation). The parasite lactate dehydrogenase assay were performed as a 48 hour test without ^{3}H-hypoxanthine addition. At the end of the incubation period, 50 μl of 1% parasitized red blood cells of each isolate were put in 96 - well microtitre plates (incubated for 20 minutes with 100μl of malstat® reagent). Equal volumes of nitroblue tetrazolium (NBT) and phenazine ethosulphate (PES) were mixed and 10 µl of fresh mixture added to each well and incubated in the dark for 20 minutes. The optical density was read at 650nm wavelength after the reaction was stopped with 5% acetic acid (Makler *et al.*,1993). The blue formazan product was evaluated by end point analysis at 650 nm wavelength using ELISA reader (Molecular Devices Thermomax ®). The pLDH data was then analysed by log-logit method using softmax™ software that directly extrapolated the titration data of the drugs (Makler *et al.*, 1993). The IC50 values were expressed in nanograms per mililitre (ng/ml) of test culture. The pLDH assay was run for each isolate with or without the drugs in the 96-well culture plates. The reference strains with known drug sensitivity profiles for chloroquine and mefloquine D6 (chloroquine sensitivity, mefloquine resistant) and W2 (chloroquine resistant, mefloquine sensitive) served as controls (Makler and Hinrichs, 1993).

3.19 Determination of correlation between microscopy and pLDH using field isolates KS021, KS041, KS140 and KS608.

The four Kisumu isolates, were culture-adapted for three weeks using standard procedures (Trager and Jensen, 1976). When the parasitaemia steadily increased (KS140, up to 10.24 %), both thick and thin blood smears were prepared and parasites counted. The isolates were then serially diluted in a 96-well microtitre plate up to the 11th well while the 12th well was served with red blood cell control at 1% haematocrit. The optical density (OD) reading was assessed using an ELISA plate reader (Molecular Devices Thermomax). The same procedure was carried out for the Kisumu field isolates KS021 (3.04), KS041 (3.92 %) and KS608 (5.16 %). A correlation analysis was carried out for the various drug study methods.

3.20 Determination of correlation between diagnostic tools: microscopy, pLDH and ^{3}H-hypoxanthine for 48 hours, using field isolates KS021, KS041, KS140 and KS608.

The four field isolates KS021, KS140, KS041 and KS608 were culture adapted until the growth rates became steady. Sensitivity assays were initiated by adjusting the initial parasitaemia to 1 - 2% with matching type of human red blood cells suspended in complete tissue culture medium (RPMI 1640, 25mM Hepes, 25mM bicarbonate and 10% normal matched type human serum - Makler *et al.*,1993). The suspensions were dispensed in triplicates at 0.2 ml/well into 96-well, flat bottomed microtitre plates. The cultures were incubated at 37° C for 48 hours in a gas mixture (92% Nitrogen, 5% CO_2 and 3% O_2 -East Africa Oxygen). In the hypoxanthine experiments, the incubation was interrupted after 24

hours for the addition of 0.5 μCi of ^{3}H- hypoxanthine. At the conclusion of the incubation period, the cultures were carefully resuspended and aliquots were removed for the analysis of pLDH activity, for the preparation of thin blood smears, and for the measurement of 3H - hypoxanthine uptake using liquid β- scintillation counter using standard procedures described in section 3.12 above

3.21 ^{3}H- hypoxanthine assay: standardization using culture adapted malaria parasites and antimalarial drugs by probit analysis method.

Standardization of the assay was necessary since the drug plates used in the study were prepared in the laboratory (KEMRI - immunology - malaria laboratory). A standard parasite dilution sheet was finally produced which was used in all experiments (Appendix 3). The antimalarial drugs were run at varying starting concentrations depending on their performances during the standardization process (Chloroquine, starting conc. 250 ng/ml; Quinine, starting conc.

1 000 ng/ml and Mefloquine, starting conc. 500 ng/ml). The controls included the higher drug concentrations, (A_1), no drug, parasitised RBC (A_{11}) and the red blood cell - unparasitized RBC's (A_{12}). The IC50 concentrations were measured by probit analysis for hypoxanthine assay while for pLDH the data was directly extrapolated by the use of ELISA reader (Makler and Hinrichs, 1993).

3.21.1 Monitoring blood clearance of parasitaemia by microscopy for different follow-up days after the use of antimalarial drugs.

For the pLDH blood clearance with treatment, the recruits were students and workers inEgerton University health clinic, Rift- Valley, who had travel history to malaria endemic regions of Kenya and who were blood slide positive for malaria. Those who met the recruitment criteria were enrolled into the study using malaria investigation form (Appendix 2) signed by the doctor or clinical officer attached to the study patient. Finger pricks for the determination of parasitaemia were done daily for seven days follow up for both ambulatory and in - patient on the pLDH kinetics study. Study code was given to each patient, history of the patient recorded in the sheet, both thick smear and thin smear parasitaemia taken and the drug given during treatment course recorded. The number of follow ups was recorded at the end of the study as well as the patients position, whether ambulatory or in - patient. During each day of visit, about 0.5 ml of blood was collected into the ACD tubes, frozen immediately and transported to Nairobi for pLDH blood clearance future studies using monoclonal antibodies. A total of 68 patients (39 males and 29 females) were enrolled into the study. The types of drugs and drug combinations administered were also recorded.

3.21.2 Mean IC 50 (ng/ml) of drugs which failed to give reproducible results by pLDH using reference strains D6 and W2.

The study was carried out using culture adapted reference strains D6 and W2. The four drugs, doxycycline, azithromycin, pyrimethamine and sulfadoxine failed to give consistent results except with the reference strains D6 and W2. The drug plates were set in the same way as for the previous ones (see to section 3.11). These plates (B- plates)

were slow acting drugs which were set at 0.4 % initial parasitaemia, for 66 hours. These included doxycycline 1000 ng/ml, azythromycin 10,000 ng/ml, sulfadoxine 10,000 ng/ml, and Pyrimethamine 250 ng/ml. The Thermomax ® log -logit method using soft-max ™ software that directly extrapolated IC50 and the correlation coefficient was used and the results of the obtained mean IC50 in ng\ml were recorded.

3.3 STATISTICAL ANALYSIS

The pLDH assay results were subjected to analysis of variance (ANOVA) and least significant difference (LSD) tests to determine differences among means for the cut-offs. Where appropriate, student-t test was used for analysis.

CHAPTER 4

4.0 RESULTS

4.1 Comparison of pLDH and light microscopy in school children and `hospital clinical cases from Western Kenya.

4.1.1 Relationship between pLDH optical density (OD) and parasitaemia.

In all the six culture adapted malaria parasite isolates tested for pLDH in the laboratory, there was a strong positive correlation between the optical density values and parasitaemia except in purified gametocytes of the International Amsterdam strain NF 54 for which there was negative (-0.74) correlation (Table 1). The Mombasa isolate, M24 showed the lowest correlation in the asexual isolates tested 0.76. Table 2 shows the results of the linear regression of OD on parasitaemia and vise versa. All the slopes were significant ($p < 0.05$) indicating a significant linear relationship between OD and parasitaemia. When the isolates are combined the coefficient of determination is low (0.58) and even lower in NF54 gametocytes (0.54). Linear relationship is consistent irrespective of OD and parasitaemia in Y or X axes (Section A and B in Table 2). The coefficient of determination (R^2) was however less than 60% for the isolates M24 and NF54 gametocytes, indicating low prediction power of the linear relationship for these two isolates. For the two isolates which were collected from the study area and culture adapted, linear correlation between pLDH OD and parasitaemia was significantly positive, (K012-0.86; K030-0.81 Table 3). The reference strain, D6 indicated a correlation value 0f 0.92 while the pooled samples had a correlation of 0.88. Table 4 shows the results of the linear regression analysis of field adapted malaria isolates (K012 and K030) compared with the reference strain D6. The slopes were highly significant

and the coefficients of determination higher than 65% for all the relationships (Section A and B in Table 4). The results also indicated that the isolates had lower predicitve values in comparison to the cloned D6- chloroquine sensitive Sierra Leone isolate (0.85).

Table 1: Correlation coefficients between pLDH optical density of red blood cell and parasitaemia in culture adapted laboratory malaria parasites.

Isolate	# tested	Mean OD±SD	Mean % parasitaemia	Correlation
D6	22	0.146±0.03	0.54± 0.91	0.90***
FCB	22	0.164± 0.04	0.3± 0.55	0.98***
K39	33	0.174± 0.04	0.3± 0.5	0.91***
M24	22	0.176±0.06	0.65± 1.1	0.76***
NF54	22	0.186± 0.05	0.41± 0.7	0.89***
S104	22	0.153± 0.02	0.1± 0.17	0.89***
ALL Isolates pooled	154			0.77***
NF54 Gametocytes	11	0.139± 0.06	0.19± 0.32	-0.74**

** Significant at 1% level
*** Significant at 0.1% level
Level of significance determined using the conventional t- test for correlation values.

The laboratory isolates were grown in culture until parasitaemias were above 1%. Each isolate was serially diluted in 96-well plate and tested in triplicate. The pLDH optical density was read and extrapolated by the reduced form of APAD at 650nm using a spectrophotometer (UV_{max} kinetic Microplate reader; Molecular Devices Co., Menlo Park, C.A).

Table 2: Linear relationship (Y= a+bX)) between pLDH optical density (OD) of red blood cell (RBC) and parasitaemia in culture adapted laboratory malaria parasites (Standard error of the parameters, a and b, are in parentheses).

	Isolate	Y	X	# tested	a (Y- intercept) * **	b (slope) + ++	R^2 (Coefficient of determination)
	SOM A6	OD	PARASIT	22	0.13 (0.003)	0.03 (0.003)	0.82
	FCB	OD	PARASIT	22	0.14 (0.002)	0.08 (0.004)	0.96
	K39	OD	PARASIT	22	0.15 (0.004)	0.073 (0.006)	0.82
	M24	OD	PARASIT	22	0.15 (0.01)	0.039 (0.008)	0.57
A	NF54	OD	PARASIT	22	0.16 (0.006)	0.064 (0.008)	0.78
	S104	OD	PARASIT	22	0.14 (0.002)	0.093 (0.011)	0.79
	Combined	OD	PARASIT	154	0.15 (0.003)	0.046 (0.003)	0.58
	NF 54 (gam)	OD	PARASIT	22	0.14 (0.002)	-0.015(0.005)	0.54
	SOM A6	PARASIT	OD	22	-0.34 (0.43)	27.1 (2.87)	0.82
	FCB	PARASIT	OD	22	-1.8 (0.1)	12.7 (0.59)	0.96
	K39	PARASIT	OD	22	-1.7 (0.17)	11.33 (0.95)	0.82
	M24	PARASIT	OD	22	-1.9 (0.52)	14.6 (2.8)	0.57
B	NF54	PARASIT	OD	22	-1.87 (0.28)	12.3 (1.4)	0.78
	S104	PARASIT	OD	22	-1.2 (0.152)	8.49 (0.99)	0.79
	Combined	PARASIT	OD	154	-1.71 (0.15)	12.58 (0.87)	0.58
	NF54 (gam)	PARASIT	OD	22	5.22 (1.54)	-36.3 (11.1)	0.54

* Y- intercept (a)

** Standard error of a

\+ Slope (b)

++ Standard error of b

The isolates were culture adapted for maximum of two weeks upto parasitaemia above 1%. The cultures were tested for p(LDH) in 96-well microtiter plates after serial dilution in triplicate wells. Corresponding thick and thin smear microscopy slides were prepared using standard procedures. Both optical density values and microscopy were determined from the highest to the lowest concentrations and the results compared. The gametocytes were prepared for testing using standard techniques (Ogwang' *et al.*, 1993).

Table 3: Correlation coefficients between pLDH optical density (OD) of red blood cell (RBC) and parasitaemia in culture adapted field malaria parasites and the reference strain (D6).

Isolate	#Observations	Mean OD± SD	Mean % Parasitaemia	Correlation
D6	37	0.137±0.196	1.01	0.92
K012	39	0.048±0.119	0.65	0.86
K030	37	0.07±0.11	0.64	0.81
Pooled	113			0.88

The two isolates were the only ones that gave reproducible results.
*** significant at 0.1% level.
The two isolates K012 and K030 were collected from the study site, Kisumu, transported to the Laboratory in transport media the same day using courier services and set in culture, 24-hours after venipuncture. The isolates were culture adapted for three weeks, serially diluted in 96-well microtiter plates, and tested in triplicate for correlation between optical density and microscopy using reference strain, D6, as the standard.

Table 4: Linear relationship (Y= a +b X) between pLDH optical density (OD) of red blood cell (RBC) and parasitaemia in culture adapted field malaria parasites (Standard error of the parameters, a and b, are in parentheses).

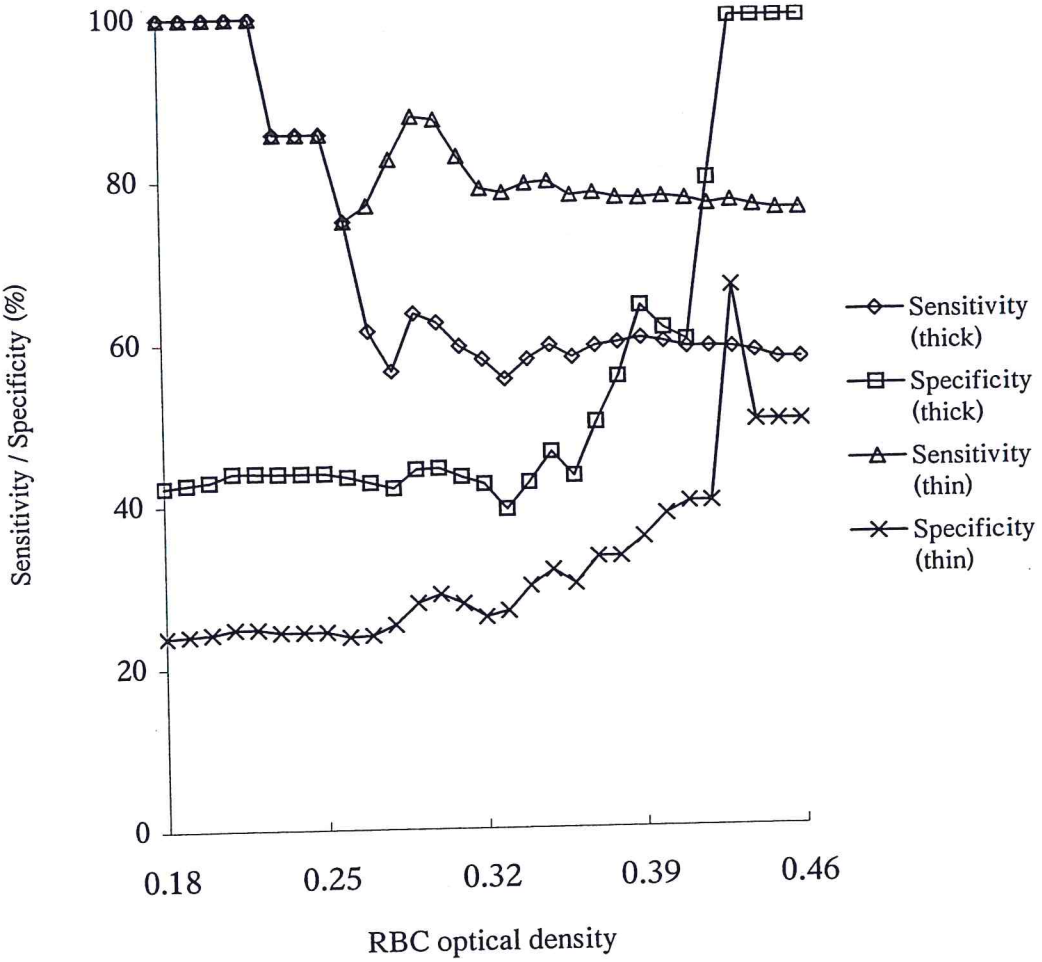
	ISOLATE	Y	X	Sample size	a	b	R^2
A	D6	OD (rbc pLDH)	PARASIT Thin smear (B)	37	0.014 (0.015)	0.123 (0.009)	0.85
	K012	OD (rbc pLDH)	PARASIT Thin smear (B)	39	-0.02 (0.012)	0.104 (0.01)	0.75
	K030	OD (rbc pLDH)	PARASIT Thin smear (B)	37	0.01 (0.013)	0.094 (0.011)	0.66
	Combined	OD (rbc pLDH)	PARASIT Thin smear (B)	113	-0.0028 (0.008)	0.114 (0.006)	0.78
B	D6	PARASIT thin smear (B)	OD (rbc pLDH)	37	0.052 (0.115)	7 0 (0.49)	0.85
	K012	PARASIT thin smear (B)	OD (rbc pLDH)	39	0.304 (0.087)	7.2 (0.68)	0.75
	K030	PARASIT thin smear (B)	OD (rbc pLDH)	37	0.151 (0.109)	7 0 (0.85)	0.66
	Combined	PARASIT thin smear (B)	OD (rbc pLDH)	113	0.185 (0.059)	6.8 (0.34)	0.78

The same procedure was used as in Table 3.
The two isolates K012 and K030 were collected from the study site, Kisumu, transported to the Laboratory in transport media the same day using courier services and set in culture, 24-hours after venipuncture. The isolates were culture adapted for three weeks, serially diluted in 96-well microtiter plates, and tested in triplicate for correlation between optical density and microscopy using reference strain, D6, as the standard.

4.1.2 Red blood cell pLDH cut -off sensitivity and specificity in field trials, using thick smear (A) and thin smear (B) as gold standards.

The enzyme assay, pLDH, gave the highest sensitivity (100%) with thin smear microscopy and lower sensitivity (< 60%) with thick smear microscopy. The sensitivity with thin smear decreased with increase in optical density cut-offs, stabilizing at about 75%. With thick smear, the sensitivity decreased with increase in OD stabilizing at about 60% (Figure 1). The specificity for the assay was lower when thin smear was used as gold standard. This increased with rise in optical density of pLDH cut-off, reaching maximum value of about 68% at OD =0.42. When thick smear was used, the specificity rose upto a maximum value of 100% at pLDH readings of about 0 .4 (Figure 1). These results suggest that OD values of between 0.39 and 0.43 were optimal cut-offs to achieve a reasonable sensitivity (60 to 80%) and specificity (60 to 70%), using both thick and thin smear microscopy as standards.

FIGURE 1:Determination of red blood cell pLDH cut-off, sensitivity and specificity using thick (A) and thin (B) smear as gold standards

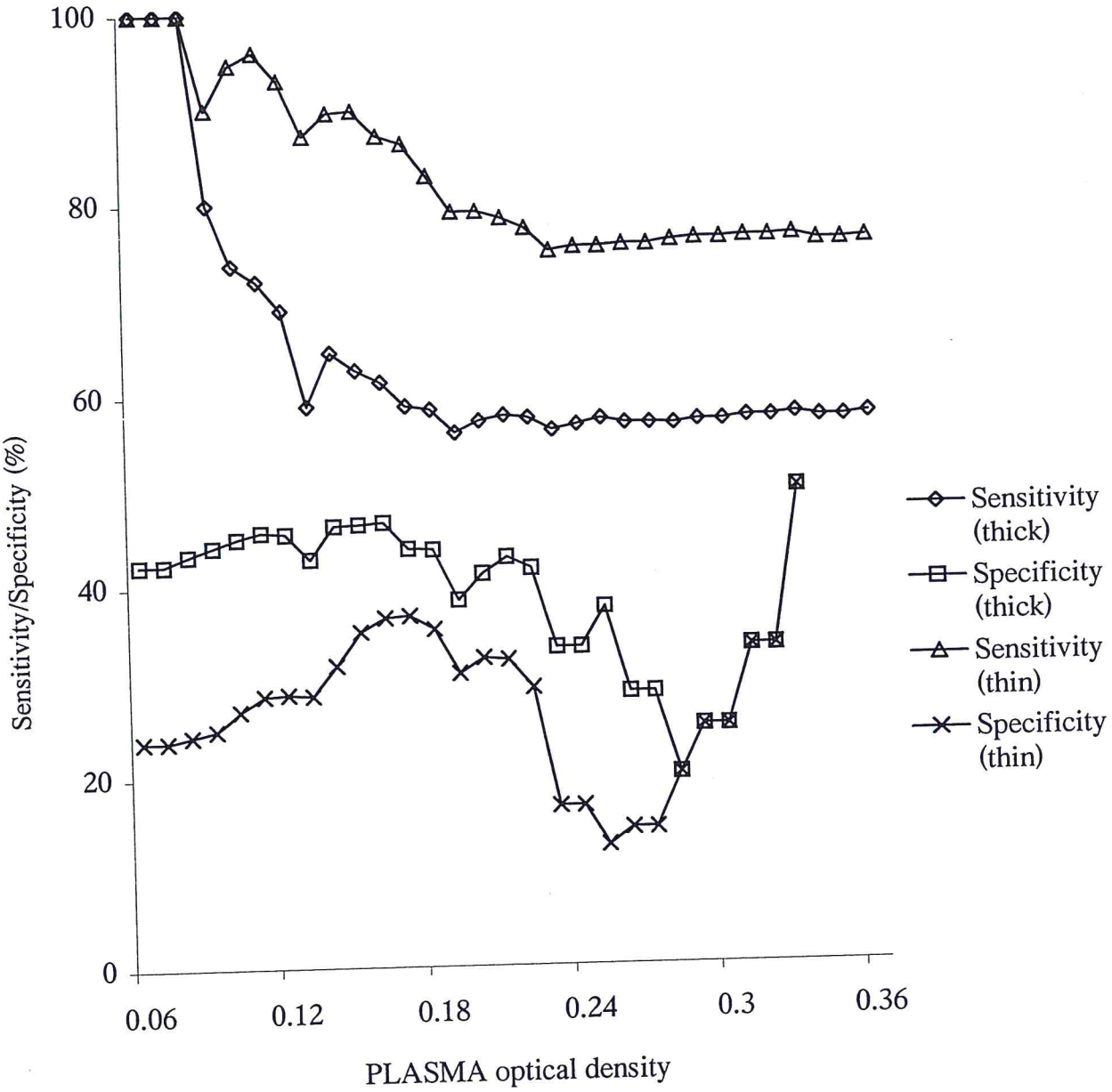


Venous blood was collected from healthy children of school going age living in malaria endemic region and immediately put in eppendorf tubes with anticoagulant. The plasma and red blood cells were then separated and immediately put in cold storage and transported to the laboratory for pLDH analysis. Both thick and thin smears were made and read independent of pLDH results

4.1.3 Plasma pLDH cut-off, sensitivity and specificity in field trials by using thick smear (A) and thin smear (B) as gold standards.

The enzyme assay, pLDH, showed a similar trend for plasma as already described for the red blood cell in which the highest sensitivity (100%) was obtained with thin smear microscopy whereas the lowest sensitivity (55%) was recorded with thick smear microscopy. The sensitivity decreased with increase in optical density cut-off, stabilizing at about 75%. The specificity for the assay was lowest (14%) when thin smear was used as a gold standard. The plasma cut-off specificity value reached peak (48%), a value lower than the lowest sensitivity (55%), using the two microscopic techniques (Figure 2). The results suggest that OD values of between 0.1 and 0.15 were the optimal cut-off for plasma to achieve a resonable sensitivity (70 to 90%) and specificity (35 to 45%), using both thick and thin smear as standards.

FIGURE 2: Determination of plasma pLDH cut-off sensitivity and specificity using thick smear (A) and thin smear (B) as gold standards.

