EFFECTS OF TWO NEEM INSECTICIDE FORMULATIONS ON THE TROPHIC INTERACTIONS BETWEEN THE DIAMONDBACK MOTH, *PLUTELLA XYLOSTELLA* (L.) (LEPIDOPTERA: PLUTELLIDAE) AND ITS PARASITOIDS

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April 2002

DECLARATION BY CANDIDATE

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

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DEDICATION

To my mother, Ms. Angelica K. Nyakana; thank you.

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I acknowledge my family for their love, support and absolute faith in my abilities over the years.

I thank all my friends and in particular: Patricia, Marjorie, Laila, Cheryl, Clare, Wilson and Matthew whose love, support and encouragement I relied upon when the going got tough and despondency threatened. I also thank my fellow ARPPIS scholars for their constant encouragement and collegiality especially when the 'dudus misbehaved'.

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So many other people have helped, encouraged and contributed in numerous ways to the commencement and completion of this work; my sincere thanks to each and everyone of you.

ABSTRACT

The diamondback moth (DBM), *Plutella xylostella* (L.) is a pest of Cru cifers and, of worldwide importance. Reliance on chemical insecticide cor rol is not a sustainable option therefore, new pest control initiatives em hasise the use of parasitoids and biorationals such as neem ins cticides. Neemros® (a powder formulation) and Neemroc EC® (an oil for ulation) are two neem insecticides that are registered in Kenya for use ag nst vegetable pests, including the DBM. However, the much needed inf rmation on their impact on DBM parasitoids is lacking. The aim of this stu y was to assess the responses of DBM parasitoids to the neem for ulations when exposed to them directly or through the plant or host lar ae. The formulations were applied as foliar sprays using either a kna sack sprayer or a hand-held mist sprayer fitted with a cone nozzle.

In field experiments, conducted during 1999 at Juja (Kenya), Ne mroc EC® checked DBM infestation but Neemros® was not as eff ctive. Three parasitoid species namely *Diadegma mollipla* (Holmgren), *Oo myzus sokolowskii* (Kurdjumov) and *Cotesia plutellae* (Kurdjumov) were rec vered. Parasitism by all three species did not differ significantly bet ween Neemros® treated and control plots but was significantly lower in the Neemroc EC® treated plots during two weeks ($F= 3.09$, $d.f= 8, 36$; $P<0.05$). The individual parasitoid species also had different parasitism lev ls and also responded differently to the neem insecticide treatments.

Cause of mortality analysis in DBM cohorts revealed that larval parasitoids accounted for <1% of the total mortality during the experimental period while the neem formulations accounted for between 15-26% of the total mortality.

In laboratory tests undertaken with *D. mollipla,* parasitoid adults that had direct topical contact with the neem formulations showed comparable longevity to those exposed to water (control), while exposure to Karate®, a synthetic pyrethroid caused 100% mortality in less than six hours. There were no significant differences in the proportion of hosts parasitised by the neem- and water-sprayed adults ($F= 2.40$, $d.f= 2, 75$; $P>0.05$) and their foraging patterns were also similar (ANOVA, $P>0.05$). Also evaluated were the olfactory responses of *D. mollipla* to plants/plant-host complexes sprayed with the neem formulations. The parasitoids were significantly more attracted to odours from the Neemros®-sprayed plants/plant-host complexes than to a blank (clean air). In contrast, parasitoid response to odours from the Neemroc EC®-sprayed plants/plant-host complexes did not differ significantly from the blank. In choice tests between infested plants sprayed with water or a neem formulation, parasitoids showed no preference for odours from water-sprayed plants over those from Neemros®-sprayed plants but showed a preference for the odours from the water-sprayed plants over those from Neemroc EC®-sprayed plants. In host acceptance and suitability tests, parasitism rates in neem- and water-

sprayed hosts exposed to parasitoids at 24 hrs and 48 hrs after treatment were, with one exception, not significantly different. A high mortality level in the neem-treated parasitised hosts suggested their unsuitability for parasitoid development. It is concluded that the neem formulations may be relatively safe to adult DBM larval parasitoids at the doses used in the study. The establishment of plant refugia that would harbour untreated DBM larvae and, thus help to perpetuate the parasitoid population could offset the deleterious effects observed on parasitoid development.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AVRDC	Asian Vegetable Research and Development Centre
AZA	Azadirachtin
DBM	Diamondback moth
d.f	Degrees of freedom
IGR	Insect growth regulator(s)
KARI	Kenya Agricultural Research Institute
NKCP	Neem kernel cake powder
NSKE	Neem seed kernel extract(s)
NSO	Neem seed oil
SAS	Statistical Analysis System software
s.e.	Standard error
SNK	Student-Newmann-Keuls multiple range test
WAT	Week(s) after transplanting

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Crucifers are important crops wherever they are cultivated but their production is severely constrained by the diamondback moth (DBM), *Plutella xylostella* (L.) and other pests. The development of insecticide-resistance in DBM populations has resulted in a more intensive and often inappropriate use of the chemical insecticides that are currently available for containing the pest (Osterman & Dreyer, 1995). This has given rise to concerns about the health risks posed by insecticide residues on vegetables and calls for the development of integrated pest management (IPM) strategies that farmers can use to combat the pest problem without compromising product quality.