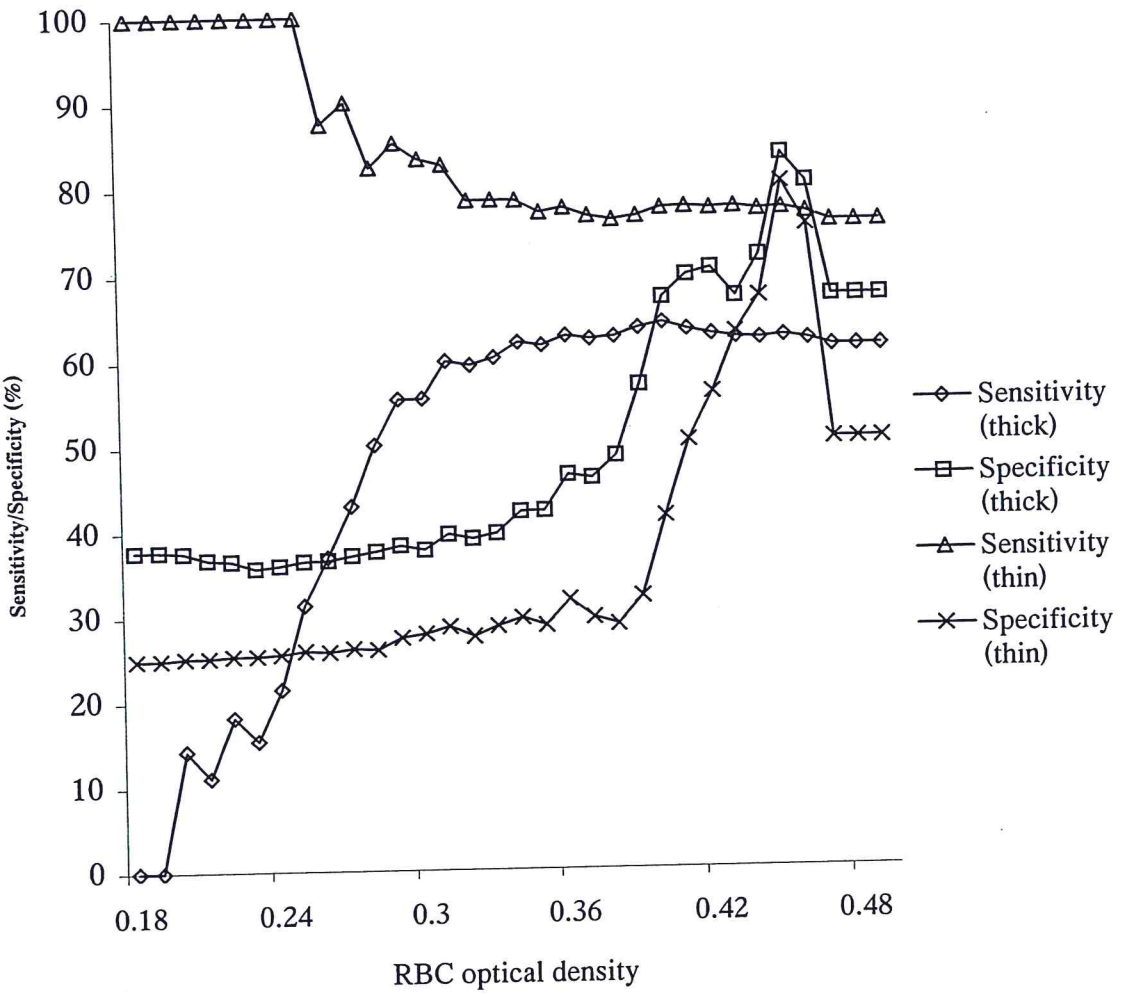


Venous blood was collected from healthy children of school going age living in malaria endemic region and immediately put in eppendorf tubes with anticoagulant. The plasma and red blood cells were then separated and immediately put in cold storage and transported to the laboratory for pLDH analysis. Both thick and thin smears were made and read independent of pLDH results

4.1.4 Red blood cell pLDH cut–off, sensitivity and specificity in clinical trials by using both thick smear (A) and thin smear (B) microscopy as gold standards.

In clinical trial the sensitivity was highest with thin smear microscopy as standard. The two microscopy methods were consistently different in all the trials. Whereas, the sensitivity with thin smear decreased with increase in OD cut-off, the sensitivity with thick smear increased with increase in the OD cut-off. Both however, started to stabilize at a cut-off of about 0.30 OD, with sensitivity of about 80% for the thin smear and about 60% for the thick smear. The specificity values were higher when thick smear was used, but at higher optical density cut- off (about, 0.45), the two microscopic methods gave similar specificity (Figure 3). The results of the clinical studies suggest that OD values of between 0.41 and 0.45 were the optimal cut-off for red blood cell to achieve a reasonable sensitivity (60 to 80%) and specificity (60 to 80%), using thick and thin smear as standards.

FIGURE 3: Determination of RBC pLDH cut-off, sensitivity and specificity in clinical trials by using both thick smear (A) and thin smear (B) microscopy as gold standards.

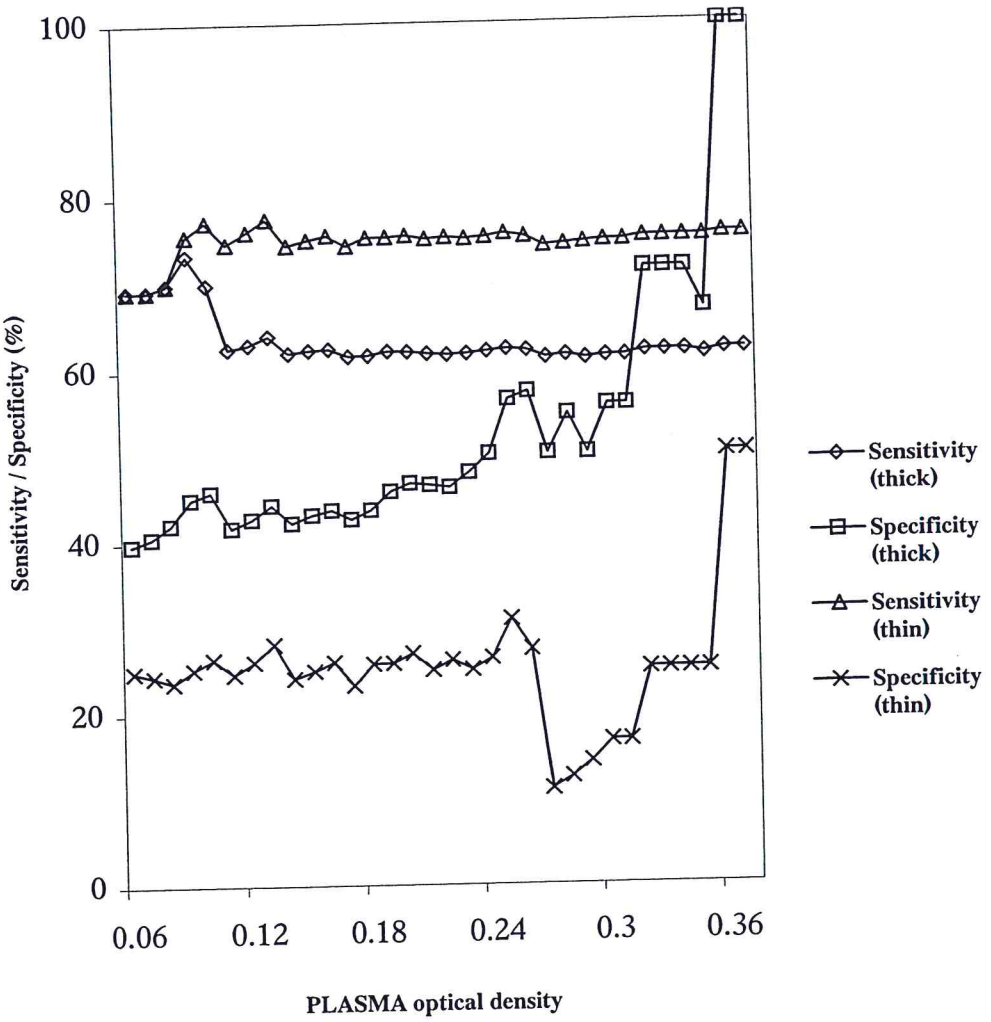


Study subjects were individuals from the study area with clinical symptoms of malaria. Blood samples were drawn and processed for plasma and red blood cells (by spinning in a serofuge at 500g). The assay was performed four hours after storage at 40°C. Blood smears were prepared from whole blood samples prior to processing.

4.1.5 Plasma pLDH cut-off, sensitivity and specificity in clinical trials by using both thick smear (A) and thin smear (B) microscopy as gold standards.

In clinical trials, plasma pLDH sensitivity value was highest when thin smear microscopy was used, and remained about constant between 70 and 78% as pLDH OD cut-off increased. The same trend was observed (sensitivity stabilizing at about 60%) when thick smear microscopy was used as gold standard in this study (Figure 4). Higher specificity values were obtained when thick smear microscopy was used as standard. The results of the clinical studies suggest between 0.25 and 0.32 as the optimum cut-off for plasma OD, to achieve reasonable sensitivity (60 to 80%) and specificity (30 to 80%), using thick and thin smears as standards.

FIGURE 4: Determination of plasma pLDH cut-off, sensitivity and specificity in clinical trials by using thick smear (A) and thin smear (B) microscopy as gold standards.



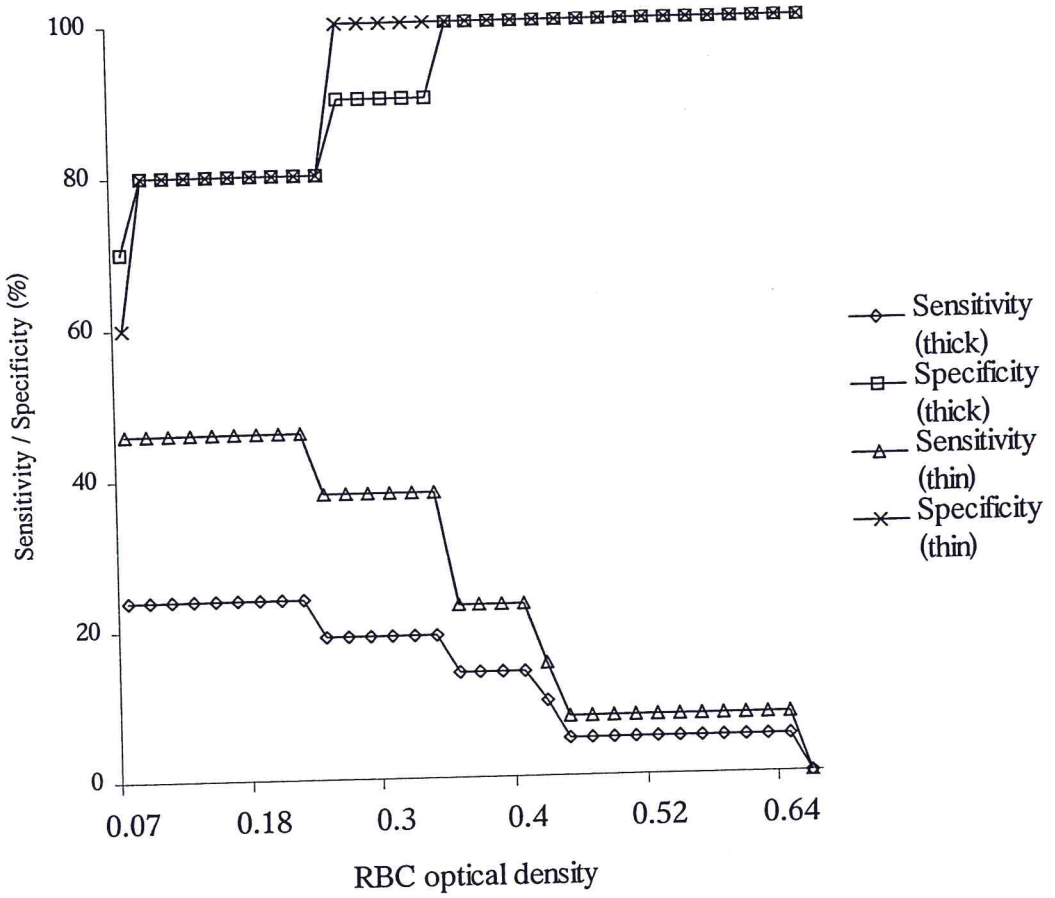
Study subjects were individuals from the study area with clinical symptoms of malaria. Blood samples were drawn and processed for plasma and red blood cells (by spinning in a serofuge at 500g). The assay was performed four hours after storage at 40°C. Blood smears were prepared from whole blood samples prior to processing.

4.1.6 Red blood cell pLDH cut-off, sensitivity and specificity in sampled clinical subjects in malaria endemic region by use of thick (A) and thin (B) smear microscopy

The sampled study subjects (n=31), all positive by thick smear, but only 18 positive by thin smear had mean percentage plasma of 85 ± SD of 27, with minimum value of 9% and maximum value of 100%.

The results of the RBC pLDH analysis in sampled clinical subjects from malaria endemic region of Kenya gave a higher sensitivity using thin smear microscopy than thick smear as standard. The highest sensitivity were 38% and 19% obtained for OD cut-off between 0.06 and 0.2, when thin and thick smear were used as standard, respectively (Figure 5). The specificity pattern was similar in both thick and thin smear (90% and 100% respectively, at RBC cut off of 0.26 optical density). At higher RBC cut-off above 0.4 optical density, sensitivity pattern was consistently low for both standards (< 20%) but specificity was maximum for both (100%). The results of the sampled clinical studies suggest that OD values of between 0.06 and 0.2 were the optimal cut-off for red blood cell to achieve a reasonable sensitivity (25 to 45%) and specificity (60 to 80%), using thick and thin smear as standards.

FIGURE 5: Determination of red blood cell pLDH cut-off, sensitivity and specificity in sampled clinical subjects in malaria endemic region by use of thick (A) and thin (B) smear microscopy.

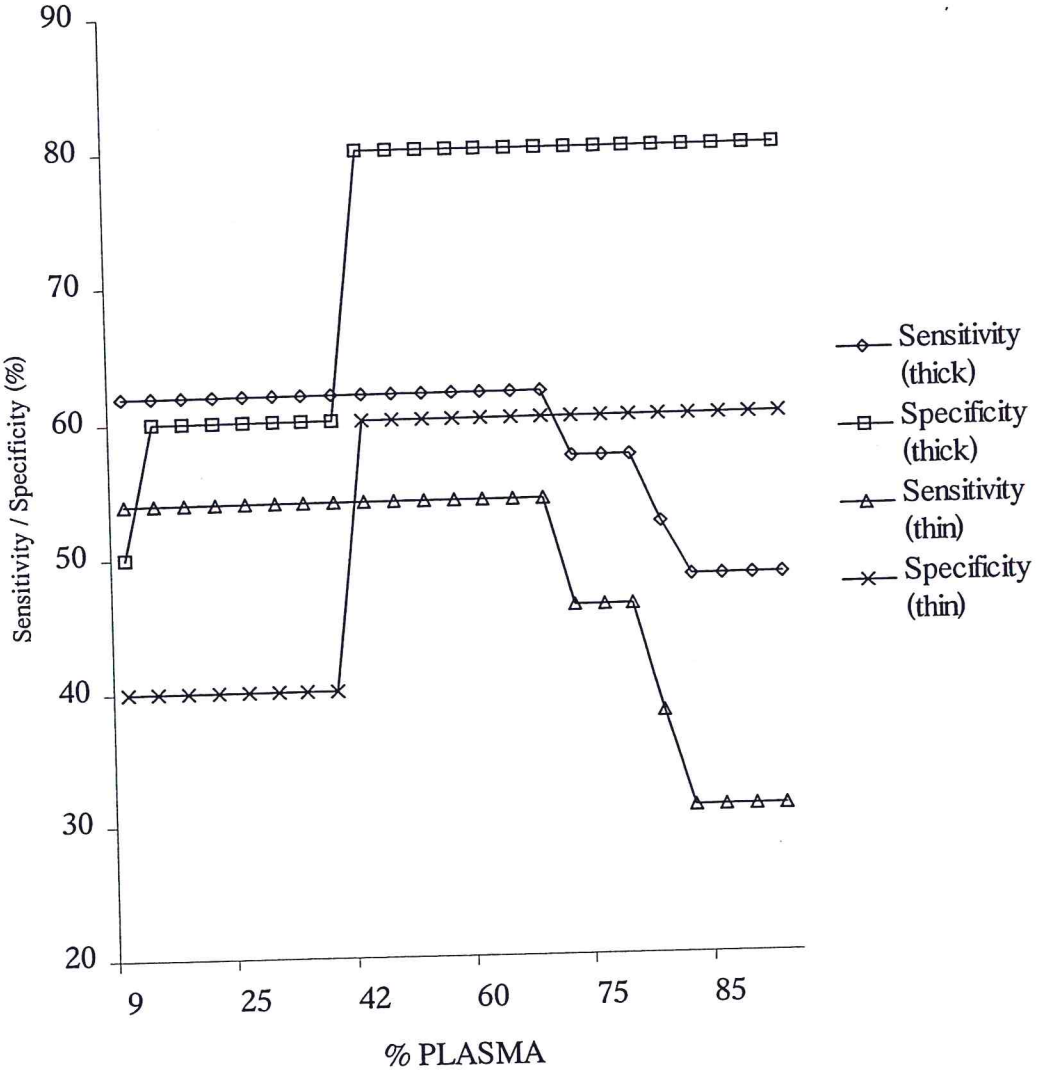


Study subjects were individuals from the study area with clinical symptoms of malaria. Blood samples were drawn and processed for plasma and red blood cells (by spinning in a serofuge at 500g). The assay was performed four hours after storage at 40°C. Blood smears were prepared from whole blood samples prior to processing.

4.1.7 Plasma pLDH cut-off, sensitivity and specificity in sampled clinical subjects in malaria endemic region by use of thick (A) and thin (B) smear microscopy.

Plasma pLDH of the sampled studies indicated a high level of sensitivity and specificity using thick smear microscopy (62%, 80% respectively), while low level was indicated using thin smear microcopy (54%, 60% respectively) at the same cut off plasma percentage of 45 (Figure 6). The sampled plasma were run as % plasma, where the lowest percentage was 9 and the highest 100%. At 45% plasma level, the optimum cut-off for sampled clinical subjects was achieved which gave sensitivity and specificity patterns near similar with the 156 clinical subjects cut-off of OD of 0.25 to 0.32 (Figure 4).

FIGURE 6: Determination of percentage plasma pLDH cut-off sensitivity and specificity in sampled clinical subjects in malaria endemic region by use of thick (A) and thin (B) smear microscopy.

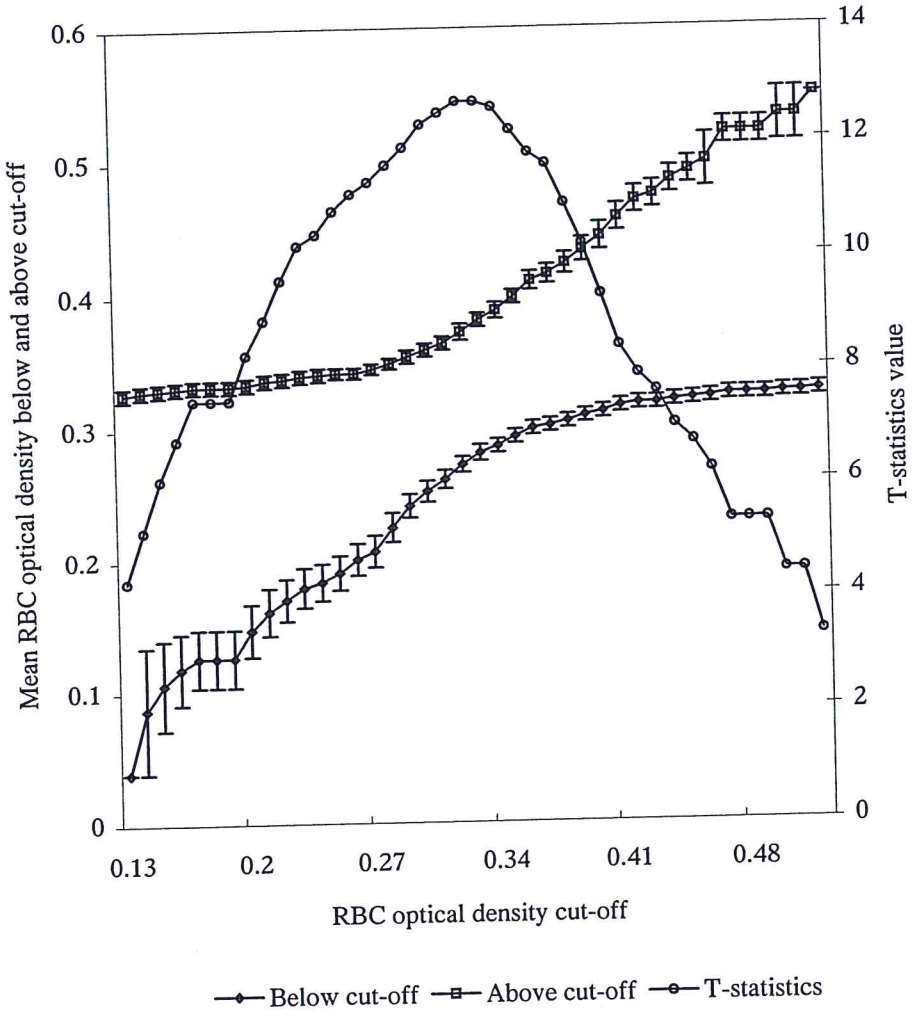


The plasma from sampled clinical subjects was serially diluted in 96-well microtiter plates and then tested for percentage plasma pLDH. The values were then compared with microscopy in sensitivity and specificity analysis

4.1.8 pLDH cut-off in field trials by using the RBC and plasma optical density readings.

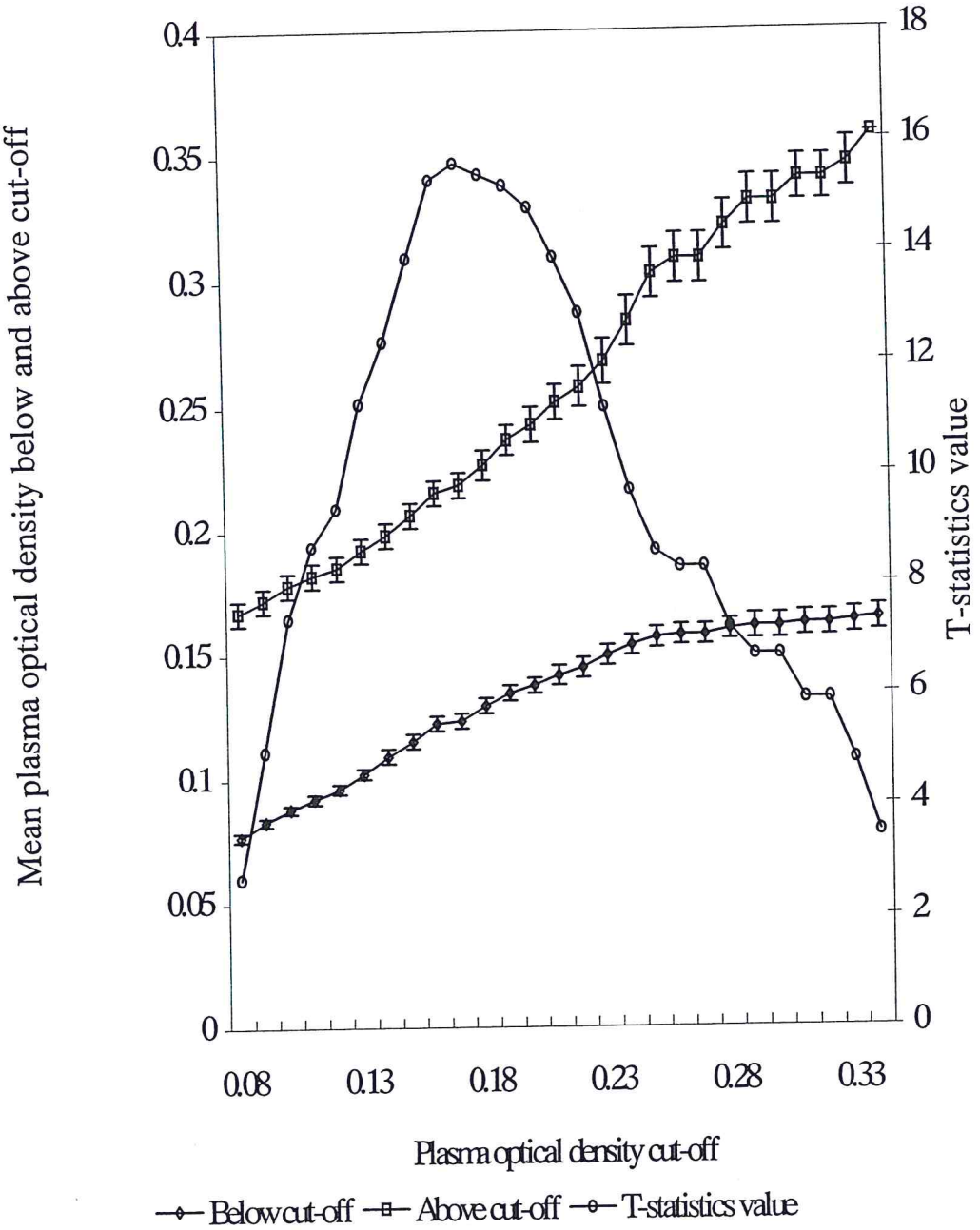
Figure 7a and 7b show the mean value of RBC and plasma pLDH for subjects above or below certain arbitrary cut-offs, determined independent of microscopy. The student t-statistic values indicate how significantly different the mean values for the two classes of subjects (above and below certain cut-off) are. The t- statistic value was highest (t= 13.8; $p<0.001$) at RBC cut-off of 0.32 (Figure 7a), giving mean RBC values of 0.28 ± 0.004 for subjects below this cut-off, and 0.361 ± 0.004 for subjects above the cut-off. For plasma, the highest t- statistic value (t= 15.6, $p<0.001$) was obtained at a cut-off of 0.17 (Figure 7b), with mean plasma optical density of 0.123 ± 0.003 for subjects below, and O.D 0.218 ± 0.005 for subjects above this cut-off.