Integrated pest management techniques seek to reduce pest-induced losses by managing the agronomic, biological and socio-economic components and interactions of the entire system. An understanding of how these different components function and interact with each other is crucial when developing an effective IPM programme. The conservation and enhancement of populations of natural enemies are cornerstones of successful IPM programmes (Stehr, 1975; Waage, 1989) as they reduce populations of primary pests, limit pest damage and keep secondary pests

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The aim of this study was to investigate whether some neem formulations used for the management of the diamondback moth have any adverse effects on the parasitoids of this cabbage pest. The parasitoids are also a component of the IPM programme for diamondback moth. It is hoped that the results of the study will provide useful information that is necessary in developing cost-effective IPM programmes that assure a more compatible use of neem insecticides and parasitoids of the diamondback moth.

1.2 LITERATURE REVIEW

1.2.1 Status of pest management in vegetable based agrosystems

Rapid human population growth in the developing countries of the tropics and the subtropics has seen a huge increase in the demand for supplies of fresh vegetables and fruits. This has facilitated the almost year round cultivation of vegetables either on small-scale peri-urban gardens or large scale commercial farms to satisfy the demand. Vegetables are important supplementary sources of food and nutrition for many urban dwellers and one of the major sources of cash for small-scale farmers who often are the major producers.

Crucifers are one of the most important vegetables grown worldwide and the most important in Kenya. Their continuous cultivation has greatly encouraged the build-up of the associated pest complex of which the

diamondback moth [DBM] is the most notorious and the most difficult to control (Talekar & Shelton, 1993; Kibata, 1996). To ensure adequate yields and produce damage-free vegetables for the urban markets, farmers have been forced to apply ever more frequently increasing dosages and/or cocktails of chemical insecticides (Kibata, 1996). In Southeast Asia, where a similar problem prevails, DBM populations have developed resistance to most of the major classes of synthetic insecticides, thereby forcing farmers into pesticide dependency (Talekar & Shelton, 1993). Other insects previously listed as minor pests of Crucifers have gained major status largely because their natural enemies have been wiped out (AVRDC, 1992). This has led to the risk of the produce carrying insecticide residues beyond the maximum residue limits (MRLs), environmental contamination with insecticides and increased production costs.

An integrated management strategy for DBM control incorporating ecological, economic and sociological considerations remains the only option and is already being actively pursued in South-east Asia (AVRDC, 1992; Mengech *et al.*, 1995).

1.2.2 Economic Significance of Crucifers in Eastern Africa

Crucifers cultivated in the tropics include common cabbage (*Brassica oleracea* L. var. *capitata* L.), kale (*B. oleracea* L. var. *acephala* (DC) Alef.), Chinese cabbage (*B. chinensis* L.), cauliflower (*B. oleracea* L.

var. *botrytis* L.), broccoli (*B. oleracea* L. var. *italica* Plenck), rapeseed (*B. napus* L.), turnip (*B. oleracea* L. var. *rapa* L.) and radish (*Raphanus sativus* L.) (Tindall, 1983; Talekar & Shelton, 1993). In East Africa, cabbage and kale are the most important and are mostly grown by resource-limited farmers on relatively small gardens (Kibata, 1996). Nevertheless, annual production of these two crops in Kenya was just under 540,000 metric tonnes in 1998, with an estimated value of approximately $2 million (MALDM, 1998).

Cabbage is an important food crop rich in vitamins (A, the B complex, C, D) and minerals (iron, phosphorus, calcium) and low in calories (Tindall, 1983). In East and Southern Africa, DBM and aphids, [*Brevicoryne brassicae* (L.), *Myzus persicae* (Sulzer), and *Lipaphis erysimi* (Kalt.)] are key insect pests but other economically important pests include the cabbage webworm (*Hellula undalis* [Fabricius]) and the bagrada bug (*Bagrada hilaris* [Burmeister]) (Anon., 1995; Nyambo & Pekke, 1995; Oduor *et al.*, 1996; Varela *et al.,* 1999). Control measures are primarily targeted at DBM because it attacks (and if not checked, skeletonises 50-100% of) the crop from the seedling stage; aphids occur later in the season and infestations only become serious in the absence of rain.