FIGURE 7A: Determination of pLDH cut-off in field trials using the RBC optical density readings



The mean of all the study subjects for field evaluation were statistically analysed in order to get mean cut-off value for field application.

FIGURE 7B: Determination of pLDH cut off in field trials using the plasma optical density readings

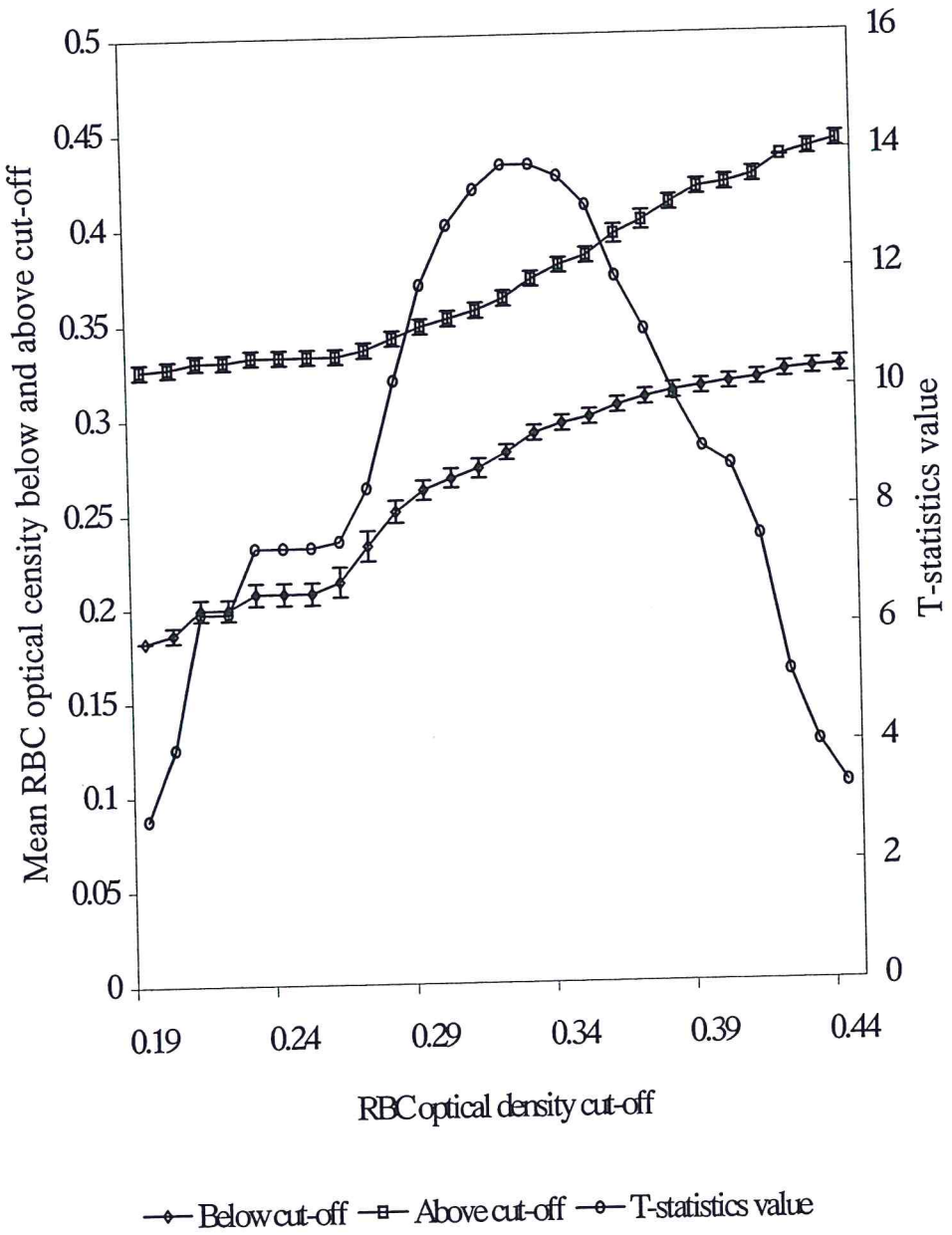


The mean plasma pLDH OD values obtained in the field study were statistically analysed for mean cut-off value for field application.

4.1.9 Parasite LDH cut off in clinical trials using the RBC and plasma optical density readings

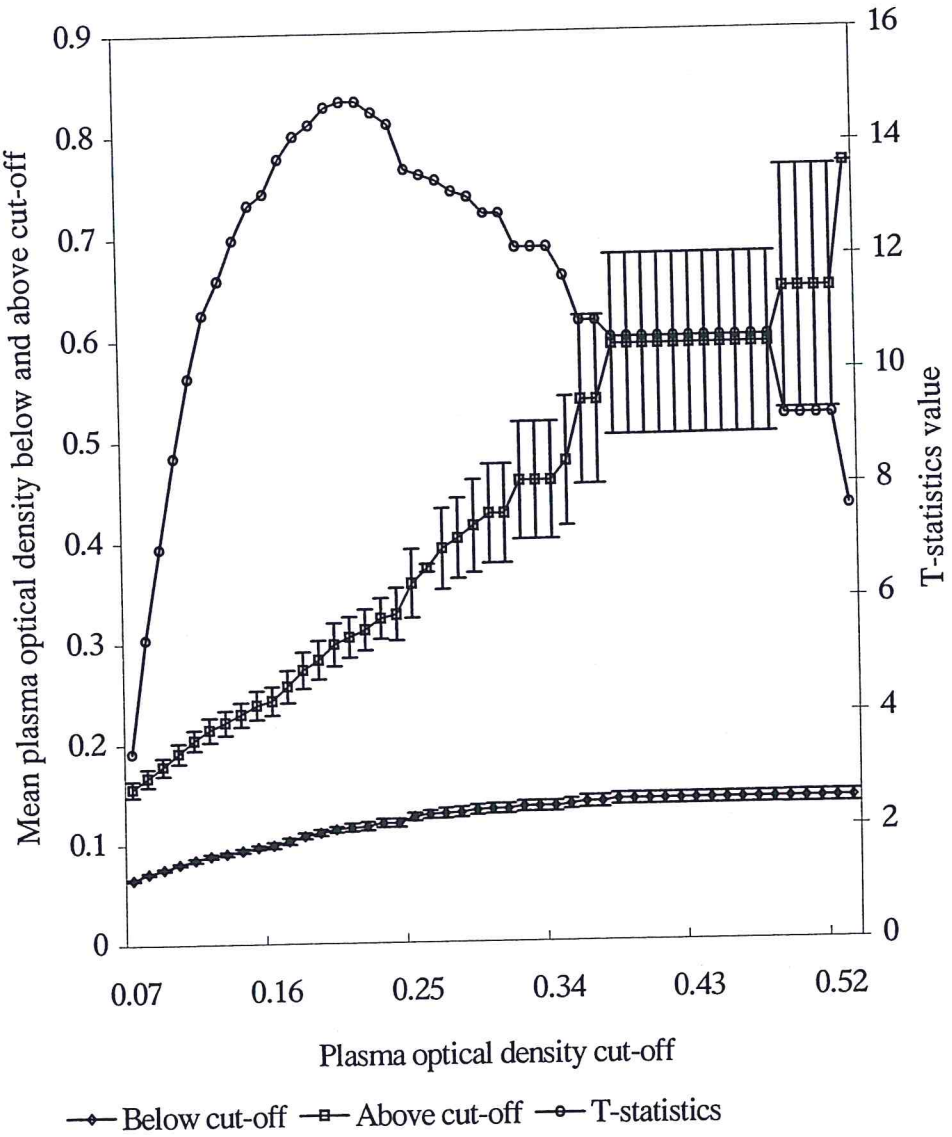
Figure 8a and 8b show the mean value of RBC and plasma pLDH for subjects above or below certain arbitrary cut-offs, determined independent of microscopy. The student t-statistic values indicate how significantly different the mean values for the two classes of subjects (above and below certain cut-off) are. The t- statistic value was highest ($t= 12.7$; $p<0.001$) at RBC cut-off OD of 0.32 (Figure 8a), giving mean RBC values OD of $0.271_{\pm}$ 0.006 for subjects below this cut-off, and OD of $0.371_{\pm}$ 0.006 for subjects above the cut-off. For plasma, the highest t- statistic value ($t= 14.8$, $p<0.001$) was obtained at a cut-off of 0.21 (Figure 8b), with mean plasma O.D reading of 0.115 ± 0.004 for subjects below, and O.D of $0.304_{\pm}$ 0.02 for subjects above this cut-off.

FIGURE 8A: Determination of pLDH cut-off in clinical trials using the RBC optical density readings



The mean red blood cell pLDH optical density values obtained in the clinical study were statistically analysed for a mean cut-off value for clinical application.

FIGURE 8B: Determination of pLDH cut off in field trials using the plasma optical density readings.

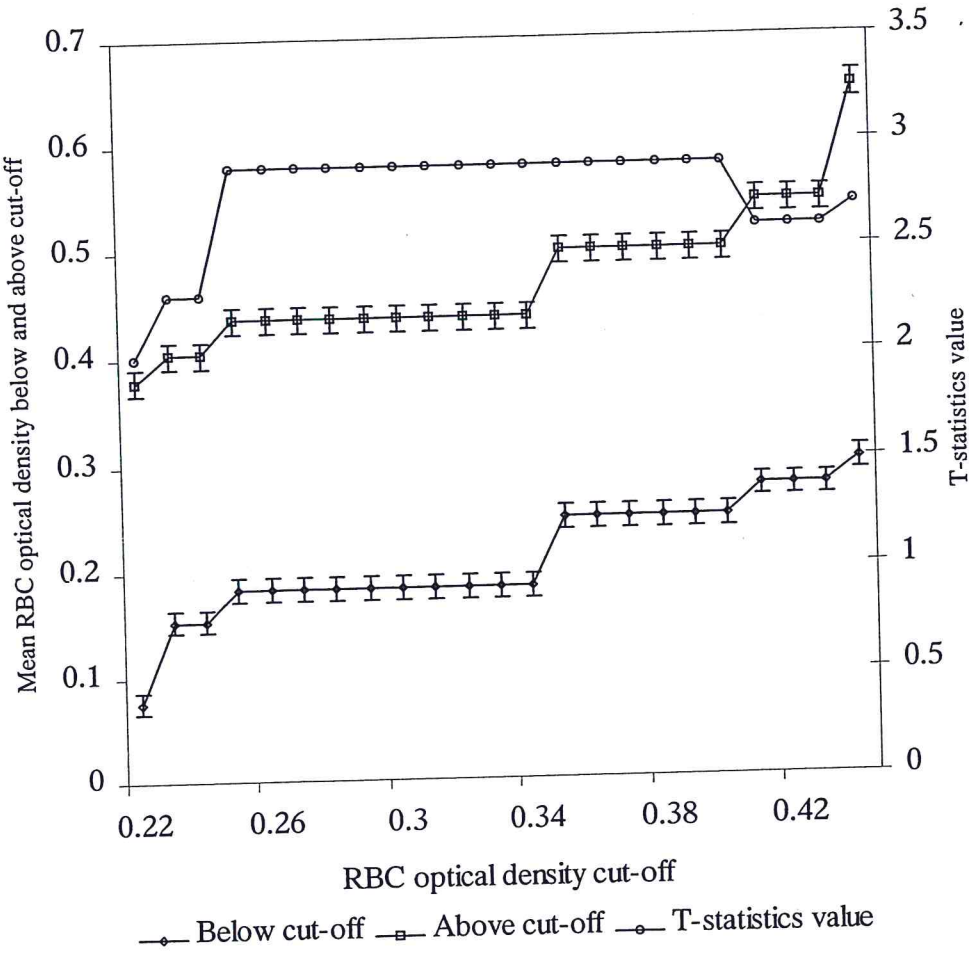


The mean plasma pLDH optical density values obtained were statistically analysed.

4.1.10 Parasite LDH cut off in sampled clinical trials by using the plasma optical density readings.

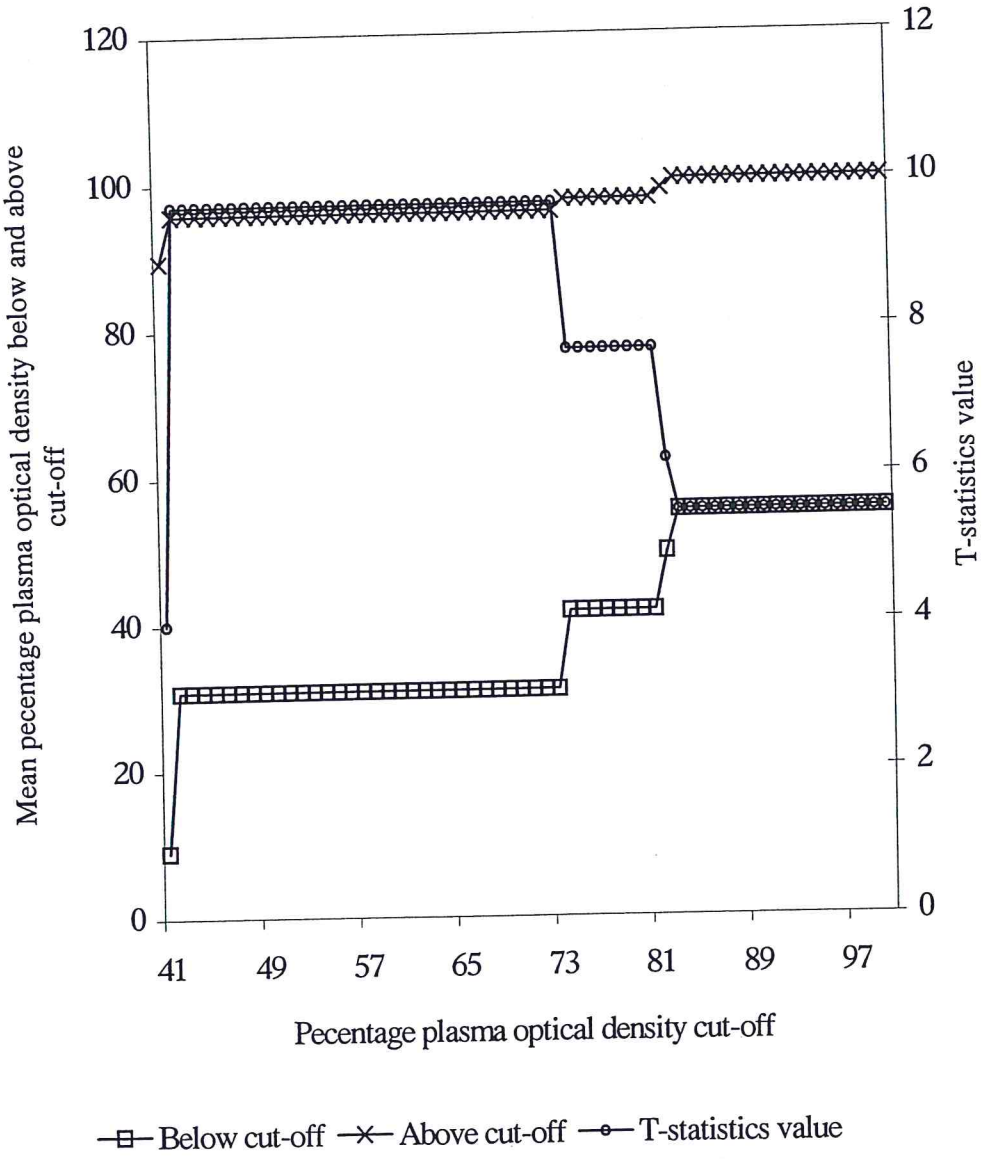
Figure 9 shows the mean value of RBC and plasma pLDH for subjects above or below certain arbitrary cut-offs, determined independent of microscopy. The student t- statistic values indicate how significantly different the mean values for the two classes of subjects (above and below certain cut-off) are. The t- statistic value was highest ($t= 3$; $p<0.02$) at RBC cut-off of 0.26 (Figure 9), giving mean RBC values of $0.183_{\pm}$ 0.05 for subjects below this cut-off, and $0.4361_{\pm}$ 0.06 for subjects above the cut-off. The t- statistic value did not indicate peak cut-off in plasma and red blood cells in this study (Figures 9 and 10).

FIGURE 9: Determination of pLDH cut -off in sampled clinical trials using the plasma optical density readings.



The mean red blood cell pLDH OD values obtained in the sampled clinical study were statistically analysed in order to come up with mean cut-off value for clinical application. The highest t- statistic value was not achieved in this study.

FIGURE 10: Determination of pLDH cut-off in sampled clinical trials using the red blood cell optical density readings.



The mean percentage plasma OD values obtained in the sampled clinical study were statistically analysed in order to come up with mean cut-off value for clinical application.

The highest t- statistic value for this study was not achieved.

4.1.11 Comparison between optimum optical density for plasma and red blood cell pLDH by using thick smear (A), thin smear (B) microscopy, independent red blood cell and plasma values in field (school subjects) and clinical (hospital ambulatory patients) trials.

The minimum value of the RBC or plasma that gave considerable sensitivity and specificity were used as the optimum cut-offs for the purpose of comparison. These values, together with the sensitivity and specificity, are presented in Table 5.

In the field study, the cut-off optical density for plasma was lower when thick smear was used as standard, compared to thin smear (0.1and 0.15 respectively). The cut off-value obtained by plasma independent of microcopy was higher (0.17) than that obtained by both thick and thin smear microscopy. These values suggest a cut-off of between 0.1 and 0.2 for field study plasma. The red blood cell cut-off optical density was also lower when thick smear was used compared to thin smear (0.39and 0.43 respectively). The cut -off value obtained by red blood cell independent of microscopy was lower (0.32) than that obtained by both thick and thin smear microscopy. These suggest a cut-off of between 0.3 and 0.45 for field study red blood cells pLDH.

In clinical studies, the cut off optical density for plasma was higher when thick smear was used compared to thin smear (0.32 and 0.25 respectively). The cut off value obtained by plasma independent of microscopy was lower (0.22) than that obtained by both thick and thin smear microscopy, suggesting a cut-off of between 0.2 and 0.35 for clinical study plasma pLDH. The red blood cell cut- off optical density was also lower when thick smear was used compared to thin smear (0.41 and 0.45 respectively). The cut off value obtained by red blood cell independent of microscopy was lower (0.33) than that obtained

by both thick and thin smear microscopy, suggesting a cut-off of between 0.3 and 0.45 for clinical study red blood cell pLDH.

In field trials the highest sensitivity was obtained for plasma by using thin smear microscopy (89.8%) while the highest specificity was obtained by using thick smear microscopy (45.01%). The red blood cell (RBC) highest sensitivity was reached when the cut off was determined independently and compared to thin smear (78.95%) while the highest specificity was achieved by using the thin smear cut-off of (OD= 0.43; specificity= 66.67%).

In clinical studies the highest sensitivity was obtained for plasma by using thin smear microscopy (75.57%) and independent plasma cut off with respect to thin smear (75.21%) while the highest specificity for plasma was obtained using thick smear microscopy (71.43%). The red blood cell (RBC) highest sensitivity was obtained when the cut-off was determined independently and compared to thin smear (78.38%) while the highest specificity was achieved by using the thin smear cut off (OD=0.45;specificity= 80.0%) as summarised in Table 5.

Table 5:Optimum OD cut-off for plasma and red blood cell pLDH by using thick smear (A), thin smear (B) microscopy, independent red blood cell (RBC) and plasma values in field (school subjects) and clinical (hospital ambulatory patients) trials.

p(LDH)	Method	OD Cut-Off		Sensitivity		Specificity	
		Field	Clinical	Field	Clinical	Field	Clinical
PLASMA	A	0.10	0.32	73.68	61.74	45.01	71.43
	B	0.15	0.25	89.00	75.57	35.2	30.77
	plasma A	0.17	0.22	58.90	61.54	43.9	46.15
	plasma B	0.17	0.22	86.30	75.21	36.8	26.09
RBC	A	0.39	0.41	60.34	62.94	64.29	69.23
	B	0.43	0.45	77.17	76.97	66.67	80
	RBC (A)	0.32	0.33	57.89	60.00	42.47	39.4
	RBC (B)	0.32	0.33	78.95	78.38	26.03	28.57

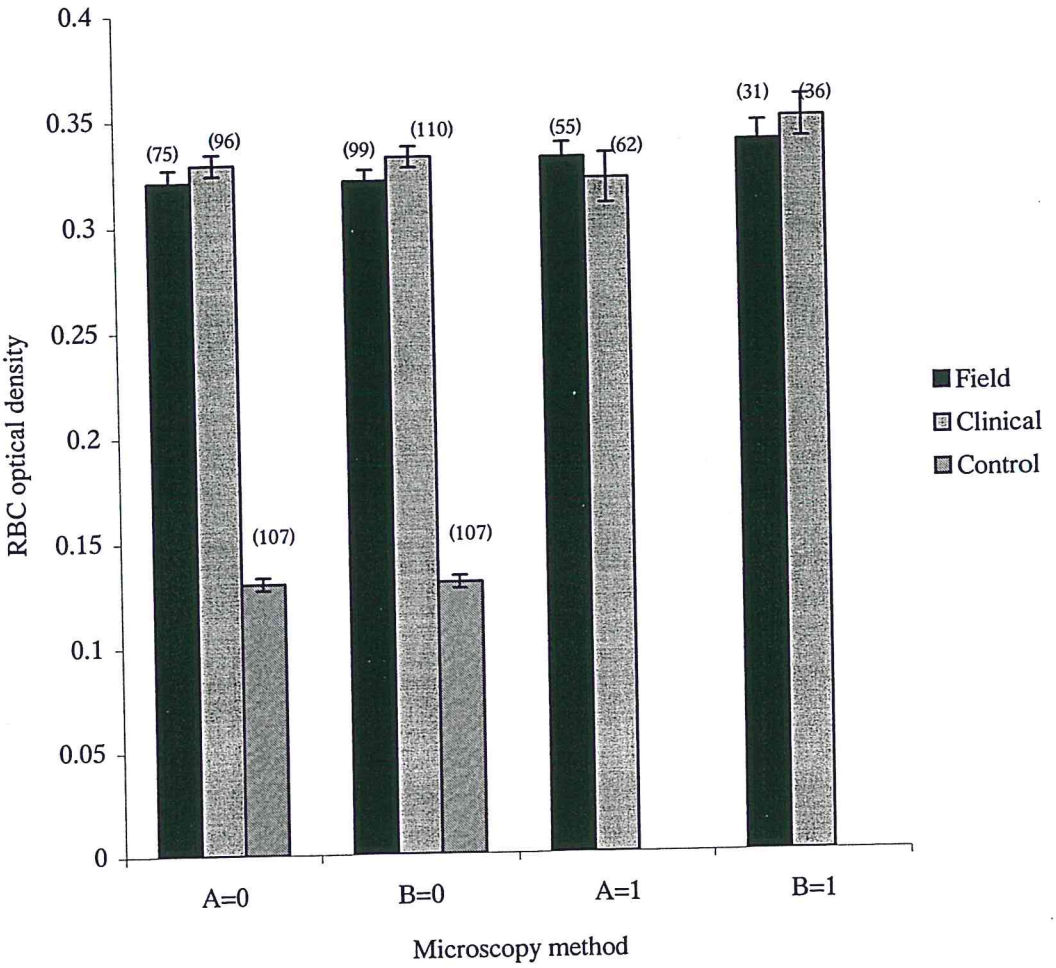
In both field and clinical studies, after venipuncture, thick and thin smears were made then plasma and red blood cells were separated immediately in eppendorf tubes with citrate dextrose anticoagulant. The samples were run for pLDH optical density The pLDH optical density was read and extrapolated by the reduced form of APAD at 650nm using a spectrophotometer (UV_{max} kinetic Microplate reader; Molecular Devices Co., Menlo Park, C.A). Cut-off O.D values were determined using thick and thin smear microscopy and the t- statistic value using ANOVA. Sensitivity and specificity values were then determined using the three cut-off methods.

Both field and clinical RBC sensitivity and specificity values obtained in this study were similar using the various cut-offs method. The plasma optical density cut-offs in the clinical subjects were higher than the field samples. The maximum sensitivity and specificity for red blood cell (RBC) field and clinical samples were obtained using the cut-off value obtained with thin smear microscopy (B) { 77.17; 66.67 and 76.97; 80%; respectively}. The maximum sensitivity for plasma in field and clinical samples was obtained using thin smear (B) {89 and 75.57 %, respectively} but the specificity values were very low (below 50%) in all the cut-off methods used, except in clinical trials which gave 71.43% specificity when thick smear microscopy was used.

4.1.12 Mean pLDH red blood cell (RBC) and plasma values when subjects are thick smear microscopy negative (A=0) and thin smear microscopy negative (B=0) , in field, clinical and control trials.

The mean pLDH values obtained for the various trials indicated that the values for red blood cell were not significantly different between the field and clinical studies, but different in control studies, when thick smear negative samples were used. For plasma studies, the values were significantly different in all the studies for thick smear negative samples. When thin smear negative samples were used, both RBC and plasma cut-off values for field and clinical samples were similar, but different in the control group, (Figure 11).

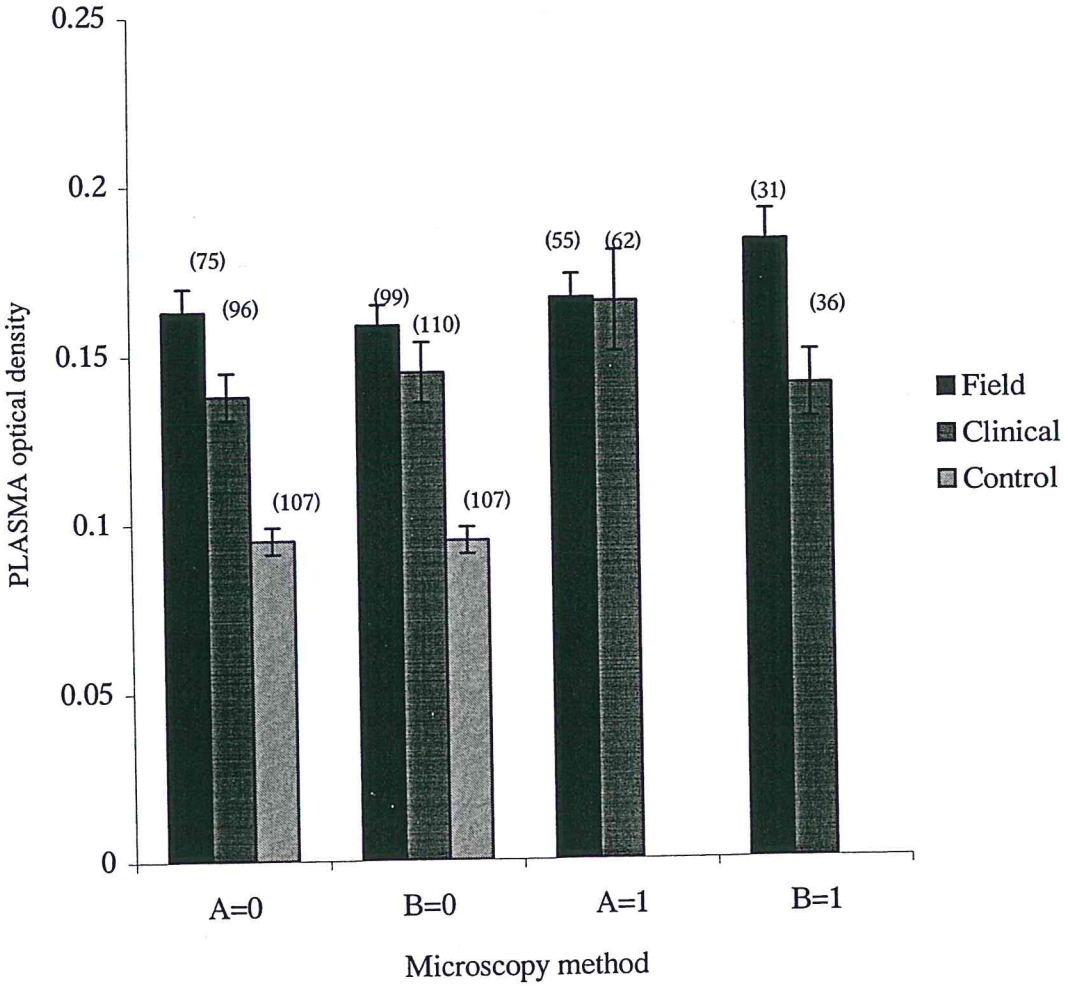
FIGURE 11: Mean parasite LDH values for red blood cells (RBC) when subjects are thick and thin smear microscopy negative (A or B=0) and positive (A or B=1), in field, clinical, and control trials.



In the groups A=0, B=0, the red blood cell cut-off for field and clinical trials were similar but different from the control group; In A=1 and B=1, the two trials for field and clinical trials indicated significantly the same red blood cell cut-off values. All control subjects were microscopy negative.

The mean RBC and plasma using thick smear positive subjects, were similar for field and clinical trials. The values were not significantly different between field and clinical trials for the thin smear positive subjects, but there was a significant difference in the plasma values between the two trials. The control subjects were all negative by thick and thin smear hence were not considered in this comparison (Figure 12).

FIGURE 12: pLDH mean for plasma when subjects are thick and thin smear microscopy negative (A or B=0) and positive (A or B=1), in field, clinical, and control trials.

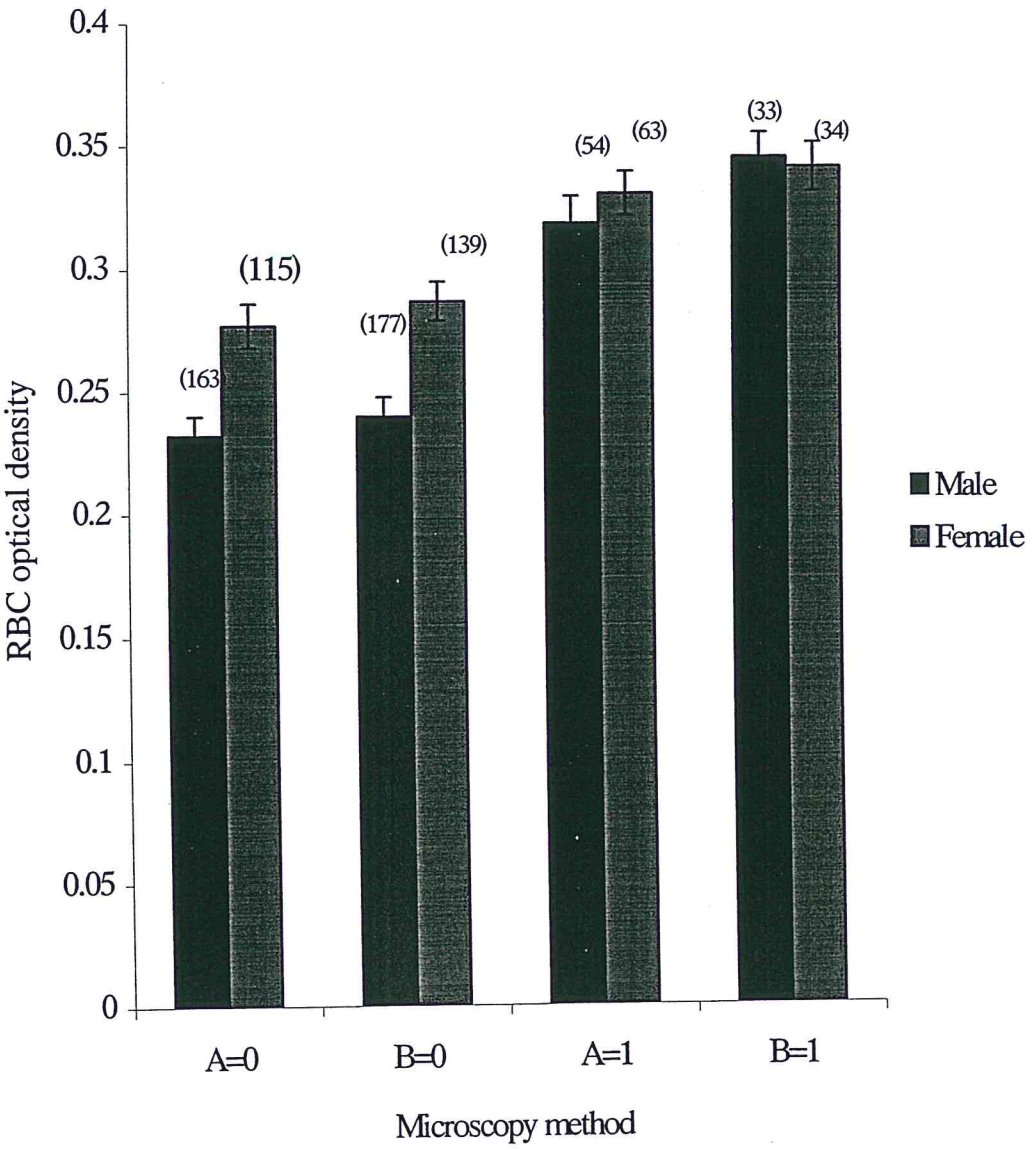


In the groups A=0, all the plasma cut-offs were significantly different, in B=0 field and clinical cut-off were significantly similar but both were significantly different from the control group. In A=1, the plasma levels in the two trial groups were significantly different. All control subjects were negative by microscopy. The numbers in bracket are the sample sizes.

4.1.13 Effect of sex on red blood cell and plasma pLDH

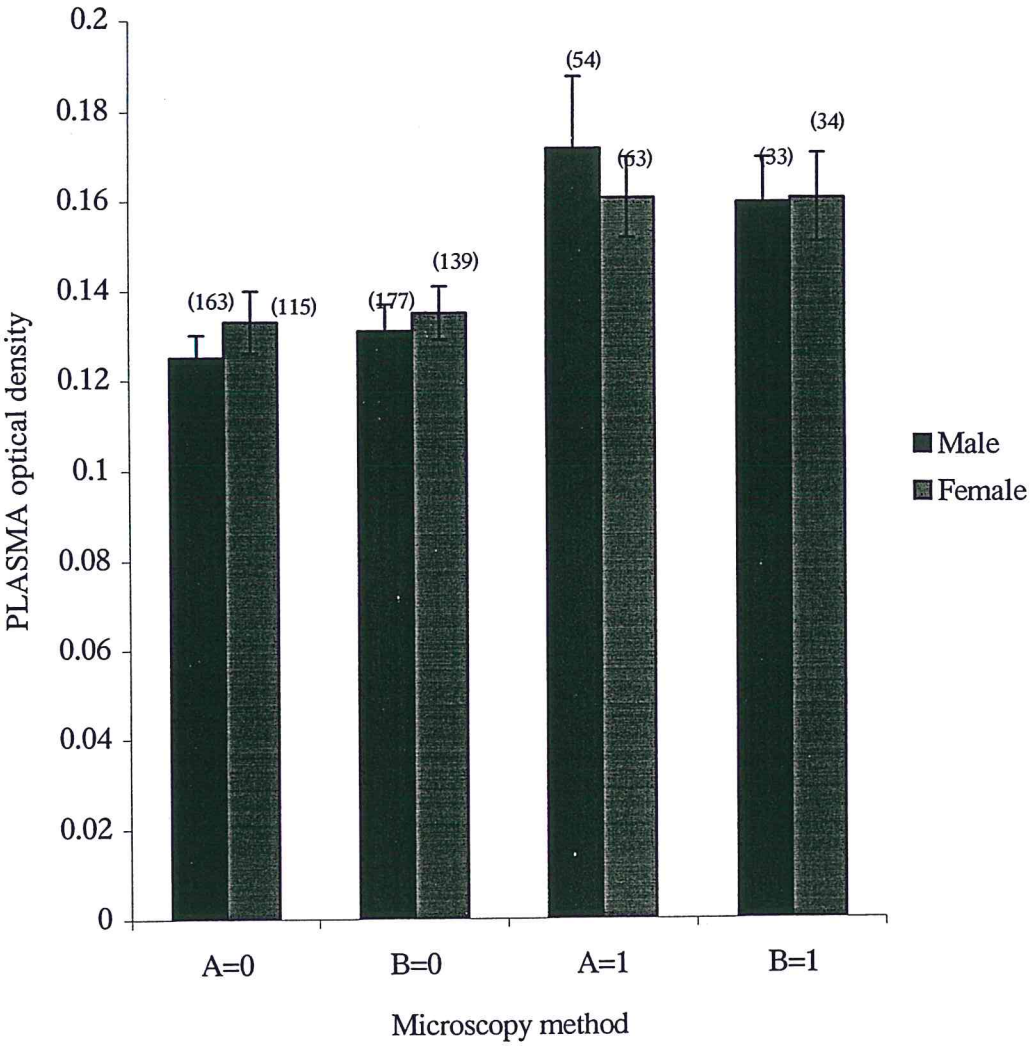
The interaction between sex and all the parameters tested (treatments) was not significant ($p>0.05$) in all the four groupings (A=0,B=0,A=1,B=1) of the subjects. Therefore, the sexes were compared in each grouping for all trials combined (Figure 13). Females had significantly higher RBC optical density pLDH values when microscopy negative, but were not significantly different from males when microscopy positive. Both males and females had similar mean plasma O.D in all the four groupings when microscopy negative and positive (Figure 14).

FIGURE 13: Effect of sex on mean pLDH red blood cell (RBC) for microscopy thick and thin smear negative (A or B=0) or positive (A or B=1) in all trials combined.



When the subjects were separated by sex, the red blood cell pLDH values were significantly higher in thick (A=0) and thin (B=0) smear microscopy negative female subjects than males, while the positive subjects (A=1;B=1) had significantly similar RBC pLDH values.

FIGURE 14: Effect of sex on mean plasma pLDH for microscopy thick and thin smear negative (A or B=0) or positive (A or B=1) in all trials combined.



When subjects were separated by sex, the plasma levels were similar in microscopy negative and positive male and female subjects. Numbers in bracket are the sample sizes.

4.1.14 Effect of age for all groupings: microscopy results of subjects in both clinical and field trials.

No significant correlation was found between age and pLDH RBC or plasma O.D (Table 6). A plot of pLDH values versus age showed no definite relationship pattern. This suggests that pLDH is independent of age whether study subjects is microscopy positive or negative. The results obtained in this study can therefore be generalized for all ages of subjects.

pLDH in field and clinical trial groups.

4.1.15 Relationship between clinical presentation and pLDH RBC and plasma levels in symptomatic ambulatory patients in a malaria endemic zone.

The clinical symptoms of the ambulatory patients were recorded on the first day of attendance of the outpatient clinic at the Kisumu District Hospital. The clinical signs were then coded as follows:

1- Headache
2- Headache + weakness or aches (pains)
3- Headache + weakness or aches (pains) + fever
4- Headache + weakness or aches (pains) + fever + vomiting or nausea
5- Headache + weakness or aches (pains) +fever + vomiting or nausea + diarrhea
6- Headache + weakness or aches (pains) + fever + vomiting or nausea + diarrhea + restlessness or convulsions
0- Other non-specified symptoms- questioning celebral malaria, like violent, destroying property

For analysis, group 0 was removed and group 6 was combined with group 5 because the subjects in these groups were very few. Analysis of varience (ANOVA) was used to compare the mean RBC and plasma O.D pLDH values for the different clinical signs

groups. RBC pLDH was not significantly different between the clinical symptoms, whereas significant differences were obtained between the symptoms for plasma (Table 7). This suggests that the symptoms in patients may be related to plasma pLDH level but not RBC pLDH level.

Table 6. Correlation coefficiets between age and RBC or plasma pLDH levels for field and clinical trials.

Trials	Microscopy	# Observed	pLDH RBC	p(LDH) plasma
Clinical	A=0	55	0.167 ns	-0.164 ns
	A=1	33	0.215 ns	-0.0262 ns
	B=0	62	0.162 ns	-0.118 ns
	B=1	14	0.231 ns	-0.14 ns
Field	A=0	75	-0.112 ns	0.0284 ns
	A=1	55	0.220 ns	0.062 ns
	B=0	99	-0.066 ns	0.034 ns
	B=1	31	0.265 ns	0.166 ns

ns = correlation not significantly different from zero at p=0.05.

In both field and clinical cases, age effect was determined in different categories of individuals: the asymptomatic, non- parasitaemic (A=0; B=0 field); the asymptomatic parasitaemic (A=1, B=1 field); the symptomatic non parasitaemic (A=0; B=0 clinical) and the symptomatic parasitaemic (A=1, B=1 clinical) subjects. In all the cases, age had no effect on pLDH levels.

Table 7.Mean (± S.E) pLDH RBC and plasma levels in symptomatic ambulatory patients displaying different clinical signs, in a malaria endemic zone.

Clinical signs	Number Observed	Mean (RBC)	Mean (plasma)
1	13	0.345 ± 0.019	.156 ± 0.02
2	43	0.328 ± 0.007	0.123 ±.0.009
3	62	0.330 ± 0.008	0.137 ± 0.009
4	24	0.302 ± 0.022	0.187 ± 0.033
5	14	0.317 ± 0.025	0.207 ± 0.034

LSD, p(0.05) for plasma=0.055

LSD, p(0.05) for RBC=0.042

1- Headache

2- Headache + weakness or aches (pains)

3- Headache + weakness or aches (pains) + fever

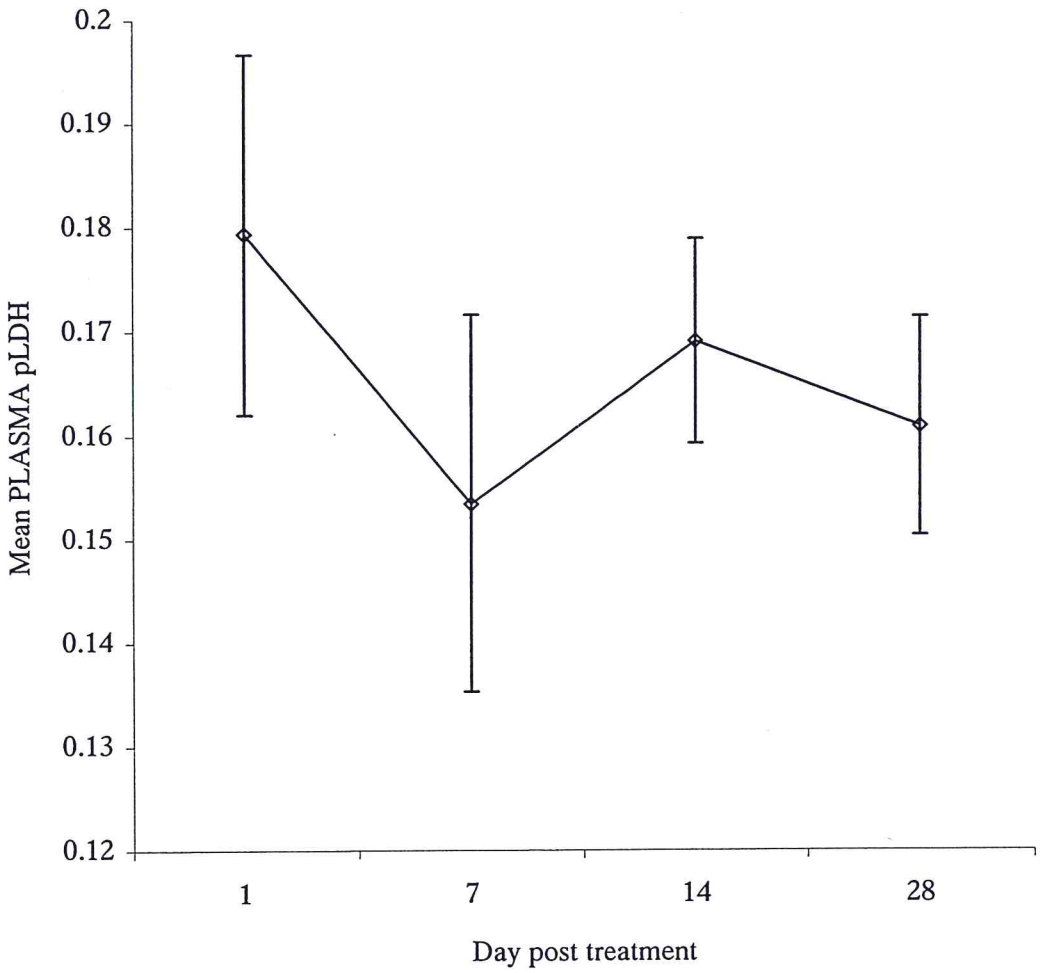
4- Headache + weakness or aches (pains) + fever + vomiting or nausea

5- Headache + weakness or aches (pains) +fever + vomiting or nausea + diarrhea
The clinical signs were correlated with mean plasma and red blood cell optical density values.

4.1.16 Progression of plasma pLDH levels post treatment using Fansidar ®single dose in malaria endemic clinical trial (Retrospective study).

Patients were monitored while within the malaria endemic zone on days 1,7,14 and 28 after antimalarial dosage of Fansidar®. ANOVA was used to compare the plasma levels in patients on the different days after treatment. There was no significant difference in mean plasma pLDH levels between the days (Figure 15).

Figure 15: Progression of plasma pLDH levels post treatment using Fansidar® single- dose in malaria endemic clinical trial (Retrospective study).



The study subjects were volunteers who were on antimalarial (Fansidar®) drug studies. The plasma were removed on the first day of treatment (day 0) before administration of the drug. The patients were followed on days 7, 14, 28. The samples were kept in storage at –80oc and tested 24 months later for pLDH plasma levels. The results indicated no significant difference in plasma pLDH levels during the follow-up.

4. 2. 0 Determination of sensitivity and specificity of immunochromatographic (ICT) Parasight- F and parasite lactate dehydrogenase pLDH optimal dipstick using light microscopy as gold standard.

4.2.1 Sensitivity and specificity of immunochromatographic (ICT) Parasight- F and parasite lactate dehydrogenase pLDH optimal dipstick, using light microscopy as gold standard.

The pLDH dipstick gave the highest specificity but the lowest sensitivity (Table 8). In all the tests, the two species, *P.ovale* and *P. malariae,* were detected as positive only as mixed infections with *P. falciparum,* except in one case where the infection was picked as positive pure infection by pLDH but detected as negative by ICT and Parasight -F. In that case the sample was indicated by pLDH dipstick as *Plasmodium vivax.* Using *Plasmodium falciparum* positive samples only (pRBC-f), the positive and negative predictive values obtained were, 0.712, 0.755 for ICT; 0.336, 0.97 for pLDH dipstick, and 0438, 0.943 for Parasight-F respectively. The sensitivity and specificity values were calculated using the formular in section 3.8 and the results obtained in Table 8. The sensitivity and specificity values obtained using the other non-*falciparum* malaria species were detected as mixed infections with *P. falciparum.*

Table 8.Sensitivity and specificity of immunochromatographic (ICT), Parasight- F and parasite lactate dehydrogenase pLDH optiMAL dipstick using light microscopy as gold standard.

Test	Species	Sensitivity	Specificity
Parasight-F	*P. ovale*	40.00	76.80
	P. falciparum	43.70	93.20
	P.malariae	57.10	77.60
	pRBC-f	**43.84**	**94.30**
ICT	*P.ovale*	60.00	53.20
	P. falciparum	71.10	74.07
	P.malariae	71.43	54.10
	pRBC-f	**71.23**	**75.47**
p(LDH)	*P. ovale*	40.00	83.20
	P. falciparum	33.80	96.91
	P. malariae	50.00	84.14
	pRBC-f	**33.56**	**97.48**

The field survey was carried out in Ndori, village within the malaria endemic zone. The three assays were done independent of microscopic results and for more accurate parasite quantification both white and red blood cell counts were done in order to calculate total number of parasites per mm^3 of blood, pRBC-f means pure *P. falciparum* infections. The three assays were tested according to the manufacturers instruction (section 1.5.4).

4.2.2 Parasite count of infected blood on the determination of total parasite cut-off as an improvement on sensitivity and specificity (Ndori study).

Table 9 shows the mean parasite counts in thin microscopy positive or negative blood, using different test methods. When microscopy negative, the mean parasite counts range between 23 and 322, but not significantly different, for the different test methods. When positive, the mean counts were significantly lower for ICT than the other two methods. Different parasite count cut-offs were used to determine the sensitivity and specificity of the methods, using thin microscopy as the standard (Table 10). Sensitivity generally decreased with increase in cut-off value, while specificity increased. At a cut-off value of zero parasites, both sensitivity and specificity were optimum for *P. falciparum*. A cut-off value of 100 will be required to obtain sensitivity and specificity of about 60% for *P.malariae* and *P. falciparum* mixed infections while for *P.ovale* mixed infections no definite cut-off was achieved (Table 10).

Table 9.Mean (± S.E) parasite count (number of parasites per mm^3) of thin microscopy positive or negative infested blood for Parasight- F, ICT and pLDH.

Microscopy	New test	n	mean *P. falciparum* count per mm^3 of blood
Negative	Parasight- F	232	105.39 ± 68.40
	pLDH	252	322.13 ± 186.30
LSD=376	ICT	162	23.23 ± 5.11
Positive	Parasight- F	73	2519.65 ± 756.40
	pLDH	53	2400.15 ± 659.70
LSD=1714	ICT	143	1430.92 ± 411.20

(LSD, P=.05).