1.2.3 The Diamondback moth - Life History, Pest Status and Management

1.2.3.1 Lifehistory

The diamondback moth (DBM), *P. xylostella* (Lepidoptera: Plutellidae) is a cosmopolitan pest that usually feeds and oviposits on members of the family Cruciferae. Members of the Cruciferae contain mustard oils and glucosinolates or their derivatives that are usually toxic to a large majority of generalist insect feeders but stimulate gustatory and oviposition responses in the DBM (Nayar & Thorsteinson, 1963; Taleker & Shelton, 1993; Verkerk & Wright, 1996). The DBM has also been recorded feeding and ovipositing on snowpeas (Leguminosae) in Kenya (B. Löhr, ICIPE, pers. comm.). It is widely believed that the DBM evolved in Europe probably around the Mediterranean region with the indigenous Crucifers (Talekar & Shelton, 1993), but Kfir (1998) is of the opinion that the species could have evolved in Southern Africa. Whatever its ecological origins, DBM's ability to tolerate a wide range of climatic conditions has enabled the species to establish itself in the temperate, subtropical and tropical regions of the world. It is especially serious in those regions where Crucifers are cultivated continuously and chemical insecticides are relied upon heavily for control.

The adult moth is about 8mm in length, grey-brown with a characteristic diamond pattern on its back from which it derives its name (Plate 1.1). The moths are commonly active towards dusk, when they mate and/or oviposit. Under captive conditions the female moth may live for up to 16 days during which she may lay more than 400 eggs. The pale yellow eggs are oviposited singly or in clusters of 2-4 along the leaf veins on the upper and/or lower leaf surfaces and have an incubation period of 2-4 days. First instar larvae mine into the leaf where they feed on the spongy mesophyll for two days and then emerge as second instars. The second, third and fourth instars each lasts for 2 days followed by a prepupal period of one day. Pupation may last for 7-11 days (Plate 1.2). Mating takes place soon after emergence and oviposition follows immediately, with peak oviposition occurring on the day of emergence. Development times are dependent on temperature but are shorter at higher temperatures (Shirai, 2000). The lifecycle may be completed in 20 days or less in warm climates (25-30°C) but is longer (25-40 days) at cooler temperatures ($\leq$21°C) (Finch & Thompson, 1992; Keinmeesuke *et al.*, 1992; Wakisaka *et al.*, 1992; Shirai, 2000). Consequently there may be 15 or more generations per year in the tropics (Liu *et al.*, 1981; Keinmeesuke *et al.*, 1992; Varela *et al.*, 1999).

Plate 1.1: Diamondback moth adult

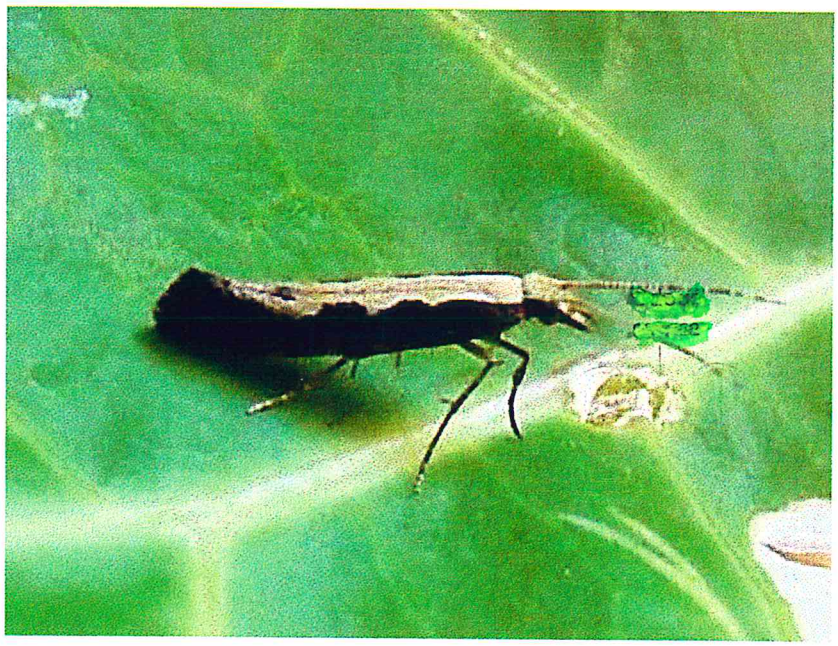


Plate 1.2:

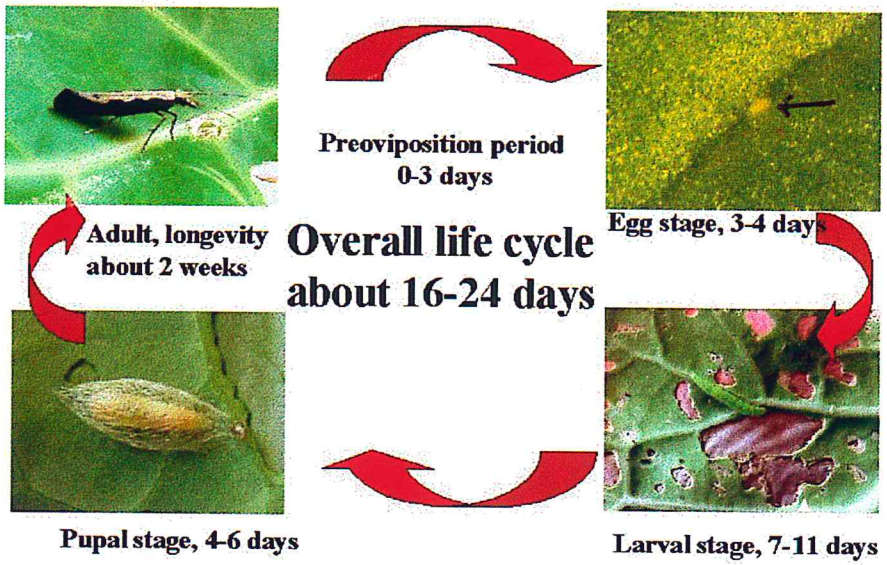


Plate 1.3: Diamondback moth larvae (arrows) feeding on cabbage leaf

Plate 1.4: Diamondback moth damage on cabbage [*note characteristic "windows" on leaves*]

1.2.3.3 Management

Management of DBM is heavily reliant o e use of chemical insecticides especially in the developing countries c tropics (Tabashnik *et al.*, 1987). Worldwide, the annual cost for aging the pest is estimated at $1 billion (Talekar & Shelton, 1993). I sive insecticide use has decimated DBM natural enemies (Idris & Gra 1993a) while DBM has developed resistance to most of the majc asses of chemical insecticides (Liu *et al.,* 1981, 1982; Idris & Grafius, 3b; Syed & Fauziah 1996). Botanicals and insect growth regulators Rs) are alternative control measures but it is possible that DBM ma velop resistance to them too (Völlinger, 1995). There are already repor resistance to some *B.t.* (*Bacillus thuringiensis*) formulations (Kirsch Schmutterer, 1988; Tabashnik *et al.,* 1990, 1992a, 1992b) and IGF Perng *et al.,* 1988; Kobayashi *et al.,* 1992). Increasing production co largely attributed to pest control of DBM have forced a reassessme f the management strategy for the species. The trend now is to a an integrated and ecologically compatible management package in orating the use of microbial pesticides such as *B.t.*, botanicals such neem insecticides, biological control and cultural methods such as rcropping with trap crops and minimising the use of chemical insecticid The components of such an integrated control package should act inte ively in suppressing the DBM population while slowing down the e ution of insecticide

resistance and conserving natural enemies (Tabashnik *et al.,* 1987). For instance, Liu *et al.* (1981) found that where insecticide pressure was low, DBM populations showed only low levels of resistance or none to synthetic pyrethroids.

1.2.4 Biological control of DBM

1.2.4.1 Parasitoids of DBM - a summary

The potential for biological control of DBM lies in the use of key parasitoids, which are important natural checks. About 40 parasitoids of DBM are known but only five show any real potential as biological control agents; these are *Diadegma* spp., *Cotesia plutellae* (Kurdjumov), *Oomyzus sokolowskii* (Kurdjumov), *Microplitis plutellae* (Muesebeck) and *Diadromus* spp. (Lim, 1986; Talekar & Shelton, 1993; Anon., 1995; Verkerk & Wright, 1996; AVRDC, 1997). Fitton & Walker (1992) reviewed the taxonomy of the hymenopterous parasitoids of DBM. *Diadegma* is an ichneumonid solitary endoparasitoid that preferentially attacks the second larval instar. *M. plutellae* and *C. plutellae* are braconid solitary endoparasitoids of subfamily Microgastrinae and prefer to attack the second and third larval instars of DBM. *C. plutellae* is the more predominant parasitoid in regions of higher temperatures (26-30°C) and could be the major biological control agent in such regions (Talekar, 1992). *Diadromus* spp. are pupal parasitoids in the ichneumonid subfamily Ichneumoninae; *Diadromus collaris* (Gravenhorst)

has been widely introduced in biological control efforts. *O. sokolowskii* is a gregarious primary and facultative hyperparasitoid that belongs to the superfamily Chalcidoidea. It is the only chalcidoid that shows any real potential for biocontrol of DBM despite its hyperparasitic tendencies. Other chalcidoids such as *Tetrastichus howardii* Olliff (also known as *T. ayyari* and *T. israeli*) and the egg parasitoids *Trichogramma* and *Trichogrammatoidea* (both of family Trichogrammatidae) also occur but show little potential as biocontrol agents for DBM (Fitton & Walker, 1992; Talekar & Shelton, 1993).

1.2.4.2 Diadegma spp. (Hymenoptera: Ichneumonidae, Campopleginae)

Wasps of the genus *Diadegma* are solitary endoparasitoids of DBM. Several species of *Diadegma* are known to occur but the taxonomic dilemma surrounding the genus suggests that many of the species are synonyms of each other. The genus includes the species *D. semiclausum* Hellén, *D. fenestrale* (Holmgren), *D. insulare* (Cresson), *and D. rapi* (Cameron) (Fitton & Walker, 1992). The species of *Diadegma* in sub-Saharan Africa has been identified as *Diadegma mollipla* (Holmgren) (Azidah *et al.,* 2000). *D. semiclausum* and *D. insulare* have shown the greatest potential for biological control in South-east Asia and the United States and Canada, respectively (Harcourt, 1986; Talekar, 1992).