The study subjects were individuals from Ndori with pure *Plasmodium falciparum* infections. I.C.T picked relatively lower mean parasite count for positive slides than pLDH optiMAL and parasight-F. The same was true for negative slides though this did not indicate significant differences.

Table 10. Sensitivity and specificity of parasite counts (per mm3 of infested blood) at different cut-off values using thin smear as standard.

Parasite cut off (numbers)	Parasite species	Sensitivity	Specificity
0	*P. malariae*	100.0	54.5
0	*P. ovale*	60.0	52.2
0	***P. falciparum***	100.0	97.5
10	*P. malariae*	100.0	54.5
10	*P. ovale*	60.0	52.2
10	***P. falciparum***	100.0	97.5
100	*P. malariae*	64.3	72.4
100	*P. ovale*	40.0	71.1
100	***P. falciparum***	60.6	98.2
1000	*P. malariae*	28.6	93.5
1000	*P. ovale*	20.0	92.6
1000	***P. falciparum***	15.5	99.4
10,000	*P. malariae*	7.1	98.3
10,000	*P. ovale*	20.0	98.3
10,000	***P. falciparum***	4.2	100

In Ndori study, total parasite count was carried out for mixed and pure infections as a measure to improve on the sensitivity and specificity of the three assays. The best sensitivity (100%) and specificity (97.5%) were achieved with *P. falciparum* non-mixed infections when parasite numbers were >10 per mm^3 of infested blood.

4.3 Relationship between the diagnostic parameters by means of pLDH and tritiated hypoxanthine uptake assays.

4.3.1 Relationship between RBC pLDH, ^{3}H-Hypoxanthine assay and, thick and thin smear microscopy.

Correlation analysis was used to determine if there was a significant linear relationship between the various diagnostic assays for four isolates (singly and combined) {Tables 11to 15}. Correlation between the diagnostic tools were highly significant for each isolate and all isolates combined. Linear regression analysis was therefore used to relate pairs of the diagnostic tools to enable the prediction of one given another (Table 16,17,18,19 and 20). All the regression slopes were positive and highly significantly different from zero. The coefficients of determination (R^2), which indicate the predictive powers of the regression models, were generally highest for isolate KS140 (98.6%; Table 18) and lowest for the combined isolates (92.3%; Table 20) for the paired optical density (OD) and hypoxanthine assays. In all the four isolates, the pLDH optical density (OD) and hypoxanthine gave the highest coefficient of determination (predictive power), while the three test method combinations, OD versus thick, thin versus thick, and hypoxanthine versus thick indicated the lowest predictive power in the four isolates tested individually and when the results were combined (Tables, 16,17,18,19,and 20). These results suggest that pLDH, thin smear microscopy, and the hypoxanthine assays could be applied for drug sensitivity studies but pLDH stands out the best being non-radiolabelled, faster, easy

to handle and with minimal training for the users. Linear regression analysis also indicated that any of the diagnostic tests paired with thick smear microscopy generally indicated lower values, suggesting that thick smear is not comparable to the other diagnostic and drug sensitivity assays, probably due to its sensitivity. The other isolates, KS021 (Table 16), KS041 (Table17), and KS608 (Table 19), gave consistently high readings for the pair OD and hypoxanthine (95.2, 95.1, and 98.3%, respectively). The results indicated a strong correlation between the four test assays and the four field isolates tested individually and in combination, suggesting a possibility of applicability under field conditions.

Table 11: Correlation between diagnostic tools using field isolate KS021

Diagnostic tool.	^{3}-H Hypoxnthine	Thin-smear microscopy	Thick-smear microscopy
Optical density	0.975 ***	0.967 ***	0.786 ***
3H-Hypoxanthine		0.932 ***	0.708 **
Thin- microscopy			0.825 **

** significant at 1% level
*** significant at 0.1% level

The isolate was culture adapted for three weeks, then serially diluted (starting parasitaemia, 3.04%) in triplicate in 96-well microtiter plates. The plate was divided into three sections: the first three wells (A.B.C.) for hypoxanthine, second (D.E.F.) for pLDH and the last two wells (G and H) for microscopy.

Table 12: Correlation between diagnostic tools using field isolate KS041

Isolate KS 041	^{3}-H Hypoxnthine	Thin-smear microscopy	Thick-smear microscopy
Optical density	0.975 ***	0.908 ***	0.736 **
3H-Hypoxanthine		0.947 ***	0.835 ***
Thin- microscopy			0.917 ***

** significant at 1% level
*** significant at 0.1% level

The isolate was culture adapted for three weeks, then serially diluted (starting parasitaemia, 3.92%) in triplicate in 96-well microtiter plates. The plate was divided into three sections; the first three wells (A.B.C.) for hypoxanthine, second (D.E.F.) for pLDH and the last two wells (G and H) for microscopy.

Table 13: Correlation between diagnostic tools using field isolate KS140

Isolate KS 140	^{3}-H Hypoxnthine	Thin-smear microscopy	Thick-smear microscopy
Optical density	0.993 ***	0.975 ***	0.915 ***
3H-Hypoxanthine		0.990 ***	0.912 ***
Thin- microscopy			0.906 ***

** significant at 1% level
*** significant at 0.1% level

The isolate was culture adapted for three weeks, then serially diluted (starting parasitaemia, 10.24%) in triplicate in 96-well microtiter plates. The plate was divided into three sections: the first three wells (A.B.C.) for hypoxanthine, second (D.E.F.) for pLDH and the last two wells (G and H) for microscopy.

Table 14: Correlation between diagnostic tools using field isolate KS608

Isolate KS 608	^{3}-H Hypoxnthine	Thin-smear microscopy	Thick-smear microscopy
Optical density	0.991 ***	0.915 ***	0.759 **
3H-Hypoxanthine		0.878 ***	0.699 *
Thin- microscopy			0.816 **

* significant 10% level
** significant at 1% level
*** significant at 0.1% level

The isolate was culture adapted for three weeks, then serially diluted (starting parasitaemia, 5.16%) in triplicate in 96-well microtiter plates. The plate was divided into three sections; the first three wells (A.B.C.) for hypoxanthine, second (D.E.F.) for pLDH and the last two wells (G and H) for microscopy.

Table 15: Correlation between diagnostic tools using field isolates KS021, KS041, KS140, KS608, all combined.

Isolates combined	^{3}-H Hypoxnthine	Thin-smear microscopy	Thick-smear microscopy
Optical density	0.961 ***	0.897 ***	0.811 ***
3H-Hypoxanthine		0.966 ***	0.819 ***
Thin- microscopy			0.815 ***

* significant 10% level
** significant at 1% level
*** significant at 0.1% level

The isolates were culture adapted for three weeks, then serially diluted in triplicate in 96-well microtiter plates. The plates were divided into three sections for each isolate; the first three wells (A.B.C.) for hypoxanthine, second (D.E.F.) for pLDH and the last two wells (G and H) for microscopy.

Table 16: Linear regression (Y= a + bX) of one diagnostic tool on another using field isolate KS021 (n=12). Figures in parentheses are the standard errors of the corresponding parameters.

Y	X	a	b	R^2
OD	thin	0.219 (0.005)	0.083 (0.007) ***	93.6
Thick	thin	0.856 (0.212)	1.41 (0.310) **	68.0
^{3}H-Hypoxan.	thin	-322.17 (360.2)	4240 (522) ***	86.9
OD	^{3}H-Hypoxan	0.228 (0.004)	0.00002 (0.000001) ***	95.2
Thick	^{3}H-Hypoxan	1.07 (0.240).	0.0003 (0.00008) ***	50.2
Thin	^{3}H-Hypoxan	0.126 (0.072)	0.0002 (0.00003)***	86.9
OD	thick	0.198 (0.017)	0.04 (0.01) ***	61.8
Thin	thick	-0.266 (0.182)	0.48 (0.1) **	68.0
^{3}H-Hypoxan.	thick	-1210 (1033)	1880 (592) **	50.2
thick	OD	-2.55 (1.02)	15.8 (3.9) **	61.8
thin	OD	-2.46 (0.244)	11.3 (0.94) ***	93.6
^{3}H-Hypoxan.	OD	-11743 (967)	51934 (3704) ***	95.2

** means significant at 1% level
*** means significant at 0.1% level

Table 17: Linear regression (Y= a + bX) of one diagnostic tool on another using field isolate KS041 (n=12).

Y	X	a	b	R^2
OD	thin	0.201 (0.02)	0.08 (0.01) ***	82.5
thick	thin	1.05 (0.16)	0.66 (0.09) ***	84.1
^{3}H-Hypoxan.	thin	-520.76 (597)	3141 (337) ***	89.7
OD	^{3}H-Hypoxan	0.212 (0.01)	0.00003 (0.000002) ***	95.1
thick	^{3}H-Hypoxan	1.26 (0.2).	0.0002 (0.00004) ***	69.8
thin	^{3}H-Hypoxan	0.278 (0.17)	0.0003 (0.00003)***	89.7
OD	thick	0.131 (0.05)	0.09 (0.03) ***	54.2
thin	thick	-1.15 (0.366)	1.28 (0.18) ***	84.1
^{3}H-Hypoxan.	thick	-3847 (1676)	3878 (807) **	69.8
thick	OD	0.07 (56)	6 (1.8) **	54.2
thin	OD	-1.87 (0.48)	10.4 (1.5) ***	82.5
^{3}H-Hypoxan.	OD	-7690 (846)	37000 (2645) ***	95.1

Figures in parentheses are the standard errors of the corresponding parameters.

** significant at 1% level
***significant at 0.1% level

Table 18: Linear regression (Y= a + bX) of one diagnostic tool on another using field isolate KS140 (n=12).

Y	X	a	b	R^2
OD	thin	0.238 (0.01)	0.04 (0.003) ***	95.1
Thick	thin	1.06 (0.24)	0.37 (0.05) ***	82.1
^{3}H-Hypoxan.	thin	452 (446)	2327 (102) ***	98.1
OD	^{3}H-Hypoxan	0.231 (0.006)	0.00002 (0.0000006)***	98.6
Thick	^{3}H-Hypoxan	0.991 (0.23).	0.0002 (0.00002) ***	83.2
Thin	^{3}H-Hypoxan	-0.14 (0.19)	0.0004 (0.00002)***	98.1
OD	thick	0.17 (0.03)	0.08 (0.01) ***	83.7
Thin	thick	-1.9 (0.82)	2.24 (0.33) ***	82.1
^{3}H-Hypoxan.	thick	-4129 (1860)	5299 (753) ***	83.2
Thick	OD	-1.37 (0.5)	10.3 (1.4) ***	83.7
Thin	OD	-6.3 (0.69)	27 (1.9) ***	95.1
^{3}H-Hypoxan.	OD	-14809 (863)	64632 (2432) ***	98.6

Figures in parentheses are the standard errors of the corresponding parameters.

*** significant at 0.1% level

Table 19: Linear regression (Y= a + bX) of one diagnostic tool on another using field isolate KS608 (n=12).

Y	X	a	b	R^2
OD	Thin	0.219 (0.01)	0.05 (0.007) ***	83.7
thick	Thin	1.06 (0.18)	0.59 (0.13) **	66.6
^{3}H-Hypoxan.	Thin	108 (516)	2224 (383) ***	77.2
OD	^{3}H-Hypoxan	0.219 (0.003)	0.00002 (0.0000008) ***	98.3
thick	^{3}H-Hypoxan	1.14 (0.22).	0.0002 (0.00007) *	48.9
thin	^{3}H-Hypoxan	0.13 (0.2)	0.0004 (0.00006)***	77.2
OD	Thick	0.17 (0.03)	0.05 (0.02) **	57.7
thin	Thick	-0.94 (0.43)	1.12 (0.25) **	66.6
^{3}H-Hypoxan.	Thick	-1882 (1344)	2435 (787) *	48.9
thick	OD	-1.22 (.76)	10.7 (2.9) **	57.7
thin	OD	-3.8 (.64)	17.7 (2.5) ***	83.7
^{3}H-Hypoxan.	OD	-10575 (527)	48490 (2018) ***	98.3

Figures in parentheses are the standard errors of the corresponding parameters.

* significant at 10% level
** significant at 1% level
*** significant at 0.1% level

Table 20: Linear regression (Y= a + bX) of one diagnostic tool result on another four. Isolates KS021, KS041, KS140, KS608 combined (n=48) from the study area which were culture adapted in the laboratory. Figures in parentheses are the standard errors of the corresponding parameters.

Y	X	a	b	R^2
OD	Thin	0.234 (0.01)	0.04 (0.003) ***	80.5
thick	Thin	1.21 (0.11)	0.41 (0.04) **	66.4
^{3}H-Hypoxan.	Thin	254 (238)	2428 (96) ***	93.3
OD	3H-Hypoxan	0.228 (0.005)	0.00002 (0.0000007)***	92.3
thick	^{3}H-Hypoxan	1.18 (0.11).	0.0002 (0.00002) ***	67.0
thin	^{3}H-Hypoxan	-0.01 (0.1)	0.0004 (0.00002)***	93.3
OD	Thick	0.16 (0.02)	0.07 (0.008) ***	65.8
thin	Thick	-1.55 (0.35)	1.64 (0.17) ***	66.4
^{3}H-Hypoxan.	Thick	-3783 (866)	4139 (428) ***	67.0
thick	OD	-.81 (0.29)	8.9 (0.94) ***	65.8
thin	OD	-4.4 (0.43)	19.7 (1.4) ***	80.5
^{3}H-Hypoxan.	OD	-11833 (681)	53135 (2257) ***	92.3

** significant at 1% level
*** significant at 0.1% level

4.4. *In vitro* drug sensitivity profiles for both used and candidate antimalarial drugs.

4.4.1 Inhibitory concentrations (ICs) for two field isolates (KS021 and KS140) using ^{3}H- hypoxanthine assay

The drug concentration and the mean radioactivity count per minute (cpm) values were used for calculating the IC50s using the modified method of Sixsmith *et al;* (1984).

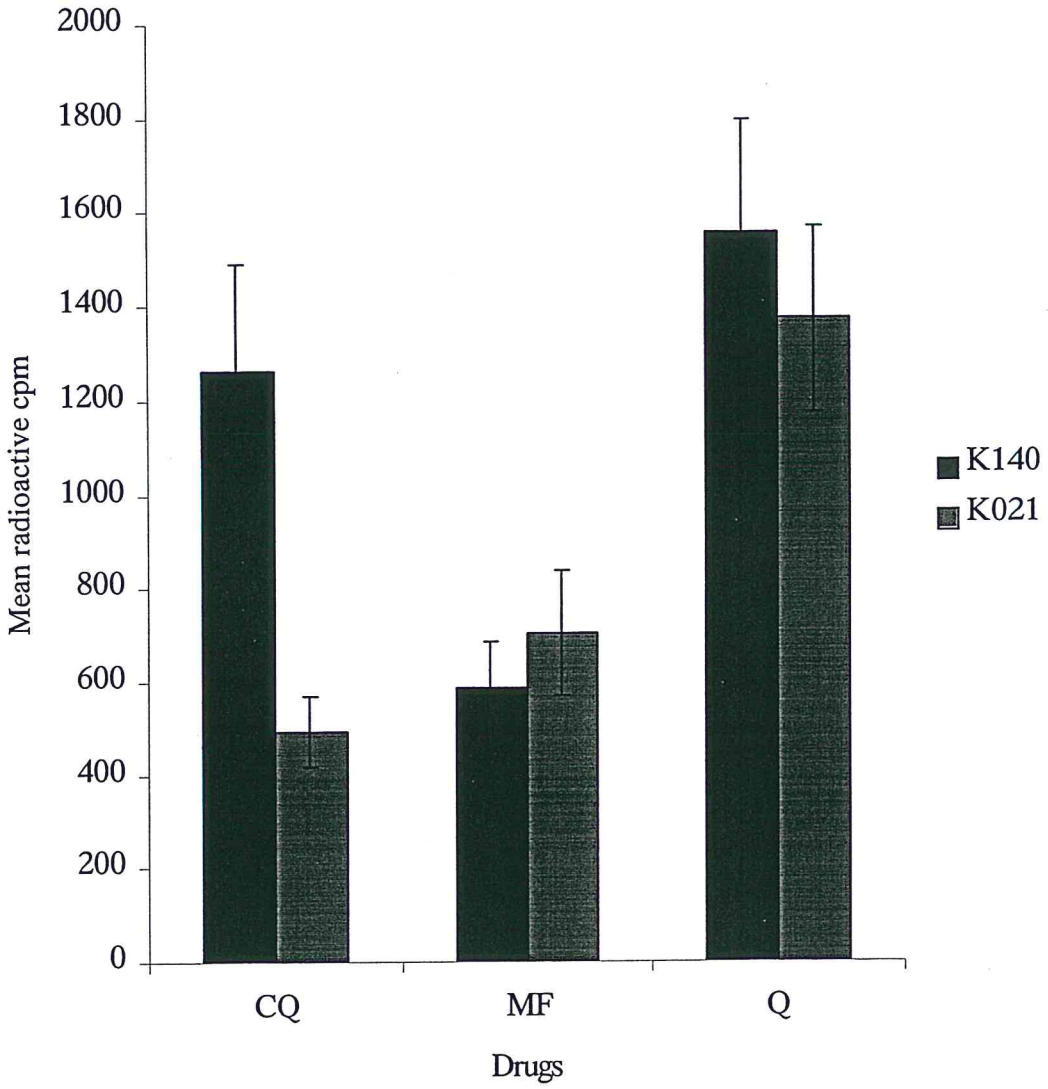
The two field isolates were culture adapted to parasitaemias above 3% and then tested for hypoxanthine uptake using three drugs chloroquine, mefloquine, and quinine. Using probit analysis method, 50% inhibitory concentration (IC50), 75% inhibitory concentration (IC75), and 95% inhibitory concentration (IC95) were calculated (Table 21). Student- t statistic test was used to compare the mean radioactive cpm between the two isolates, for each drug (figure 16). For chloroquine, mean cpm was significantly higher in isolate KS140. The mean cpm were not significantly different between the isolates for the other two drugs (figure 16). Figure 16 is a representation of over 33 replicate results indicating mean cpm counts for the two isolates from the same study area, varying but having the same sensitivity pattern. The IC, 50, 75 and 95 values for the two isolates, using the three drugs were not significantly different when the fiducial lower and upper limits were considered (Table 21).

Table 21: Inhibitory concentration of 3H- tritiated hypoxanthine (ng/ml) for two culture adapted malaria parasites (KS021 and KS140) and three drugs, using probit analysis method.

Isolate	Drug	IC50	IC75	IC95
KS021	Chloroquine	6.66	35.89	405.20
KS140	Chloroquine	5.22	14.40	62.35
KS021	Mefloquine	4.71	22.90	222.90
KS140	Mefloquine	15.90	98.50	1353.00
KS021	Quinine	54.73	182.19	1028.00
KS140	Quinine	38.00	131.50	785.00

The two isolates were culture adapted for three weeks and test by 3H-hypoxanthine assay (48hr.) in 96 well drug plates set at KEMRI. The CPM counts were then subjected to probit analysis for the IC values for the three drugs.

FIGURE 16: Mean radioactive cpm for two field isolates (KS021 and KS140) in three different drugs using ^{3}H- hypoxanthine assay .



A correlation analysis was carried out using two isolates and the three drugs. The results indicated that the two isolates had different mean cpm counts for chloroquine, though the ic 50 values, were the same in both isolates. For mefloquine and quinine, the two isolates indicated no significant difference in mean radioactivity counts per minute (cpm). The number of observations was the same in both isolates (n=33).

4.4.2. Inhibitory concentrations (ICs) for two field isolates (KS030 and KS012) using 3H- tritiated hypoxanthine assay.

The two field isolates were culture adapted to parasitaemias above 3% and then tested for hypoxanthine uptake using three drugs chloroquine, mefloquine, and halofantrine. Using probit analysis method, 50% inhibitory concentration (IC50), 75% inhibitory concentration (IC75), and 95% inhibitory concentration (IC95) were calculated (Table 22). The IC, 50, 75 and 95 values for the two isolates, using two drugs, halofantrine and mefloquine, were not significantly different when the fiducial lower and upper limits were considered, that is both were sensitive to the two drugs, except at IC 75 for mefloquine, when the two isolates, KS012 and KS030 had different lower and upper fudicial limits (7.7-10.6 and 13.5-34.0 respectively), meaning that, at IC value of 75, the two isolates indicated different values, KS012 indicating a lower reading (mefloquine sensitive) than KS030 (mefloquine resistant). For chloroquine, the two isolates KS012 and KS030 had significantly different IC 50 and IC75 values (lower and upper fudicial limits: 17.7-26.3 and 0.13-6.4 for IC50s; 39.2-59.4 and 1.6-13.5 respetively). This indicates that the two isolates, KS 30 is chloroquine sensitive, while KS012 is resistant at IC 50 and 75 but at IC95 both are resistant to chloroquine. This suggests that the two isolates from the same study area responded differently to the three antimalarial drugs hence had different sensitivity patterns. These results indicate that the reported IC value is dependent on the level of inhibition tested for each drug and isolate tested. In Table 22, the reults indicate that isolate KS012 is chloroquine resistant but mefloquine sensitive while KS030 is chloroquine sensitive but mefloquine resistant. The reference strain used

here (Table 22) also indicates chloroquine sensitive and mefloquine resistance. The field isolates already indicate signs of resistance to chloroquine and mefloquine, but no resistance to halofantrine using the hypoxanthine assay.

Table 22: Inhibitory concentration of 3H- tritiated hypoxanthine (ng/ml) for two culture adapted malaria parasites (KS012 and KS030), the reference strain D6, and three drugs, using probit analysis method.

Isolate	Drug	IC50	IC75	IC95
D6	Chloroquine	7.55	12.25	24.58
KS012	Chloroquine	21.80	47.48	145.47
KS030	Chloroquine	2.84	7.70	32.37
D6	Halofantrine	0.94	1.42	2.58
KS012	Halofantrine	0.75	1.48	3.94
KS030	Halofantrine	1.33	3.24	11.75
D6	Mefloquine	14.02	19.51	31.39
KS012	Mefloquine	6.11	8.87	15.19
KS030	Mefloquine	13.53	18.34	28.40

The three isolates were culture adapted for three weeks and tested by 3H-hypoxanthane assay (48hrs.) in 96 well drug plates set. The CPM counts were then subjected to probit analysis for the IC values for the three drugs. The results of hypoxinthine calculation of the inhibitory concentration could be compared to the to the reference isolate included in the test (D6) which has known chloroquine sensitive and mefloquine resistant properties.

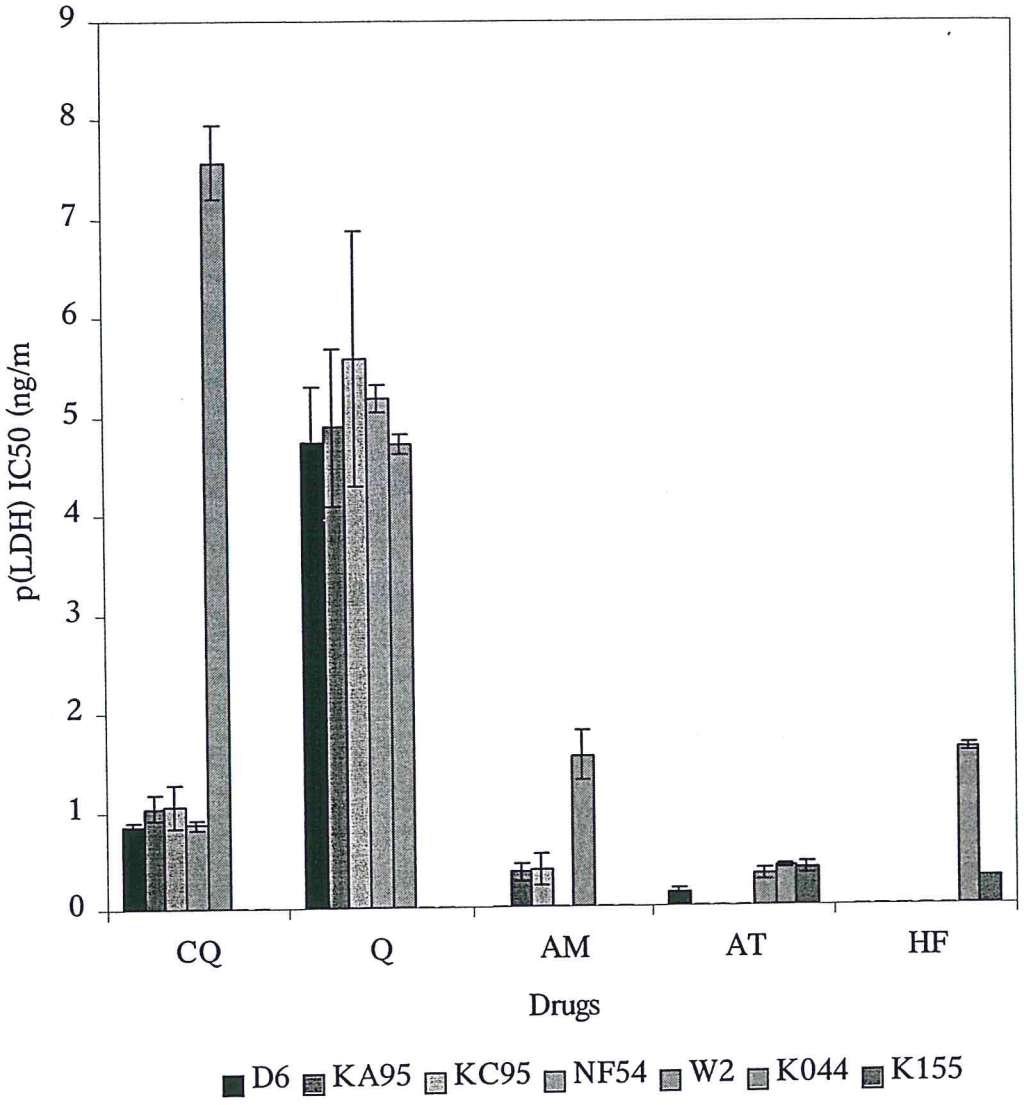
4.4.3 IC50 (in ng/ml) pLDH values for three antimalarial drugs chloroquine (CQ), amodiquine(AM)and quinine(Q),using three laboratory (D6, W2, NF54) and two field (KA95, KC95) adapted malaria parasites on WHO standard plates.

Figure 17 shows the mean pLDH IC 50s for the different isolates.

The two reference strains D6 and W2 indicated different mean IC 50 values for chloroquine, D6 being sensitive and W2 being resistant. These were used as markers to indicate sensitivity or resistance. The field isolates, KA95, KC 95 and the laboratory isolate, NF54 were all chloroquine sensitive by pLDH. The five isolates tested for quinine (D6, KA95, KC95, NF54 and W2) were all sensitive. For amodiaquine, W2 was resistant while KA95 and KC 95 were sensitive. No resistance was reported for atovaquone and halofantrine for the isolates tested (W2, D6, KS155 and KS 044).

The other drug tested, Fansidar (F), failed to give significant reproducible results hence was excluded from the report.

Figure 17: Mean pLDH IC50s for the different isolates.



The drugs were tested using drug plates freshly prepared at KEMRI laboratories, Kenya.

4.4.4 IC50 (ng/ml) pLDH values for six antimalarial drugs.

The laboratory isolates, W2 and D6 are resistant and susceptible standards respectively, against which the field isolates were compared. Tables 23, 24, 25, 26, and 27 show the IC50s of these isolates for the different drugs. For chloroquine (Table 23), only isolate KS044 was significantly higher than the resistant standard (W2), suggesting that this isolate is highly resistant. Isolates KS063 and KS155 were also resistant. Other isolates were not significantly different from the susceptible D6. For mefloquine (Table 24), all the isolates tested were sensitive to mefloquine. Though there was difference in the mean pLDH IC50 between D6 and W2 values, this was not significant (p=.885). For quinine (Table 25), the eleven isolates tested indicated three categories of response to quinine. Isolates, KS012, KS193, KS031, and D6 were the first group: most sensitive in decreasing order; the isolates, W2, KS157, KS168, KS068, KS211, KS047 and KS044 were in group two category: nearly sensitive though not significantly different from the first group; and the third group, KS155, had the highest reading though not significantly different from the second group. All the isolates tested were sensitive to quinine though at different levels of IC50 values, indicating that all the isolates tested were quinine sensitive hence there was no significant variation in response to the drug among the isolates (p=0.22; Table 25). The two reference strains, D6 and W2 have been reported as quinine sensitive thus the tested field isolates were not significantly different from the reference strains. For dehydroartemisinin (Table 26), isolate KS211 had the

highest mean IC50 ($1.38_{\pm}$ 0.99), though this was not significantly different from KS193 ($1.26_{\pm}$ 0.5) and KS044 ($0.56_{\pm}$ 0.29). The other isolates, including the reference strains, D6 and W2 were highly sensitive to very low doses of dehydroartemisinin (lower than $0.01_{\pm}0.19$ ng/ml; Table 26). For atovaquone (Table 27), only two field isolates and the reference strains could be replicated. The three isolates, KS044, KS155, and W2 were sensitive to atovaquone, though D6 gave the lowest sensitivity level ($0.137_{\pm}0.037$) with this drug.

Table 23: Mean ± S.E. IC50 (ng/ml) pLDH values for chloroquine (CQ) for different field isolates and two reference isolates.

Isolate	# observations	Mean IC50 (± S.E.)
D6	19	6.33 ± 0.76
K044	4	67.82 ± 50.90
K063	2	36.65 ± 9.95
K155	8	34.21 ± 6.40
K001	2	23.40 ± 7.80
K168	6	3.32 ± 0.80
K157	8	14.96 ± 7.11
K012	4	14.37 ± 4.40
K211	5	7.93 ± 2.74
K031	6	7.83 ± 1.74
K193	5	4.74 ± 1.13
K047	6	4.08 ± 0.84
W2	15	16.57 ± 2.28

LSD p(0.05)=29,02

Stock solution of Chloroquine was prepared in sterile distilled water. Stock solution of Quinine, Mefloquine, Dehydroartemisinin, and Halofantrine were prepared in methanol. Two-fold serial dilutions of the drugs were distributed in the 96 well plates in triplicate. 10 wells contained diluted drug, one well (No. 11) had no drug but had parasitised RBCs and the last well (No. 12) had no drug, no parasitised RBCs, but had uninfected RBCs as control.

Table 24: Mean ± S.E. IC50 (ng/ml) pLDH values for mefloquine (MF) for different field isolates and two reference isolates.

Isolate	# observations	Mean IC50 (ng/ml)
D6	19	14.08 ± 5.54
K044	6	9.95 ± 3.44
K063	2	17.88 ± 10.12
K155	5	7.41 ± 1.06
K001	2	1.90 ± 0.60
K168	6	5.44 ± 2.22
K157	3	3.92 ± 2.46
K012	3	18.07 ± 2.77
K211	5	15.09 ± 5.70
K031	6	9.38 ± 2.32
K193	3	7.48 ± 2.09
K047	5	8.02 ± 4.44
W2	23	9.34 ± 1.76

LSD p(0.05)=19.14

Stock solution of Chloroquine was prepared in sterile distilled water. Stock solution of Quinine, Mefloquine, Dehydroartemisinin, and Halofantrine were prepared in methanol. Two-fold serial dilutions of the drugs were distributed in the 96 well plates in triplicate. 10 wells contained diluted drug, one well (No. 11) had no drug but had parasitised RBCs and the last well (No. 12) had no drug, no parasitised RBCs, but had uninfected RBCs as control.

Table 25: Mean ± S.E. IC50 (ng/ml) pLDH values for quinine (Q) for different field isolates and two reference isolates.

Isolate	# observations	Mean IC50 (ng/ml)
D6	16	41.33 ± 6.10
K155	6	87.57 ± 15.65
K044	6	58.45 ± 18.70
K047	5	53.36 ± 27.00
K211	6	52.55 ± 5.40
K063	2	47.30 ± 10.70
K168	7	46.82 ± 16.60
K157	3	44.70 ± 7.40
K031	6	35.68 ± 14.00
K193	4	24.80 ± 9.70
K012	5	22.26 ± 5.00
W2	19	45.10 ± 8.40

LSD p(0.05)=43.39

Stock solution of Chloroquine was prepared in sterile distilled water. Stock solution of Quinine, Mefloquine, Dehydroartemisinin, and Halofantrine were prepared in methanol. Two-fold serial dilutions of the drugs were distributed in the 96 well plates in triplicate. 10 wells contained diluted drug, one well (No. 11) had no drug but had parasitised RBCs and the last well (No. 12) had no drug, no parasitised RBCs, but had uninfected RBCs as control.

Table 26: Mean ± S.E. IC50 (ng/ml) pLDH values for dehydroartemisinin (DH) for different field isolates and two reference isolates.

Isolate	# observations	Mean IC50
D6	23	0.48 ± 0.089
K211	3	1.38 ± 0.99
K193	3	1.26 ± 0.50
K044	10	0.56b ± 0.29
K047	5	0.56 ± 0.17
K168	5	0.54 ± 0.29
K012	9	0.50 ± 0.032
K031	6	0.33 ± 0.042
K157	8	0.22 ± 0.05
K155	5	0.20 ± 0.095
K063	2	0.19 ± 0.01
W2	20	0.54 ± 0.12

LSD p(0.05)=0.69

Stock solution of Chloroquine was prepared in sterile distilled water. Stock solution of Quinine, Mefloquine, Dehydroartemisinin, and Halofantrine were prepared in methanol. Two-fold serial dilutions of the drugs were distributed in the 96 well plates in triplicate. 10 wells contained diluted drug, one well (No. 11) had no drug but had parasitised RBCs and the last well (No. 12) had no drug, no parasitsed RBCs, but had uninfected RBCs as control.

Table 27: Mean ± S.E. IC50 (ng/ml) pLDH values for atovaquone (AT) for two field isolates and two reference isolates.

Isolate	# observations	Mean IC 50
D6	7	0.137 ± 0.037
K044	3	0.413 ± 0.020
K155	3	0.390 ± 0.065
W2	3	0.330 ± 0.059

LSD p(0.05)=0.154

Table 28a and b show the IC50 values of each drug for all the field isolates combined. Quinine had the highest overall IC50, which was significantly different from all others. Chloroquine was next highest, but not significantly different from mefloquine. All the other drugs tested had very low over-all IC50 values which were not significantly different from one another (Table 28a). Table 28b shows the combined IC50 values for all the field isolates tested. The different drugs were also compared for each of the reference strains, D6 and W2 (Tables 29 and 30). The results were similar and follow the same pattern as of the reference strain W2 (Table 30). Quinine gave a significantly higher IC50 value from the other drugs. Both chloroquine and Mefloquine were similar. Mefloquine was significantly different from the other two drugs (dehydroartemisinin and atovaquone) for D6 (Table 29) but not for W2 (Table 30).

Table 28a: Summary of measurements of mean IC50 (in ng/ml) pLDH values for six antimalarial drugs using field adapted malaria parasites (all field isolates combined including reference strains D6 and W2).

Drugs	# Observations	Mean IC50 ± S.E.
Chloroquine	102	16.66 ± 2.87
Mefloquine	96	10.55 ± 1.34
Quinine	91	44.60 ± 3.66
Dehydroartemisinin	103	0.50 ± 0.06
Atovaquone	20	0.28 ± 0.03
Halofantrine	8	1.47 ± 0.44

LSD p(0.05)=11.84

Table 28b: Summary of measurements of mean IC50 (in ng/ml) pLDH values for six antimalarial drugs using field adapted malaria parasites (all field isolates combined excluding reference strains D6 and W2).

Drugs	# Observations	Mean IC50 ± S.E.
Chloroquine	68	19.56 ± 4.17
Mefloquine	54	9.83b ± 1.19
Quinine	56	45.37 ± 4.95
Dehydroartemisinin	60	0.49 ± 0.08
Atovaquone	10	0.36 ± 0.03
Halofantrine	8	1.47 ± 0.44

LSD p(0.05)=15.52

Table 29: Summary of measurements of mean IC50 (in ng/ml) pLDH values for six antimalarial drugs using reference strain D6 (chloroquine sensitive, mefloquine resistant).

Drugs	# Observations	Mean IC50
Chloroquine	19	6.33 ± 0.75
Mefloquine	19	14.08 ± 5.50
Quinine	16	41.33 ± 6.10
Dehydroart.	23	0.48 ± 0.09
Atovaquone	7	0.14 ± 0.04

LSD p (0.05)=11.84

Table 30: Summary of measurements of mean IC50 (in ng/ml) pLDH values for six antimalarial drugs using reference strain W2 (chloroquine resistant, mefloquine sensitive).

Drugs	# observations	Mean IC50
Chloroquine	15	16.57 ± 2.27
Mefloquine	23	9.34 ± 1.76
Quinine	19	45.1 ± 8.4
Dehydroartemisinin	20	0.54 ± 0.12
Atovaquone	3	0.33 ± 0.06

LSD p (0.05)=17.62

4.4.5 Drugs which failed to give reproducible results by pLDH.

Four drugs, doxycycline, azithromycin, pyrimethamine, and sulfadoxine failed to give reproducible IC50 results using reference strains, they were therefore not tested further for pLDH drug sensitivity IC50 because of lack of standardization using D6 (chloroquine sensitive, mefloquine resistant strain) and W2 (chloroquine resistant, mefloquine sensitive strain). In most experiments the drugs failed (F) even to produce a standard curve by the log-logit method using softmax software that directly extrapolated the data by end point analysis at 650nm using ELISA reader (Molecular Devices Thermomax ®).

Some of the results that were observed are reported in Table 31 below. The four drugs were observed to be slow acting hence the incubation period was extended to 66 hours but still no reproducible results were obtained (Table 31).

Table 31: IC50s of drugs which failed to give reproducible results by pLDH.

Reference strain	Doxycycline (1000ng/ml)	Azithromycin (10,000 ng/ml)	Pyrimethamine (250ng/ml)	Sulfadoxine (10,000 ng/ml)
D6	F	10000	0.0137	6090
	8300	2900	F	F
	391	2970	267.00	F
	1690	1700	30.40	F
	1560	546	9400.00	F
	F	786	3.820	F
	2190	391	0.840	F
	2490	1800	34.500	F
W2	4490	3300	249.000	110
	F	2490	2.270	0.00013
	39500	25000	9.180	F
	19200	10800	2.280	19500
	2300	6250	0.083	F
	0.0059	3130	62.500	F
	2360	2070	.0091	F
	F	F	F	F

4.5 Determination of clearance period of parasitaemia and correlation with microscopy parasitaemia and pLDH as an indication of *in vivo* rational use of antimalarial drugs within parts of Kenya.

The Rift Valley follow-up study was designed to determine the clearance period of parasitaemia and correlate this with plasma and RBC pLDH for *in vivo* drug susceptibility studies. After realising the limitation of the colourimetric enzyme pLDH assay in field and clinical studies, it was clear that one would need a more purified form of the assay, like monospecific monoclonal antibody, in order to come up with specific drug susceptibility report. A more purified monoclonal pLDH has been produced (Makler, personal communication). This will be used with the samples (now in storage at -80 ^{0}C) to produce more specific IC50s and also to reveal antimalarial susceptibility or resistance in these patients. The microscopy study revealed a positive correlation between parasitaemia and the day of patients follow-up after treatment (P=0.0011). There was a general trend for the parasitaemia to reduce significantly from day 3 of follow- up irrespective of the drug administered (Table 32).

Table 32: Mean ± S.E. microscopy parasitaemia for different follow-up days after use of antimalarial drugs within parts of Kenya where the patients contracted the malaria infections.

Day after drug was used	Sample size	Mean parasitaemia
1	61	1.21 ± 0.4
2	59	0 .99 ± 0.36
3	50	0.15 ± 0.03
4	47	0.06 ± 0.01
5	39	0.06 ± 0.02
6	29	0.01 ± 0.006
7	21	0.02 ± 0.01

LSD p (0.05)=0.83.

The study subjects were patients attending hospital in Rift Valley, were malaria positive and had travel history to malaria endemic regions of Kenya. The patients were followed for a maximum of seven days after treatment with anti-malarials. Each day blood was taken for plasma, RBC pLDH analysis, thick and thin smears were made, and Giemsa stained for microscopy.

CHAPTER 5

5.0 DISCUSSION

5.1 Use of parasite lactate dehydrogenase pLDH and light microscopy as diagnostic tools for malaria in Western Kenya.

In the culture adapted malaria parasite isolates tested for pLDH in the laboratory, there was a strong correlation between pLDH optical density of red blood cells and microscopy except for Mombasa isolate M24 and the purified gametocytes of the Amsterdam strain, NF54. Sanderson *et al.*, (1981) while carrying out enzyme typing of *Plasmodium falciparum* from African and some other developing countries, reported the isolate M19 as representative of parasites from East Africa. The Mombasa isolate was tested at remarkably higher parasitaemias (above 1%) that pLDH is known to be detectable (2.5-4.5%). The variation is an indication that there may be differences in the isolates of *P. falciparum* from the same region of the world, though they may appear to possess remarkably similar LDH enzyme forms as previously reported (Sanderson *et al.*, 1981). This study, the isolates were set at the same time and only tested on the second day after the appearance of young rings, a stage when the parasites are at high metabolism, hence increased lactate production (when mainly asexual trophozoite and schizont stages were present). Similar studies were reported for stage specific production of metabolism of *Plasmodium falciparum* (Scheibel *et al.*, 1979; Pfaller *et al.*, 1982; Basco *et al.*, 1995) and in *Plasmodium knowlesi* (Shakespeare *et al.*, 1979).

The results of the study of the six isolates suggested that the isolates may vary in their production of pLDH depending on the region of the country it emanates from.

Jenelik *et al,* (1996a) while testing pLDH as a diagnostic test in Uganda, reported very low sensitivity and specificity for the assay, an indication that some factors influence this assay in field application. The variation in parasite isolate pLDH production is one factor that may directly influence the sensitivity and specificity of this assay as observed in this study. The first report of the diagnostic properties of pLDH by using (3-acetyl pyridine NAD) APAD as co-enzyme (Makler and Hinrichs, 1993), was carried out using strains from the American Type culture collection (50005, 50028, 30932, 30992), which could have missed the pLDH isolate variation observed in this field study.

The gametocytes produced a negative correlation with microscopy, an indication that they may not be producing detectable pLDH within the hosts red blood cells and within the parasites themselves. The sexual stage parasites were prepared using standard techniques (Ogwang *et al.*, 1993; Kariuki *et al.*,1998), hence could not have been exfagellated or contaminated before testing.

Having achieved such high correlation between pLDH OD and microscopy in the laboratory except in the Mombasa Isolate and the gametocytes, it was necessary to test some field isolates from the study area. The results suggested that pLDH would be a successful diagnostic tool in this region. The two culture-adapted field isolates also indicated that there was variation in correlation between the field isolates, as was suggested in the laboratory studies, an indication that the measurement of pLDH in field studies for diagnostic purposes might be limited. Even though it was tempting to believe that one could produce a standard curve using the reference strain D6 to use for field diagnosis, the results indicated that this would be faulty, as opposed to what Makler and Hinrichs (1993) proposed in *in vitro* studies using laboratory isolates. Since the

correlations between parasitaemia and pLDH optical density (OD) was high for all the field isolates tested, it was sufficient to test for the sensitivity and specificity of pLDH in the field.

The enzyme assay indicated high sensitivity values (100%) when thin smear microscopy was used as opposed to thick smear (< 60.0%). This was a clear indication of the limitation of the assay to detect low parasitaemias, supporting the findings of Basco *et al*; (1995) that lactate dehydrogenase activity was detectable at a parasitaemia >0.4% and at a haematocrit of 1.5%. In the present study the sensitivities were set at 1% haematocrit, when the background effect of human red blood cells was minimal (Ofulla *et al.*, 1994). In the Uganda study (Jenelik *et al.*, 1996a), sensitivity and specificity for whole blood pLDH was reported as 58.8% and 62.2% using thick film microscopy. Recent experience with the measurement of pLDH activity seems to indicate that the test performs better when plasma is analysed instead of whole blood, and this would improve the sensitivity and specificity of the test (Jenelik *et al.*, 1996a). The present study incorporated skills and equipment necessary for red blood cell and plasma separation to come up with full report about this potential candidate as a field diagnostic assay. Field studies using thick and thin film microscopy, as well as t- statistic test (for thick and thin films) to determine cut-off optical density for red blood cells, gave the optimum sensitivity values as 60.3, 77.2, 57.9, and 79.0% respectively and specificities of 64.29, 66.7, 42.5 and 26 % respectively. For plasma, the sensitivity values were 73.7, 89.0, 60.0, 86.0% respectively and specificities of 45.0, 35.0, 44.0, and 37.0% respectively. The results of this study indicate that plasma pLDH gives higher sensitivities but lower specificities. The results of the sensitivity and specificity studies is an indication that the

low values obtained in field trials could be as a result of low parasitaemias missed by pLDH, or due to the fact that pLDH activity is a reflection of the adherent as well as the freely circulating asexual stage of the *P. falciparum* parasite (Makler and Hinrichs,1993).

The study carried out in Uganda focused on patients reporting in an out-patient hospital. After obtaining the results of the field study (healthy school children residing in a malaria endemic zone), the present study was set to find out the sensitivity and specificity patterns for this assay in clinical subjects. The clinical studies, using thick and thin film microscopy, as well as the t- statistic test (for thick and thin films), to determine cut off optical density for red blood cells, gave the optimum sensitivity values as 62.9, 77, 60, and 78.4% respectively and specificities of 69.0, 80.0, 39.4 and 28.6 % respectively. For plasma, the sensitivity values were 61.7, 75.6, 61.5, and 75.2% respectively and specificities of 71.4, 30.7, 46.2, and 26.1% respectively. In comparison to the Ugandan study for whole blood on clinical subjects (sensitivity of 58.8, specificity of 62.2%), this study indicated sensitivity and specificity of 62.9 and 69% respectively for red blood cells and 61.7 and 71.4% respectively for plasma. From the results, it is clear that separation of plasma from the red blood cells gives higher specificity values but lower or similar sensitivity values in clinical subjects.

The use of the t- statistic test to determine cut-off consistently gave lower specificity (46.2 vs 71.4% for plasma, 39.4 vs 69% for RBC) but similar values for sensitivity (61.5 vs 61.7% for plasma, 60.0 vs 62.9% for RBC) in clinical samples. In field studies, the methods were largely agreeable in specificity (45.0 vs 44.0% for plasma, 64.3 vs 42.5 for RBC) and sensitivity (73.7 vs 89 for plasma, 60.3 vs 57.9 for RBC). Since the method was an independent way of calculating cut-off values without reference

to microscopy for pLDH sensitivity and specificity analysis, this appears to be the best cut-off optical density method for field application. The use of the t- statistic test to determine cut -off sensitivity would remove the technician bias in pLDH studies when microscopy is used as the standard, and hence will give more accurate sensitivity and specificity values in field application.

In this study, the cut-off optical density values achieved when thin and thick smears were used in the two trial groups were the same, except in the control group which consisted of malaria negative volunteers living out of the endemic region (in Nairobi) for consecutive three months prior to the study and not on any antimalarial drugs. The control plasma and RBC pLDH were significantly lower in the control subjects than in the study subjects, a strong indication that malaria attack causes raised levels of pLDH both in red blood cells and plasma (Sanderson *et al.*, 1981; Pfaller *et al.*, 1982; Makler and Hinrichs 1993; Basco *et al.,* 1995). This is the only *in vivo* study trying to correlate red blood cell and plasma pLDH with microscopy using subjects of three different categories: the healthy non-infected individuals staying out of malaria endemic region (controls group 1), the non-parasitaemic healthy individuals in endemic region (field A=0, B=0), the parasitaemic non-symptomatic individuals in endemic region (field A=1, B=1), {both field study group 2}the non-parasitaemic symptomatic individuals in endemic region (clinical A=0, B=0) and the parasitaemic symptomatic individuals in endemic region (clinical A=1, B=1), {both clinical study group 3}.

The interaction between sex and trial groups was not significant, indicating that the sexes compared similarly in all the trial groups. For the non-parasitaemic individuals, the pLDH red blood cell cut-off values for males was lower than that of females, while

plasma level was similar for both sexes when thick and thin film microscopy were used. The reason for non-parasitaemic females having higher red blood cell pLDH was not clear. The most important fact that come out of the sex studies is that when individuals were malaria positive (both thick and thin film microscopy) then both plasma and red blood cell pLDH were similar in both sexes. This is a strong qualifier for pLDH as a field diagnostic tool, the fact that sex has no influence on the assay when individuals are parasitaemic. The effect of age was monitored in clinical and field trial groups and it was clear that age had no significant correlation with the pLDH values, for both parasitaemic and non-parasitaemic individuals. Recent studies on pLDH for malaria diagnosis never indicated the effect of sex or age on pLDH values obtained in the field (Jenelik *et al.*, 1996a). The field studies focused on the school going ages (10 - 15 years), the clinical studies focused on all age groups (from over 1yr to 56 years), while the controls were adults away from the endemic region on employment out, spanned the ages of 23yr - 49yr. This study took into consideration separation of plasma from red blood cells and it was important that both indicated no significant correlation between pLDH and age. Jenelik *et al.*, (1996a) did not prepare plasma which they claimed would require laboratory skills and equipment which usually are not available at peripheral health units, making the test unsuitable for this setting. However, if the field applicability of the assay is to be determined, there is the need to take the pains to come up with full assessments, which is presented in this study. Despite the limitations sited in this study, it is clear that sex is not one factor that limits this assay in field application. Considering that light microscopy also has limitations in diagnosing malaria at primary health care level (Payne, 1998; Patnaik *et al.*, 1994), it is true that some limitations of pLDH could be due to the

fact that it was microscopy that was used as gold standard. This is because pLDH measures not only circulatory parasites but sequestered ones or those in deep tissues as well. The pLDH assay may not be fully applicable in hospitals and clinics but it may be useful for epidemiological or research purposes as the laboratory results, even using field isolates as indicated in the present studies.