Diadegma spp. attacks any of the four larval instars but more readily parasitises the second instar (Waage & Cherry, 1992). The parasitised host continues to feed and develop until pupation, when the developing parasitoid within it finally kills it and emerges to spin its own cocoon within that of the host. The developmental period is temperature dependent but is about 10-15 days at 25°C and the female parasitoid may live for more than 20 days when fed (Grafius, 1997).

Diadegma spp. is most effective at cooler temperatures (15-25°C) such as those characteristic of highland areas (Talekar & Shelton, 1993). Parasitism varies from less than 10% to over 80% in various parts of the world (Harcourt, 1986; Idris & Grafius, 1993b; Talekar & Shelton, 1993; Grafius, 1997). The parasitoid is very susceptible to chemical insecticides but readily establishes itself when chemical insecticides are applied infrequently.

1.2.5 Neem insecticides

Neem-based insecticides are promising candidates for pest management in many ecosystems, including cabbage. The insecticides are derived from the evergreen neem tree, *Azadirachta indica* A. Juss, which belongs to the Meliaceae (Mahogany) family. The tree is believed to have its origins in Southern and South-eastern Asia but today occurs throughout tropical and subtropical Asia, Africa, Australia, and Central and South

America (Schmutterer, 1990, 1995). The bark, leaves and seeds (drupes) all contain the active compounds found in neem insecticides but the seed kernels have the highest concentration of these, and are the main source. The most potent of these active compounds is Azadirachtin (aza), a steroid-like tetranortriterpenoid, which occurs as eleven isomers, but other chemical groups also occur (Schmutterer, 1990, 1995). This complex of active compounds in neem insecticides is responsible for the range of deleterious effects, which include killing, repellence, feeding and oviposition deterrence and growth disruption, against insects.

Azadirachtin's mode of action at the cellular and molecular level has been elucidated (Rembold *et al.*, 1984; Rembold, 1995). Within the insect body, azadirachtin is rapidly excreted and only a small fraction is retained. This fraction concentrates in the neurolemma of the brain and associated endocrine glands (the corpora allata and corpora cardiaca) and works by depressing the synthesis of neurohormones and their release into the haemolymph. The documented effects of neem insecticides (disrupted moulting, inhibited oogenesis, morphological defects) are a consequence of this disruption to the insect's neuroendocrinal controls.

Although neem insecticides have been used by farmers for pest control in Asia for centuries, their use has until recently been perceived as a 'backward practice' especially by the more affluent farmers in the region (Raheja, 1995). With increased awareness of IPM, consumer concerns

about pesticides residues in food and problems associated with excessive use of synthetic chemical pesticides, neem insecticides are increasingly regarded as an important input to IPM systems. Large-scale adoption of use of neem insecticides is however severely constrained by the limited availability of effective and standardised formulations that a farmer can rely on for pest control. As these become more readily available, neem insecticides are likely to become more widely adopted as viable alternatives to the use of synthetic chemical insecticides (Raheja, 1995).

1.2.6 Effects of neem formulations on natural enemies

Neem insecticides are known to adversely affect about 413 species/subspecies of insect pests in 15 orders (Schmutterer & Singh, 1995) but compared to synthetic insecticides are relatively harmless to their natural enemies, especially the adult stage (Schmutterer, 1990, 1997).

The antifeedant, anti-ovipositional, repellent, mortality and/or insect-growth regulating properties of neem insecticides have been demonstrated in various species of natural enemies. Spiders and mites are either not susceptible or are the least susceptible to neem insecticides [neem seed oil (NSO), neem seed kernel extracts (NSKE)] at concentrations ranging from 0.5-5% and azadirachtin-enriched products (Saxena *et al.*, 1984; Mansour *et al.*, 1987; Saxena, 1987). However, the development and the

fecundity of the predatory mite, *Phytoseiulus persimilis* (Athias-Henriot) was negatively influenced when its eggs were treated with various neem products.

No or only negligible mortality has been recorded in predators such as beetles (coccinellids, carabids), lacewings and ants exposed as adults to sprays of neem extracts (NSO, NSKE) or azadirachtin-enriched products (AZT-VR-K, MTB/H_2O-VR-K). Exceptions are syrphid flies which are more sensitive to the toxic effects of neem (Schmutterer, 1995). Studies in which larvae, pupae or nymphs have been exposed to direct sprays and/or residues of neem insecticides or through their food have recorded such adverse effects as increased mortality, disrupted development and morphogenetic defects. For example, Margosan-O (an oil-based neem formulation) applied to third instar nymphs of the heteropteran stinkbug, *Perillus bioculatus* (F.) caused delayed molting and morphogenetic defects (Hough-Goldstein & Keil, 1991). Larvae of the coccinellid, *Coccinella septempunctata* L. sprayed with Neemix, an enriched neem product, showed increased mortality and morphogenetic defects (Banken & Stark, 1997). In other parasitoid species, the adults are not killed when exposed to neem insecticides but their progeny are more susceptible especially when their hosts are fed on a diet containing the insecticides (Lamb & Saxena 1988; Raguraman & Singh, 1998).