In the clinical studies, clinical presentation could not be accurately used to predict malaria infection. In a study carried out by Svenson *et al.*, (1995), it was also reported that no clinical criterion could accurately predict the presence of malaria parasites in all symptomatic patients with a history of travel to a malaria - endemic area. The study subjects were individuals staying in the endemic area with clinical symptoms similar to malaria attack. Even after coding of the symptoms, this study indicates that there was no clear significant differences between the clinical symptoms (signs), especially in relation to red blood cell and plasma pLDH levels. No such study has been reported elsewhere. The near accurate clinical diagnosis for plasma pLDH was group 4 - (headache, weakness or aches/pains, fever, vomiting or nausea), though this was not significantly different from all other groups. This study indicates that the level of pLDH may not be a good indication of severity of malaria infection, except the level of parasitaemia, and that use of clinical signs to predict parasitaemia may be faulty.

The level of plasma pLDH on day0 (first day of treatment), day 7, day 14 and day 28 after treatment were not significantly different in this study, an observation which could not be explained.

5:2 Sensitivity and specificity of immunochromatographic (ICT), Parasight-F and parasite lactate dehydrogenase pLDH optiMAL dipstick by use of light microscopy as gold standard.

Optimal® dipstick assay has been developed by Flow inc.Portland, Oregon, USA. This assay was described as a sensitive and simple method that permits the diagnosis and speciation of the major forms of human malaria, *P. falciparum* and *P vivax*(Quintana, *et al,*1998*)*. The OptiMAL® dip stick assay was also said to permit health personnel to monitor patient therapy. The optimal® assay (Flow Inc 6127 W Corbett, Portland, OR 97201 USA), which was intended for use in research and development, was also tested in the field in malaria endemic region for its potential use in malaria diagnosis in this study, having noticed the limitations of the pLDH enzyme assay as a monitoring system for detecting the presence of pLDH.The optimal assay was compared with the other two assays, immunochromatographic (ICT) and parasight-F, which use basically similar principles but are manufactured from different companies (3/14 Roseberry st. Balgowlah, Australia and Becton Dickinson Company, U.S.). The three new diagnostic techniques were compared in a field set up for sensitivity and specificity by using microscopy as gold standard.

The pLDH optimal dipstick, which is a modification of the enzyme assay, has been reported to be a better form of the assay in that it is a rapid, easy alternative to existing tests, recognizes all major forms of human malaria, differentiates between *P. falciparum* and *P.vivax*, operates in all clinical settings, provides ability to monitor drug therapy and detects drug resistant malaria (Nguyen-Dinh, *et. al*,1994). The outcome of

my field diagnostic studies in endemic region indicated that the OptiMAL® dipstick has low sensitivity (33.6%) and high specificity (97.5%) in *P. falciparum* infections in comparison to ICT (71.2% and 75.5% respectively) and parasight-F (43.8% and 94.3% respectively). The effect of parasitaemia still featured as an important factor influencing sensitivity and specificity of the OptiMAL® assay. The mean parasitaemia for pLDH microscopy negative subjects was the highest of the three assays, though not significantly different from the other two, while for the positive, the mean cut-off was significantly higher than that obtained by ICT (2400 VS 1431 respectively). Among the three tests, it was only pLDH that speciated pure infection of *P.malariae* in the field (though as *P. vivax*), a case that was missed by parasight-F and ICT. It was of interest to note that the single pure infection of *P.malariae* was picked by optiMAL® as a *P.vivax* infection, a case which prompted further examination of the slide under microscope for confirmation as *P. vivax* has not been reported in this region. Further DNA tests are required for confirmation apart from morphological features (Snounou *et al.*,, 1993a). The fact that pLDH picked this infection that was missed by the other two assays, (at high parasitaemia) was an indication that this assay has high potential for field application, a case which limits the other two assays to *falciparum* diagnosis only. The three assays picked the other infections of non *falciparum* type only in mixed infections with *falciparum* malaria. The field study is an indication that pLDH may have a wider field application due to the fact that it recognizes major forms of human malaria. The specificity of the assay is also limited by low parasitaemias, and it attained the maximum value (100%) at 10,000 parasites per mm^3 of infected blood. The sensitivity of the assay was highest (100%) when there were >10 parasites per mm^3 of blood. From this field

study, it is true that specificity of the assay is quite promising for field applicability as opposed to what Jenelik *et al.,* (1996a) reported in the Ugandan study, but its sensitivity needs to be improved as low parasitaemias greatly influence the assay. The sensitivity of the assay is currently being looked into by various researchers by using, polyclonal antibody capture (Wang-Fuyong *et al.,* 1996), monospecific polyclonal antibodies (Kaushal *et al.,* 1995), PCR assays, to improve the detection of lactate dehydrogenase-elevating virus (Logt *et al.*, 1994). This study has strongly incriminated low parasitaemia as a limiting factor in the use of this assay for field diagnosis. In another study (Basco *et al.*, 1995), it was reported that for an optimal performance against fresh clinical malaria isolates, the pLDH enzymatic assay requires an initial parasitaemia between 1 and 2% at a haematocrit of 1.5%. In a separate study, ICT gave high sensitivity (100%) and specificity (96.2%) in field trials. This study with ICT gave sensitivity of 71.2% and specificity of 75.5%, due to its limitation in identifying other plasmodial species and gametocytes. These are some factors that have been found to also influence the performance of pLDH in the field. The fact that pLDH optimal dipstick detected *P.malariae* pure infection in the field as *P.vivax* should be of concern to the developers and manufacturers of the optiMAL® dipstick. The fact that the pLDH dipstick picked this parasite as *P.vivax* is an indication that *P. vivax* may be existing in this part of Western Kenya or that a special sub-species of *P. malariae* may be existing. This needs further specific identification like using PCR analysis (Snounou, *et al.*, 1993b). This is an important observation which needs to be addressed especially in areas where the two species co-exist.

In the field study for optiMAL® assay, it was apparent that the subjects showed

varied white blood cell and red blood cell counts due to frequent malaria attacks and other infections. The total parasite count per mm^3 of blood was therefore calculated using a uniform base (including the people with elevated white cell counts) before calculating the sensitivity and specificity of the assay. In field application, there is a lot of variability in the cell counts, unlike the laboratory situation, hence the need to measure individual counts before the results could be compared. Of interest in the study was the fact that Parasight-F and ICT, though using the same principle, gave different sensitivity and specificity values (43.8%, 94.3% and 71.2%, 75.5% respectively) considering that the two methods use the same principle, except for the difference in the manufacturing companies, the explanation for these differences may lie with the manufacturers. Shiff *et al.,* (1993), using Parasight-F test on patients in a holoendemic malaria area of coastal Tanzania, reported a sensitivity of 88.9% and specificity of 87.5%. The parasight-F, which is an antigen capture test, detecting trophozoite-derived histidine rich protein2, may indicate variation in test results. These field studies, both haematocrit and differential blood counts were carried out. The parasites were counted against 200 leucocytes, and the counting and species identification was confirmed by an independent malariologist. The difference in the Tanzanian study and this study is probably due to the fact that the latter included *P. falciparum* mixed infections in the analysis, a common condition in this study area. This was carried out to indicate the limitation of this assay in field application. This probably explains the low levels of sensitivity reported, but is necessary considering that one requires a simple, rapid test for field applicability. Its use in mixed infections may be of interest in areas where most of these species co-exist. The fact that sexual stage (antigen) parasites were included in the analysis could be one factor

that lowered the sensitivity of the assay, and this is important for field application. Modification of the field diagnostic system to accommodate the diagnostic assay in field trial, like exclusion of other infections, removal of sexual stage antigens, may ultimately give high sensitivity values which may not be achievable under normal field applications. This could explain why previous studies with Parasight-F gave very high sensitivity levels (Parra *et al.*, 1991; Taylor and Voller, 1993), as opposed to the present study.

5.3 In vitro drug sensitivity profiles for both used and candidate antimalarial drugs by means of pLDH and tritiated hypoxanthine uptake assays.

In order to test pLDH for drug susceptibility, the assay was first correlated with microscopy and the 3H-hypoxanthine assay techniques. The results of the laboratory assay indicated that pLDH had a strong positive correlation with hypoxanthine assay and thin smear microscopy, a condition which was encouraging for field application in drug sensitivity studies. Although there was a positive correlation with thick smear microscopy, the values were consistently low. This was a clear indication of the fact that the two drug assays are also influenced by parasite growth and low parasitaemias which may limit their application. This study indicated that there is a strong correlation between pLDH optical density and the 3H-hypoxanthine assay in culture adapted field isolates and for combined isolates. Makler *et al.*, (1993) reported a strong positive correlation ($r=0.976$) between the two assays. pLDH was applied in most of my field studies because it is technically easy to perform, rapid (giving IC50 value within 30 minutes of completion of the incubation phase), can be stored frozen at -20° C until it is convenient to perform the assay, the result reproducible, and is less expensive than the 3H-

hypoxanthine uptake method (Makler *et al.*, 1993). The reference strain (D6) was used as a standard during the hypoxanthine uptake assay. The results of radioactivity count per minute (cpm) was analysed using Probit to calculate levels of inhibition concentration of the drugs. Of the four field isolates that were culture-adapted and tested, and the reference strain (D6), KS021 exhibited chloroquine sensitivity (IC50 =6.66ng/ml) but high resistance (IC95 = 405.2 ng/ml); KS140 exhibited chloroquine sensitivity; KS012 was chloroquine resistant, whereas KS030 was chloroquine sensitive. KS021 exhibited mefloquine sensitivity (IC50 =4.7 ng/ml) but high resistance (IC95 = 222.9 ng/ml); KS140 exhibited mefloquine resistance; KS012 was mefloquine sensitive, whereas KS030 was mefloquine resistant. The drug pattern exhibited by the four isolates: (KS140, chloroquine sensitive, mefloquine resistant; KS030, chloroquine sensitive, mefloquine resistant; KS012, chloroquine resistant, mefloquine sensitive; and KS021, chloroquine sensitive and mefloquine sensitive) has a lot of significance to the malaria drug studies in this area. Evaluating the isolates at various IC values could indicate whether the isolate was going to remain sensitive to the drug at various levels of inhibition, which is of importance when studying drug sensitivity profiles of these isolates, and the future performance of the drug. Such a report as obtained in this study has not been published anywhere, and is an important component of drug sensitivity surveys. This study is relevant especially in areas where drug-resistant *P. falciparum* exists because chemotherapy is different or might need modification (WHO, 1988). By using the reference strain (D6) with known chloroquine sensitivity and mefloquine resistance, the tested field isolates could easily be grouped into categories of chloroquine and mefloquine susceptibility. The results of the study clearly indicate the sensitivity

patterns of the isolates to chloroquine, mefloquine, quinine and halofantrine by use of 3H-hypoxanthine assay. The drug sensitivity assay carried out using pLDH and WHO plates gave consistently low IC50 values compared to 3H-hypoxanthine assay which was done using plates made at KEMRI laboratories. Although the isolates tested could indicate the sensitivity patterns, it was clear that the readings were lower than the ones obtained by 3H-hypoxanthine. Using WHO plates, the isolates D6, KA95, KC95 and NF54 were chloroquine sensitive while W_2 was chloroquine resistant. The isolates KA95 and KC95 were also amodiaquine sensitive but W_2 was amodiaquine resistant. All the isolates, D6, KA95, KC95, NF54 and W_2 were quinine sensitive. Due to low IC50s obtained and lack of drug variety and concentrations, new plates made at KEMRI were also tested for pLDH drug sensitivity studies. The results of the pLDH studies directly correlated with 3H-hypoxanthine assay (D6: $6.33_{\pm}$ 0.76; 7.55 and K012: $14.37_{\pm}$ 4.4; 21.8) for Chloroquine. Since pLDH was faster, cheaper and easily available, it was prefered for further field studies for the remaining drugs using eleven field isolates. From these studies, it was clear that pLDH could be used successfully to predict drug sensitivity profiles of parasites to antimalarial drugs. The 50% inhibitory concentration (IC50) was defined as the concentration at which the pLDH produced by the parasites (positively correlated with the number of viable parasites) was inhibited by 50% as compared with the drug-free control wells. Failure of some of the isolates to give successful pLDH IC50 was probably due to a low sensitivity of the assay making it impossible for the extrapolation of the IC50 using log-logit by Soft Max™ software (Molecular Devices) that directly extrapolates the titration data of the drug IC50 values (Makler *et al.*, 1993; Basco and Bras, 1994). There were also some field parasites which

failed to grow, probably due to an inherent incapacity of certain isolates to adapt to *in vitro* culture conditions (Brockelman *et al.*, 1985). In the isotopic assay (3H-hypoxanthine assay) the parasite growth was indicated by the incorporation of 3H-hypoxanthine in the drug wells for the two field isolates, KS140 and KS021. The significant difference observed in growth rates in the wells for the two isolates did not translate to significant difference in the IC50 values. That is, the difference in the mean cpm counts did not affect the concentration at which 50% inhibition was achieved in both isolates. This puts credence on the use of the ICs as an indication of drug susceptibility. Since some field isolates failed to give IC50 values by pLDH and 3H-hypoxanthine uptake, it was evident that a microscopic evaluation is necessary when setting the tests to count the number of parasites for lower levels of parasitaemia. For field isolates in this study, setting cultures with initial parasitaemia lower than 1% did not give reproducible IC50 values by pLDH as reported in other studies (Basco *et al.*, 1995). The study also cited the major limitation of the enzymatic test as its level of sensitivity to detect malarial LDH. This study was carried out at a haemotocrit of 1%, and a significant LDH activity was not observed at a parasitaemia of below 1%. In the culture adapted field isolates, the initial parasitaemia of which a sigmoid curve could be plotted for LDH activity was <0.4%, the same cut-off reported by Basco and Le Bras (1994) in *in vitro* studies. This study has also confirmed that the presence of gametocytes *in vitro* decrease production of LDH and also diminish the incorporation of tritium-labelled hypoxanthine. The drug sensitivity studies were carried out using haematocrit of 1%, 2% and 3%. By increasing haematocrit, the human LDH background effect was also increased making it difficult for one to visualize the pLDH readings. The results suggest that the enzymatic assay can be

applied for *in vitro* drug screening using culture adapted *P. falciparum* clones (D6, W_2), and also to an extent, by using culture adapted *P. falciparum* field isolates.

5.4 A pLDH summary of drug susceptibility studies using the eleven field culture adapted isolates.

The results of the culture adapted isolates indicated that the parasites are generally Chloroquine resistant, near mefloquine sensitive, quinine sensitive, dehydroartemisinin sensitive, atovaquone sensitive, and halofantrine sensitive. The IC50 values obtained for quinine, mefloquine, halofanthrine, chloroquine, dehydroartemisinin and atovaquone against *P. falaparum* were within susceptible range (Roth *et al.*, 1998). This suggests application of the drug atovaquine, a broad spectrum anti-infective agent with activity against malaria and opportunistic infections in HIV/ AIDS patients (Hudson *et al.*, 1991). Carrying out the same study in other parts of the country like the coastal part and the highlands, is likely to give the drug susceptibility pattern of these parasites, and hence, patient treatment may be made easy by selecting the kind of drug that the patient is likely to respond depending on where the patient travelled to, and therefore is likely to have picked the infection from. No study has reported on drug sensitivity profiles of Kenyan isolates by use of pLDH studies and the six antimalarial drugs. Studies by Ofulla (1994), reported pLDH values obtained from field samples directly tested by pLDH without culture adaptation and without use of reference strains. The present study, by incorporating the reference strains (D6, and W_2) and culture adapting the field isolates, gave a reference point for drug sensitivity studies for the field isolates where the pLDH diagnostic surveys were carried out. Since the adaptation takes an average of 2 to 3

weeks, it is expensive in time and materials to attain large numbers that can be used for the susceptibility studies. However, this is not comparable to the cost involved in patient treatment and administration of wrong drugs in the wake of drug resistance in these parts of the country where the isolates emanated from. By coming up with the susceptibility profile, it would save time and money to prescribe to the patients the right type of drugs during treatment. This would minimize drug pressure and increase in drug resistance to the new and candidate antimalarial drugs like halofantrine, dehydroartemisinin and atovaquone (Price, *et al.*, 1996; WHO, 1994). Rational use of antimalarial drugs is now being advocated especially in the third world countries, where *El nino* related rains have worsened the spread of malaria. The enzyme assay pLDH may influence further knowledge of these parasite enzymes and their genetic basis may aid both the designing of new chemotherapy and understanding the evolution of these parasites (Roth *et al.*, 1998). It could be of use to demonstrate that nearly all pLDH enzymatic activities are increased in infected erythrocytes and that the increase is proportional to the parasitaemia (Roth *et al.*, 1998). The drug sensitivity results are then likely to yield clues for useful chemotherapeutic agents as well as insights into the evolution of plasmodia species. The red blood cells used in malaria cultures were blood group matched and were old erythrocytes (14 - 28 days) hence were free of reticulocytes and had no protein synthetic capability. This rules out the idea that the cells used for culture were also undergoing metabolic activities that could have interfered with parasite lactate dehydrogenase activity. The same was reported by Roth *et al.*, (1998) when they investigated the enzymes of the glycolytic pathway in erythrocytes infected with *Plasmodium falciparum* malaria parasites.

As suggested earlier by Vander Jagt (1981), *P. falaparum* LDH represents a potential target of antimalarials because of its distinctive properties from human LDH. The composition and properties of *Plasmodium falciparum* LDH strongly suggest that this important *Plasmodium* enzyme has evolved to become well adapted to regulated specific metabolic needs of the malaria parasite during glycolysis that occurs during the asexual blood stages (Bzik *et al.*,1993). Since pLDH drug sensitivity study has suggested usefulness in field application, it would be of interest to apply this in areas where there is drug resistance and in places of highland malaria. The laboratory isolates were collected in Kenya from various parts of the country including, Kisumu, Eldoret, Mombasa, Saradidi and one isolate was from Somalia.

5.4.1 Drugs which failed to give reproducible results.

The four antimalarial drugs, doxycycline, azithromycin, pyrimethamine and sulfadoxine failed to give reproducible results by means of pLDH. Modifications of the assay was carried out, such as increasing the incubation period to 66hrs for slow acting drugs (Klotz, Personal communication), but this did not improve the pLDH IC50 results using the reference strains. More planned studies are necessary using these four antimalarial drugs in order to come up with reproducible IC50 values by pLDH method. So far, no work has been published on the IC50 values of these four drugs using pLDH and this requires urgent studies since the two drugs, pyrimethamine and sufadoxine combined (fansidar), have been recommended to replace chloroquine as first line drug for treatment in Kenya. The other drugs, doxycycline and azithromycin, are potential antimalarial drugs which have been tested in Kenya in the field (Kisumu) as potential prophylactic drugs. Being antibiotics, they may go along way in helping the local community in the

control of malaria and other infections that require antibiotic intervention. Further studies on the IC50 values will reveal the potential of these drugs as prophylactic and chemotherapeutic agents.

Clearance period of parasitaemia

The retrospective study results suggest that malaria patients in endemic regions may not indicate varied plasma pLDH levels due to continuous mosquito challenges which may result in reincfection giving rise to plasma pLDH values obtained during the follow-up period, hence an indication that one needs to follow up such study patients outside the endemic region.

From earlier studies, it is known that the enzyme assay is less sensitive when low parasitaemias are tested (Basco, *et al.*,1995). This implies that, to come up with important data on *in vivo* drug susceptibility, which can be correlated with *in vitro* assays, improvement on the pLDH enzyme assay is warranted. More purified monoclonal antibodies will give further evidence of the usefulness of the assay (Wang-Fu Yong *et al.*, 1996; Kaushal *et al.*, 1995; Logt *et al.*, 1994). Basco *et al.*, (1995) reported that, for an optimal performance against fresh clinical malaria isolates, the enzymatic assay requires an initial parasitaemia, between 1 and 2% at haemotocrit of 1.5%, a condition which limits the assay for correlation with *in vivo* drug susceptibility studies. Purification of the enzyme will give better results as has been done with *Plasmodium falciparum* malate dehygenase (Unnasch, 1992). Previous studies have indicated that *Plasmodium falciparum* produces lactate at a rate that exceeds the maximal capacity of the normal red blood cell membrane to transport lactate (Kanaani and Ginsburg, 1991).

From this study it is clear that pLDH holds future for a lot of diagnostic and drug

susceptibility studies. *P. falciparum* LDH has been reported to contain several distinctive single amino acid insertions and deletions, compared to other LDH enzymes. Most remarkably, it contained a novel insertion of 5 amino acids within the conserved mobile loop region near arginine residue 109, a residue which is known to make contact with pyruvate in the ternary complex of other LDH. These were suggestions that novel features of *P. falciparum* LDH primary structure may be correlated with previously characterised and distinctive kinetic, biochemical, immunochemical and electrophoretic properties of *P. falciparum* LDH (Bzik *et al.*, 1993). From the present and previous studies, it appears as if pLDH is destined to provide the diagnosis of Plasmodia and drug susceptibility studies.

CHAPTER 6:

A SUMMARY OF CONCLUSIONS

1. The pLDH assay as a diagnostic tool in malaria

a) pLDH red blood cell optical density had a positive correlation with microscopy in laboratory cultivated asexual malaria parasites, except in the Mombasa isolate M24. The sexual *Plasmodium falciparum* gametocytes however, did not show the same linear relationship, suggesting that *P. falciparum* gametocytes probably do not produce stage specific pLDH.

b) There was variation in correlation between microscopy and pLDH among field culture adapted malaria parasite isolates.

c) Sensitivity and specificity of pLDH assay varied, with the method used to calculate the cut-off. The pLDH assay had a stronger correlation with thin smear than thick smear microscopy, an indication that its sensitivity was lowered by its inability to pick low parasitaemias. This explains in part why pLDH indicated high sensitivity and specificity values with thin smear.

d) The cut-off pLDH plasma values in clinical and field subjects, from the t-statistic test, were 0.22 and 0.17 respectively, whereas those of red blood cell were 0.33 and 0.32, respectively for Kenyans living in the malaria endemic region.

e) In comparing thick and thin smear, the t- statistic test provided the best tool for calculating sensitivity and specificity in both plasma and red blood cell pLDH in field and clinical studies. This method was independent of observer subjectivity encountered in thick and thin smear microscopy readings.

f) In the malaria endemic region of western Kenya where the study was carried out, the aparasitaemic females had a significantly higher mean red blood cell pLDH than the aparasitaemic males whereas the plasma pLDH levels were similar in both sexes. In parasitaemic subjects, both sexes had similar plasma and red blood cell pLDH levels, underscoring the possible applicability of pLDH in field studies.

g) There was no significant correlation between age and mean pLDH values in all samples irrespective of sex and clinical status.

h) There was no significant correlation between signs/clinical presentations of malaria as a disease and pLDH values obtained.

i) In the field retrospective study, there was no correlation between plasma pLDH and the recovery from malaria attack after one curative dose of fansidar was administered. No explanation could be found for this observation.

2. The modified pLDH optiMAL® dipstick in field diagnosis

a) In field diagnosis studies, the optiMAL® dipstick was a better diagnostic tool than ICT and parasight-F because it picked single infection of *P.malariae* which was missed out by the other two assays. It also had the highest specificity for plasmodia, but low sensitivity which required further studies for improvement. The fact that *P. malariae* was picked as *P. vivax* is of concern especially in regions where the two species co-exist and in cases of *P. vivax* drug resistance.

b) b) Among the 306 volunteers 122 (40%) had malaria parasite detectable on

blood films.

3. PLDH for determining parasitaemia and drug IC50 values

a) pLDH enzyme assay was highly correlated with the 3H-hypoxanthine assay in determining parasitaemia and drug IC 50 values. Since the LDH activity is correlated with parasite growth, the inhibition of the LDH production by an increasing concentration of an antimalarial drug can be used as a measure to determine drug susceptibility patterns of malaria parasites. The advantages of the *in vitro* test based on enzymatic activity include its specificity in detecting malaria parasites.

b) The comparable results obtained by microscopic, enzymatic and isotopic tests, the rapidity and ease of performance, the use of relatively inexpensive equipment compared with a liquid scintillation counter isotopic test are some of the major features of the enzymatic test that render it practical for field use.