With respect to the repellent and oviposition deterrent properties of neem insecticides, parasitoids are generally not deterred from attacking hosts treated with neem insecticides although their progeny may fail to develop or successfully emerge from such hosts. For example, NSKEs (2-3%) applied to egg masses did not deter the egg parasitoid, *Telenomus remus* Nixon from attacking its host (Joshi *et al.*, 1982) while *Bracon hebetor* Say, a gregarious larval parasitoid of Lepidoptera, parasitised host larvae treated with NSKEs (Raguraman & Singh, 1998). Saxena *et al.* (1981a) recorded doubled rates of parasitism by ichneumonid, braconid and encyrtid parasitoids in rice fields sprayed with NSO. The study by McCloskey *et al.* (1993) on the ichneumonid parasitoid *Erioborus (=Diadegma) terebrans* (Gravenhorst) showed that it readily attacked hosts fed on a diet containing azadirachtin but the emerging parasitoids had reduced pupal and adult weights, longer developmental periods prior to emergence and higher mortality, especially after emergence.

Disrupted development, low or no emergence and subsequent reduced vitality of parasitoids developing in hosts exposed to direct sprays of neem insecticides or through neem-treated diets has been reported for several species such as *Bracon brevicornis* Westm. (Srivastava *et al.*, 1997), *C. plutellae* (Loke *et al.*, 1992), *Goniozus triangulifer* Kieffer (Lamb & Saxena, 1988), *Tetrastichus howardii* (Olliff) (Lamb & Saxena, 1988; Fernandez *et al.*, 1992). In contrast, the development and emergence of

several aphid parasitoids, *Lysiphlebus testaceipes* (Cresson), *Aphelinus asychis* Walker, *Encarsia formosa* Gahan, *E. transversa* Smith, *Diaretiella rapae* (McIntosh) and *Ephedrus cerasicola* Stary were not disrupted when parasitised mummies were dipped in or sprayed with various neem products (Margosan-O, MeOH-NR, AZT, MTB) (Feldhege & Schmutterer, 1993; other citations by Schmutterer, 1995). Schneider and Madel (1991) (cited in Schmutterer, 1995) observed normal fecundity and activity in *Diadegma semiclausum* females that had developed in hosts previously treated with NSKE (0.1-5%).

NSO formulations apparently have more pronounced effects on the natural enemies than NSKE formulations, and alcoholic NSKEs appear to give stronger effects than the water-based extracts (Schmutterer, 1995, Srivastava *et al.*, 1997). Mixtures of neem products with other compounds may also increase the side effects on beneficial organisms than with the use of neem alone (Schmutterer, 1997). For example, higher mortality was recorded in the armyworm, *Spodoptera frugiperda* (J.E. Smith) and the mosquito, *Aedes togoi* (Theobald) when a mixture of a neem insecticide and *Bacillus thuringiensis* (*Bt*) was applied than with either component alone (Hellpap & Zebitz, 1986; cited in Schmutterer, 1995).

1.2.7 Pesticide-parasitoid interactions and implications for pesticide evaluations

Parasitoids function within a multitrophic context, using cues from different trophic levels of agroecosystems to locate and attack their hosts (Vet & Dicke, 1992). These cues are often chemical substances but may also be of a physical nature such as silk, body size or movement (Quicke, 1997). The chemical cues may derive from the host (=herbivore) such as volatiles from frass, or its food (the plant) such as the volatiles emitted during herbivore feeding. Thus, any disruption to the sources of these cues could impact negatively on the efficiency and evolutionary fitness of the parasitoid species. Disruption to the normal host-parasitoid synchrony may be caused by the adverse effects of chemical toxicants on adult female parasitoids, which may either be killed, or their searching ability is severely impaired. The pesticide may also selectively eliminate the host stage that would otherwise be available and susceptible to attack by searching females and thus result in the local emigration or local extinction of the natural enemies (Doutt *et al.*, 1976; Waage, 1989). Since parasitoids have specific rules that govern attraction to and residence time within a host patch (Godfray, 1994), changes in host distribution and/or density following pesticide applications could influence whether a parasitoid will be attracted to or arrested within a host patch (Waage, 1989).