4. Drug sensitivity patterns of field isolates

a) From this study, the sensitivity pattern of the isolates studied from the field were: chloroquine resistant, mostly mefloquine resistant, quinine sensitive, dehydroartemisinin sensitive, atovaquone sensitive, and halofantrine sensitive.

b) The sensitivity patterns of the field isolates tested using reference strains, D6 and W2 and WHO (1994) IC50 cut-offs, were: chloroquine resistant and near mefloquine resistant.

c) Based on published WHO (1994) IC 50 cut-offs for the other study drugs, they were: quinine sensitive, dehydroartemisinin sensitive, atovaquone sensitive and halofantrine sensitive

5. Correlation between the *in vitro* and the *in vivo* drug response is important in any malaria control programme. Using a more purified and sensitive form of pLDH for drug studies to indicate *in vivo* rational use of antimalarial drugs within parts of Kenya where the patients contracted the infections, will give a comprehensive and conclusive report on the use of pLDH for drug susceptibility studies. Since the samples are already in safe storage, they will be analysed once the monoclonal antibodies for pLDH are available to give more conclusive evidence of antimalarial drug sensitivity in western Kenya.

CHAPTER 7:

SUGGESTIONS FOR FUTURE WORK

7.1 The study indicates that if pLDH sensitivity is improved, it has the potential of being a better diagnostic tool than the other new diagnostic techniques that are coming up like ICT and Parasight-F. It would be of interest to carry out the same study in other parts of the country, especially in coastal region and in regions of highland malaria. Documentation of other factors that may affect use of pLDH {Sherman, 1961; 1991}, such as the seasonality of malaria sporozoites (Shililu *et al.*, 1996; 1998) may indicate different pLDH levels.

7.2 Having come up with the method of calculating cut-off optical density for plasma and red blood cells infected with *Plasmodium falciparum* (using the t- statistic test), it would be valuable to try the same in another malaria endemic region. This would give a cut-off base that can be used to come up with a standard cut-off for each locality, or if the same; this can be used to evaluate the sensitivity and specificity of pLDH for field studies in various parts of the country.

7.3 pLDH seems to hold the key for future field drug susceptibility assays. If this study can be carried out in representative regions of Kenya where malaria exist, one could come up with generic ICs of the drug in use and other candidate antimalarials. This would assist clinicians in prescribing the antimalarial drugs, depending on which region the patient visited before the attack. Review of drug susceptibility is a

continuos process (Spencer 1983; Schapira *et al.,* 1993) and it is true that parasites change in response to the drug pressure exerted. With funding available, this would be the way to go in order to curb malaria spread due to drug resistance in Kenya. This would give a point of reference as has been reported of malaria situation in Zimbabwe (Taylor and Mtambu, 1986; Taylor and Taputaria, 1988).

7.4 The four antimalarial drugs, doxycycline, azithromycin, pyrimethamine and sulfadoxine failed to give reproducible results by means of pLDH. Future focus should be on more planned studies on these drugs to come up with reproducible IC50 values since the two drugs, pyrimethamine and sulfadoxine combined (fansidar) have now been recommended to replace chloroquine as first choice drug for malaria treatment in Kenya. The other two drugs, doxycycline and azythromycin, a-part from their antibiotic properties, have been tested in Kenya as potential prophylactic drugs which may go a long way in helping the locals and non-immune subjects in malaria control and the control of other infections.

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APPENDICES

Appendix 1

INFORMED CONSENT EXPLANATION

(To be read and questions answered in a language in which the patient is fluent).

Tittle of study : Determination of antimalarial drug sensitivity in *P. falciparum* clinical isolates by the p(LDH) method.

Institutions : KEMRI / WR Project and Kisumu district hospital.

Principal investigator: 1. Rose Ogwang Odhiambo - KEMRI/ Egerton university:

Participation/investigation:

You have been asked to participate in a medical research study. It is very important that you understand the following general principles which apply to all participants in our studies, whether normal or patient volunteers.

1. Your participation is entirely voluntarily
2. You may withdraw from participation in this study or any part of the study at any time. Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled.
3. After you read the explanation, please feel free to ask any questions that will help you to understand clearly the nature of the study.

Introduction: p(LDH) assay for antimalarial drug sensitivity.
Purpose of the study: (a). To determine the effectiveness of the antimalarial drugs used in the hospitals for chemotherapy.(b) To compare the new p(LDH) technique with hypoxanthine uptake drug sensitivity tests.(c) To determine the kinetics of p(LDH) blood clearance during treatment.
Procedure to be followed: Fingerprick and venous blood will be drawn using standard procedures. At each visit, less than 3 ml of blood will be drawn. For ambulatory patients a total of 15 ml, while for in-patients, 18 ml of blood drawn during the period of the study.
Experimental medication: None.
Expected duration of participation: 1 hr for ambulatory patients for 3 days and 30 minutes every day for in-patients until the day of discharge (max. 7 days)
Risk hazards and discomforts: Pain due to finger prick and bruising during venous blood withdrawal.
Benefit: Early detection of malaria and proper selection of more effective therapy based on early diagnosis and sensitivity results.
Assurance of confidentiality of volunteers identity: All data and medical information obtained about you as an individual will be considered privileged and held in confidence.You will not be identified in any presentation of the results. Representatives of the United States Government are authorized to inspect the record of this research study.
Circumstances under which your participation may be terminated without your consent: Due to health conditions under which your participation would possibly be dangerous and other conditions which might occur that would make your participation detrimental to your own health.
Significant new findings: The investigators will keep all volunteers informed of any significant new finding which may affect their participation in the study.
Number of volunteers in the study: Ninety six as calculated by statistical analysis.
Medical care for injury or illness: You are authorized all necessary medical care for injury or disease which is the proximate result of your participation in this research.
Whom to contact for answers to pertinent questions about the research study and in the event of a research-related injury to the volunteer: The investigator, Rose Ogwang' Odhiambo.
Whom to contact for answers to pertinent questions about research subjects right: The KEMRI National ethical review committee

Informed consent agreement for adults:I _____ , having full capacity to consent and having attained an eighteenth birthday, do hereby volunteer myself to participate in a research study entitled "Determination of antimalarial drug sensitivity in *P. falciparum* clinical isolates by the p(LDH) method", under the direction of Rose Ogwang' Odhiambo. The implication of this voluntary participation, the nature, duration, and purpose of the research study; the method and means by which it is conducted; and the conveniences and hazards which may reasonably be expected have been explained to me by: Rose Ogwang' Odhiambo of Egerton University/KEMRI; P. O Box. 54840, Nairobi.I have been given an opportunity to ask questions concerning this investigational study. Any such questions have been answered to my full and complete satisfaction. Should any further questions arise concerning my rights , I may contact: KEMRI National ethical review committee. I understand that I may at any time during the course of this study, revoke may consent and withdraw from the study without any penalty or loss of benefits. However, I may be requested to undergo certain examinations, in the opinion of the attending physician, if such examinations are necessary for my health and well being. My refusal to participate will involve no penalty or loss of benefits to which I am other wise entitled.

Volunteers signature: _____ Date _____Volunteers printed name: _____Village address: _____Volunteer's language: _____Identity card number: _____Study No.: _____I was presented during the above explanation, the opportunity for questions, and hereby witness the patients signature.Witness's signature: _____ Date_____Witness's printed name_____Date_____. Investigator's signature_____ Date_____Investigator's printed name_____

BEDO GI NG'EYO KUOM GIMA IDWARO NI ITIM :

Nying nonro ma itimo ni: Nono kaka yiedhe mag malaria thiedho tuo e oko mar ringruok mar dhano ka itiyo gi rieko mar p(LDH).

Kar tiegruok: KEMRI/WRP kod kar thieth mar osibtal mar district mar Kisumo.**Jo kachero maduong:** Rose Ogwang' Odhiambo- mawuok Egerton University /KEMRI

Gima onego ing'e e nonro ni: Okwayi ni mondo ichiwri kaka achiel kuom joma ni e nonro mar chieth. En gima duong' mondo ing'e tiend weche gi mabiro timore kuom ji te manie nonro, obet ng'ama jatuo kata ng'ama ngima ma oyie.

1. Donjo mari e nonro ok en achune
2. Inyalo weyo bedo achiel kuom joma niye enonro ni e migawo moro amora, e saa ma ihero. Tamruok mari bedo achiel kuom joma ni e nonroni ok ene kelni sand moro amora. Bende onge gima ok ibi yudo ma ne obedo iyudi nikech iweyo.
3. Ka ise somo weche gi abidha to ka iyie , bed thuolo mar penjo penj moro amora manyalo miyo ibed gi winjo maber kaka nonroni chalo.

Wach motelo: Ng'eyo nyanonro mar tuo mar malaria ki konyruok kod rieko manyien {p(LDH)}.

Tiend timo nonroni: (a) Rango ka yedhe mag malaria nego tuoni oko mar dend dhano kitiyo gi rieko mar p(LDH).(b) Pimo kaka teko mar yethe mitiyogo kuom thiedho midhusi mar malaria chalo ka itiyo gi rieko mar p(LDH) kod rieko mar hypoxanthine.(c) Nono kaka gima midhusi mar malaria golo e del miluongo ni p(LDH) dhi ka rumo e del ka jatuo ithiedho kod yedhe mag malaria.

Okenge ma iluwo: Ibiro golo remo matin e lith kogno kod ligewo mar luedo. Jotuo ma dok dala biro golo remo maromo ondong' (mililita) 15 to jotuo ma orwaki kendo nindo e ospital ondong' 18 kuom thuolo duto ma nonro ni biro kawo.

Timo thieth mar temo kuom ng'ato: Ma onge

Thuolo ma ibiro kawo: Saa achiel kuom jotuo ma dok e ot nyaka ndalo adek, dakika piero adek odiechieng ko diechieng ne jotuo ma onindo e kar thieth nnyak achieng' gi wuogi e osiptal (ma ok kal ndalo 7).

Gima ok kare kod winjo rach manyalo miyi luoro: Ibiro winjo rem matin sama igolo remo , kama igole remo bende biro hinyore matin nikech sindano migolo go remo.

Kony kata ohala moro amora: Pim maber biyo iyudo midhusi mar malaria piyo, kendo kaluore kod pim mar nono yath, ibiro yudo thieth mar malaria gi yath maber e thuolo ma chuok.

Adier mar rito maling' mar joma ni e nonro ni : Ma nitiere ahinya. Onge ng'ama biro ng'eyo ni in achiel kuom joma ni e nonro ni. Nying ok ni gol ka ihulo duoko gi. Jatelo ma o a e sirikal mar America to oyienegi cheko ka nonroni itimo maber e buge magwa.

Gima nyalo miyo achiel kuom joma ni e nonroni gol oko ma ok onyise sa asaya: Kanitie tuo mor ma oyud e dendi ma nyalo miyo dhiyo nyime kod nonnroni nyalo hinyo ngima ni.

Ang'o ma nyien ma inyalo ng'eyo bang' tieko nonroni : Jotelo mag nonroni biro wachoni ka po ni nitie gimoro manyien ma giyudo ka luwore kod bedoni e nonroni.

Kar kwan mar joma ni e nonroni:

Ji pier o ochiko kod achiel.

Rit e thieth kuom hinyruok kata tuo ma dimaki nikech idonjo e nonroni: Ibiro yudo thieth kuomwa kapo ni nitie hinyruok kata tuo ma iyudo nikech ne iyie bedo achiel kuom

jo chiuruok e nonroni.

Ji ma inyalo penjo kuom hinyruok ma luwore gi nonroni: Jakachero maduong' Rose Ogwang' Odhiambo.

Kuonde ma inyalo dhiye kor ka duoko penjo , kata medo ng'eyo mang'eny kuom hinyruok ka luwore gi nonroni: Jotelo mag riuruok mochung' ne ngima kod gima onego iyudi kaka dhano ma a KEMRI.I certify that this is a true and accurate translation.

Translator's signature:_____Translator's name:_____Position:_____ _____Address:_____Telephone number:_____FAX:_____ _____

CHIWO THUOLO MAR DONJO E NONRONI KUOM JOMA DONGO. Nikech an ng'at maduong' ma ose kalo higni apar kod aboro (18), an _____ asebedo gi adiera kendo ayie bedo achiel kuom joma ni e nonroni miluongo ni "Nono kaka yedhe mag malaria nego tuo e oko mar dend dhano ka itiyo gi yo mar p(LDH)", ka itime gi ja kachero maduong', Rose Ogwang'. Oselerna tiend nonroni, thuolo, yo , kendo kaka ibi time kod rach kata chwanyruok manyalobedoye osewachna abidha kod, Rose Ogwang' Odhiambo, mar Egerton University / KEMRI, P. O. Box. 54840 NAIROBI. Osemiya thuolo mar penjo penj maluwore gi fueny mar nonroni. Penjo moro a mora aseyudo duoko ma kare, kendo ayie. Kapo ni penjo moro amora many'alo bedo bang'e maluwore gi adieri mara, anyalo neno jobura mag ngima mar KEMRI, ma biro duoka. Oyiena ni e thuolo moro a moa anyalo loko paro kendo awuogi enonroni maonge kum kata lal moramora kuom yuto madine ayudo. Inyalo kwaya ni mondo otimna nonro moko, ka en e pach jathieth, kapo ni nonrogo nyalo konyo ngima mara gi chango mara. Ka adagi donjo e nonroni onge kum kata lal moro amora ma abiro yudo. Sei mar jachiure:_____ Tarik_____Ny'ing jachiure:_____Adires jachiure:_____Dhok ma jachiure wacho:_____Namba mar kipande mar jachiure:_____Namba mar nonroni:_____Asebedo janeno seche mane ilero weche man malogo, thuolo nene ayudo mar penjo, kendo aneno adier sei mar jachiure (jatuo).Sei mar janeno:_____Tarik:_____Nying janeno:_____ Tarik:_____Sei mar janonro mochung' ne somo ni:_____I certify that this is a true and accurate translation.Translator's signature:_____Translator's name:_____Position:_____ _____Address:_____ _____Telephone number._____FAX:_____ _____

INFORMED CONSENT AGREEMENT FOR CHILDREN,

_____(Name of parent or legal guardian),being' 18 or older and having the capacity to consent for _____(name patient), do here by volunteer this child to participate in a research study entitled "Determination of antimalarial drug sensitivity in *P. falciparum* clinical isolates by the p(LDH) method"; under the direction of Rose Ogwang' Odhiambo of Egerton University/KEMRI, P.O. BOX 54840, NAIROBI. I have been given opportunity to ask questions concerning this investigational study. Any such questions have been answered to my full and complete satisfaction. Should any further questions arise concerning my rights , I may contact KEMRI National Ethical Review Committee at Kenya Medical Research Institute, P. O. Box 20778, Nairobi, Telephone, 722541. I understand that I may at any time during the course of this study revoke my consent and withdraw the patient from the study without any penalty or loss of benefits. However, the patient may be requested to under go certain examinations, if in the opinion of the attending physician, such examinations are necessary for the patients health and well being. My refusal to participate will involve no penalty or loss of benefits to which I am otherwise entitled.

Parent/Guardian's signature:_____ Date _____Parent/Guardian's printed name: _____Village address:_____Identity card number:_____Study No.:_____I was presented during the above explanation, the opportunity for questions, and hereby witness the patients signature.Witness's signature:_____ Date_____Witness's printed name_____Date_____. Investigator's signature_____ Date_____Investigator's printed name_____

CHIWO THUOLO MAR DONJO E NONRO KUOM JOMA TINDO

An_____(nying janyuol kata jachung'), moseromo higa 18 kata ose kalo kendo man kod teko mar yie mondo _____(nying jatuo), nyathini obed achiel kuom jochiure e nonro miluongo ni "nono kaka yedhe mag malaria nego tuo e oko mar dend ka itiyo gi yo mar p(LDH) , ka itimo go ja kachero maduong, Rose Ogwang'. Oselerna tiend nonroni, thuolo, yo, kendo kaka ibi time kod rach kata chwanyruok manyalo bedo e osewachna abidha kod Rose Ogwang' Odhiambo, mar Egerton University/KEMRI, P. O. Box 54840, Nairobi. Osemiya thuolo mar penjo penj maluore gi fueny mar nonroni. Penjo moro amora ase yudo duoko ma kare, kendo ayie. Kapa opo ni penjo moro amora nyalo bedo bang'e maluore gi adiera mara, anyalo neno jotelo mag riuruok mochung' ni ngima kod gima onego oyudi kaka dhano ma a KEMRI, P. O. BOX 5484, NAIROBI, namba mar simu 722541, ma biro duoka. Oyiena ni e thuolo moro amora anyalo loko paro kendo awuog e nonroni maonge kum kata lala moro kuom yuto ma dine ayudo.Inyalo kwaya ni mondo otimna nonro moko, ka en e pach jathieth, kapo ni nonrogo nyalo konyo ngima mara gi chango mara.Ka adagi donjo e nonro onge kum kata lal moro amora ma abiro yudo.Sei mar janyuol/jarit:_____

Tarik_____Ny'ing janyuo/jarit:_____Adires mar dala:_____Namba mar kipande mar janyuol/jarit:_____Namba mar nonro mar nyathi:_____Asebedo janeno seche mane ilero weche man malong'o, thuolo nene ayudo mar penjo, kendo aneno adier sei mar jachiure (jatuo).Sei mar janeno:_____Tarik:_____Nying janeno:_____ Tarik:_____Sei mar janonro mochung' ne somo ni:_____I certify that this is a true and accurate translation.Translator's signature:_____Translator's name:_____Position:_____ _____Address:_____ _____Telephone number._____FAX: _____

Appendix 2
Malaria Investigation form

Date _____ A. Name _____ Sample No._____ Age _____ Sex_____ Sub-location_____ Location_____ Division _____ Chief 's Name _____ B. **Clinical Information** : OPD _____ IPD _____ (ward) _____ Date of onset of illness _____ Duration _____ Medication taken _____ for how many days _____ Please circle following signs and symptoms (Yes - Y; No - N) Headache Y N Vommiting Y N Nausea Y N Low back pain Y N Joint pain Y N Resp. symp /cough Y N Yellow eyes /jaundice Y N Abdominal pain Y N Diarrhea Y N Decreased appetite Y N Convulsions Y N Pregnant Y N Others _____ _____ _____ C.

Malaria lab Information : field stain Giemsa Thick /thin Thick /thin Blood slide Kisumu _____%_____ _____%_____ Blood slide research _____%_____ _____%_____ Confirmation investigator _____%_____ _____%_____ Hematocrit _____ Parasight F _____ _____ Additional info _____ If patient returns; Y /N: Date _____ Temp _____ MPS ____%_____ Parasight F _____ Medicaton _____ Blood Y / N, Date _____

Additional Information Difference in admission and discharge hematocrits _____Requirement of blood transmision _____Length of Hospital stay _____Names of drugs used during treatment _____Any other information _____

Appendix 3
PARASITE DILUTION SHEET

Parasite Isolate _____ABO TYPE _____PARASITAEMIA_____ # OF CELLS REQUIRED _____ 1) ASSUME 2 x 10^7 CELLS PER WELL IN 200 μl or 1% hematocrit2) ASSUME 2 x 10^9 CELLS PER 96 WELL PLATE 3) IF USING A PREMADE DRUG PLATE ADD 100 μl PARASITIZED CELL SUSPENSION AT A CELL CONCENTRATION OF 2 x 10^8 CELLS PER ml# OF PLATES REQUIRED_____ REQUIRED PARASITAEMIA _____ (0.4 % FOR 48 HOUR /0.2% FOR 66 HOUR)# OF CELLS REQUIRED_____Hemocytometer count:# of cells per 80 tiny squares _____Dilution factor _____Constant x 50Constant (converts to cells /ml) x 1,000= total cells /ml _____4) Dilution factor to convert parasitised cells to 0.4 % or 0.2 % parasitaemia_____5) Multiply parasitised cell dilution factor by total cell # required= # of parasitised cells required: _____Substract (5) from total cell # required = # of uninfected cells required to dilute parasitized cell to 0.2% or 0.4 % parasitaemia_____Volume of media required for plates (dilute cell mixture into this volume) Remeber to dilute cells into ABO matched serum and use folate free media for experiments requiring antifolate drugs.

Appendix 4

Publications, abstracts of papers for conferences, seminars and workshops.

Publications.

Ogwang, R.A; Mwangi, J.K; Githure, J; Were, J.B.O; Roberts, C.R and Martin, S.K (1993). Factors affecting exflagellation of *in vitro*- cultivated *Plasmodium falciparum* gametocytes. *American Journal of Tropical Medicine and Hygiene* 49 (1): 25-29.

Ogwang, R.A; Mwangi, J.K; Gachihi, G; Nwachukwu, A; Roberts, C.R and Martin, S.K (1993). Use of pharmacological agents to implicate a role for phosphoinositide hydrolysis products in malaria gamete formation. *Biochemical Pharmacology* 46 (9): 1601-1606.

Ogwang- Odhiambo, R.A; Orago, A.S.S; Koech, D.K and Odulaja, A (1999). Determination of cut-off pLDH optical density levels for field evaluation of malaria in Kenya. *African Journal of Health Sciences*, in press.

Ogwang- Odhiambo, R.A; Orago, A.S.S; Koech, D.K and Odulaja, A (1999). Parasite lactate dehydrogenase assay (pLDH) for susceptibility using six antimalarial drugs in Kenya. *Journal of infectious diseases*, in press.

Abstracts for conferences.

Ogwang, R.A and Oraago, A.S.S. (2nd to 4th February, 1990). Incidence of malaria in infants and children from 1980 to 1986 in South Nyanza District, Kenya. Presented at the Annual Scientific Conference of Kenya Paedriatric Association.

Ogwang, R.A; Ofulla, A.V.O; Aleman, G and Martin, S.K(28th to 4th May, 1994). Malaria diagnosis using p(LDH) enzyme assay in comparison to microscopy as gold standard in a Kenyan endemic population. Presented at the Internation cogress for infectious Diseases, Prague, Cheque Republic.

Ogwang, R.A; Ofulla, A.V.O; Aleman, G and Martin, S.K (2nd to 5 th February, 1996). Limitations of p(LDH) malaria diagnostic enzyme assay in clinical and field trials. Presented at the 17th African Health Sciences Congress, Nairobi, Kenya.c)

Ogwang- Odhiambo, R.A; Aleman, G; Mwangi, J.K; Martin , S.K; Koech, D; Klotz, F and Orago, A.S (25th to 27 th September, 1996). Determination of antimalarial drug sensitivity in *Plasmodium falciparum* clinical isolates , by the p(LDH) method. Presented at the 4th Annual Postgraduate Scientific Conference, Department of Zoology, Kenyatta University, Kenya.

Ogwang- Odhiambo, R.A; Klotz, F.; Koech, D and Orago, A.S (4th to 7th August, 1997). Use of Parasite Lactate Dehydrogenase (pLDH) Optimal dipstick assay in Kenyan Population with endemic malaria. Presented at the 5th Annual Post- graduate Scientific conference , Department of Zoology, Kenyatta University, Kenya.

Ogwang- Odhiambo, R.A; Orago, A.S.S and Koech, K (1st to 3rd October, 1997). Use of Parasite Lactate Dehydrogenase (pLDH) assay for field and clinical diagnosis in Kenyan population with endemic malaria. Presented at the 8th Conference of the association of Kenya Medical Laboratory Scientific Officers, Nakuru, Kenya.

Ogwang- Odhiambo, R.A; Koech, D.K; Orago, A.S and Klotz, F (24th to 28th April, 1998). Limitations of p(LDH) as a drug sensitivity assay using malaria isolates from endemic regions of Kenya. Presented at the 19th African Health Sciences Congress, Arusha, Tanzania

Ogwang- Odhiambo, R.A; Koech, D.K; Orago, A.S and Odulaja, A (18th to 22nd April, 1999). Determination of cut-off pLDH optical density levels for field evaluation of malaria in Kenya. Presented at the 20th African Health Sciences Congress, Noghuchi, Accra, Ghana

Seminars and workshops

1991- 21st July- Clinical Research Centre Scientific seminar on success on growth and maintenance of mature Plasmodium falciparum gametocytes in suspended animation- a break through in gametocyte biochemical studies.

1995 July – Scientific Seminar at Zoology Department, Kenyatta University Kenya on Nitric Oxide: the wonder molecule of the year, a proposed study on its role in mediated killing of cytoadherent *Plasmodium falciparum* erythrocytic stage parasites.

1995 –July 5th – Kenya Medical Research Institute, Clinical Research Centre Scientific seminar on future prospects for p(LDH) for malaria diagnosis and drug sensitivity studies.

1999-February 21st to March 11th-Entomological research partnership, PhD workshop between Kenyatta University and Swedish University of Agriculture.