The effects of pesticides on insects are a function of formulation of the pesticide, its toxicity and mode of exposure. Banken and Stark (1997) argued that a realistic assessment of the effects of a pesticide on natural enemies could only be made if they were exposed to the pesticide through the same routes of exposure as they would under field conditions. Natural enemies in the field are exposed to pesticides through two or more routes such as contact with direct sprays or indirectly through contact with surface residues and contact with or consumption of contaminated hosts/prey. The effects arising from such indirect contact and those that are expressed in the progeny of exposed natural enemies are often termed "sublethal effects".

The sublethal effects of pesticides that directly affect parasitoid functioning can be categorised into behavioural and physiological mechanisms (Elzen, 1989). Behavioural effects include alteration of foraging pattern or efficiency, disruption of sexual communication or lack of host recognition. Physiological effects manifest as altered reproduction, reduced longevity, egg viability and fitness. The fitness of a parasitoid population is maximised by the number of viable progeny that can be produced by each individual. This requires that ovipositing females not only maximise the number of hosts that they attack but also select high quality hosts. Such hosts are those capable of sustaining the development of parasitoid progeny and further ensuring parasitoid fitness by, for example,

giving rise to individuals with high fecundity. It is therefore important to take into consideration the sublethal effects of pesticides because they may ultimately result in reduced population numbers of the parasitoid or its effectiveness in suppressing host population numbers (Elzen, 1989).

For the new generation of insecticides such as botanicals and insect growth regulators (IGRs) that are slow acting and whose effects may be expressed several days and/or generations after initial exposure, the assessments should be extended to longer post-treatment periods and also to the subsequent generation. The need for such assessments was demonstrated by Rumpf *et al.,* (1997) who showed that within a 24-48 hr post-exposure period, the IGRs under evaluation could be classified as harmless but at a 120-360 hr post-exposure period, the IGRs were harmful. In contrast, the organophosphates (an example of conventional chemical insecticides) were acutely toxic within a 24 hr post-treatment period. Thus, the mode and period of exposure as well as sublethal effects in the target and subsequent generation must be taken into account when evaluating the effects of such biorational insecticides like neem-based products.

The effects of neem insecticides on insects include repellency, feeding and oviposition deterrence, disrupted development in addition to mortality (Schmutterer, 1995). Such effects have implications for parasitoid functioning and evolutionary success and their evaluation is best done within the context of trophic relations. Verkerk & Wright (1996) argued that

the effectiveness of an IPM package for DBM could be enhanced if trophic interactions were taken into consideration.

1.3 JUSTIFICATION AND RATIONALE OF THE STUDY

Sole reliance on chemical insecticide control of DBM is no longer viable because of the DBM's high capacity for developing insecticide resistance, spiralling costs of developing new insecticides, environmental risks caused by insecticide residues in the environment and near-zero tolerance for pesticide residues on food items in the markets. An ecologically compatible DBM management strategy that incorporates the use of parasitoids, biorational insecticides such as botanicals and rational use of synthetic chemical insecticides (Schmutterer, 1992; Taleker & Shelton, 1993; Verkerk & Wright, 1996) is urgently required.

The efficacy of neem insecticides against the DBM has been confirmed in a number of studies (Dreyer, 1987; Fagoonee, 1987; Schmutterer, 1992). So too has the efficacy of DBM larval parasitoids, particularly in North America and Southeast Asia (Ooi, 1992; Poelking, 1992; Talekar & Shelton, 1993). Incorporating neem insecticides and parasitoids into an IPM programme for the DBM in Eastern Africa requires information on the effects of neem insecticides on DBM larval parasitoids but only very limited information exists on this aspect. For instance, Schmutterer's reviews (1990, 1995, 1997) on the non-target effects of

neem insecticides include 17 citations on parasitoids out of which only three (McCloskey *et al.*, 1993; and Schneider & Madel, 1991; Klemm & Schmutterer, 1993 cited in Schmutterer, 1995) are specifically on DBM parasitoids.

Many studies assessing the non-target effects of neem insecticides have concluded that they are harmless to natural enemies, largely based on the International Organisation of Biological Control's [IOBC] protocols for judging insecticide safety (Hassan, 1989). The IOBC protocols were, however, designed to evaluate the effects of faster-acting chemical pesticides and may therefore be inappropriate for neem-based insecticides that have a more subtle effect on insect physiology and life history parameters.

The concentrations of the active ingredients in the raw materials from which neem insecticides are formulated differ from region to region. Additionally, different neem insecticide products are formulated differently. The azadirachtin content in commercial neem formulations is often standardised but since no universal standard exists, it is best to evaluate new formulations as and when they appear. Thus, the results of studies that assessed the non-target effects of neem insecticides using one neem product [or on organisms in a different region] are not readily applicable to a differently formulated product [or organisms in an entirely different region]. The goal of the present study was to investigate the effects of

Neemroc EC® and Neemros® on DBM larval parasitoids in Kenya. These neem insecticides are approved for use in Kenya against horticultural pests including the DBM. Field studies were conducted to obtain an overview of parasitism trends in the field under a neem insecticide application regime, and generate hypotheses for testing under more controlled laboratory conditions. The laboratory tests investigated specific properties of neem insecticides as they relate to parasitoid behaviour and functioning within the context of trophic interactions.

1.4 HYPOTHESES

1. Neem does not affect the level of field parasitism in DBM larval populations.
2. There is no difference in the proportion of hosts parasitised, host searching behaviour and survival of neem-treated and untreated parasitoids.
3. Parasitoid response to stimuli from neem-treated and untreated host plants is the same
4. There is no difference in the proportion of hosts parasitised in neem-treated and untreated hosts.
5. Neem has no effect on the survival, host searching behaviour, development and fitness of parasitoid progeny.

1.5 GENERAL OBJECTIVE

Assess the effects of two neem insecticide formulations on DBM parasitoids at different trophic levels in cabbage.

1.5.1 Specific Objectives

1. To evaluate the effect of neem insecticide applications on parasitism as a larval mortality factor in DBM populations under field conditions.
2. To assess the effects of the neem insecticides on the survival, parasitisation ability and host searching behaviour of *D. mollipla*.
3. To assess the [host-finding] response of *D. mollipla* to stimuli from neem-treated cabbage plants.
4. To establish whether *D. mollipla* attacks and develops normally in neem-treated DBM larvae (= host acceptance and suitability).
5. To assess the effects of the neem insecticides on the development and fitness (longevity, sex ratio, egg viability and parasitisation ability) of *D. mollipla* progeny.

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

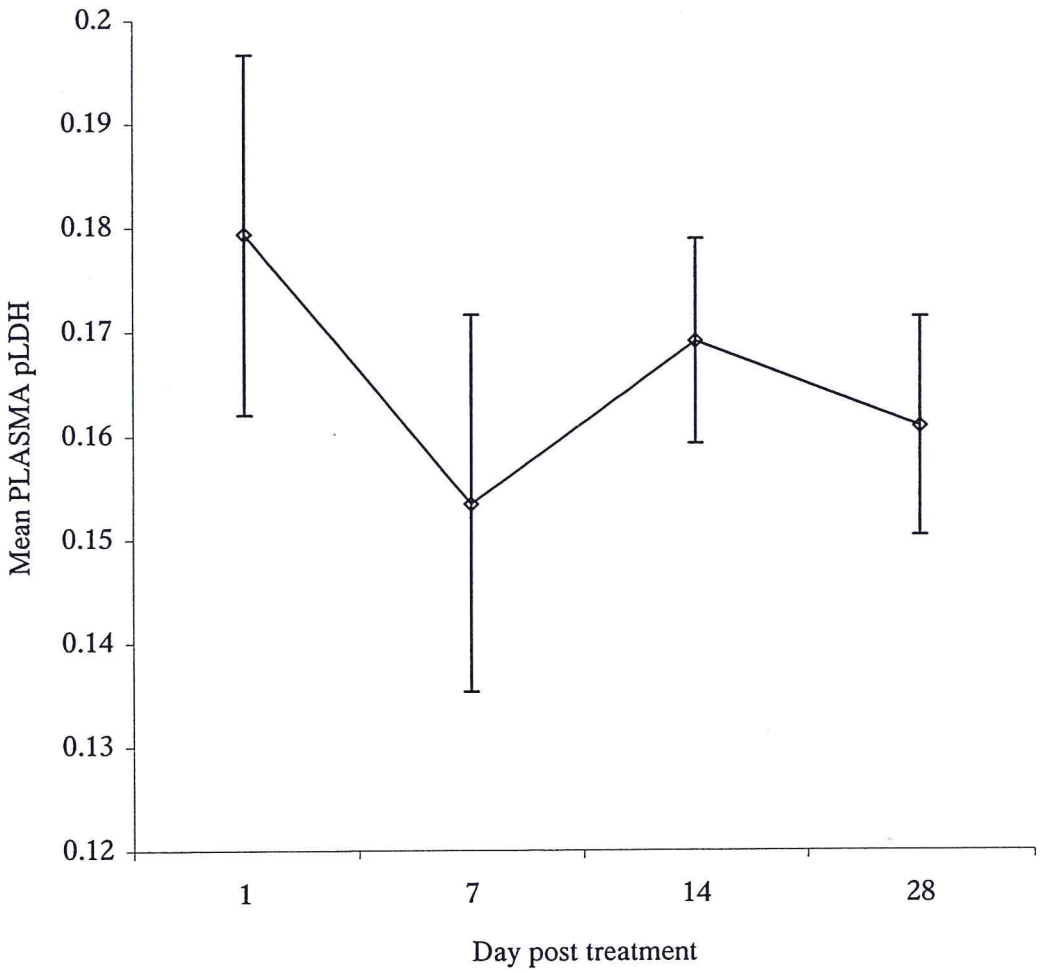
5- Headache + weakness or aches (pains) +fever + vomiting or nausea + diarrhea

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

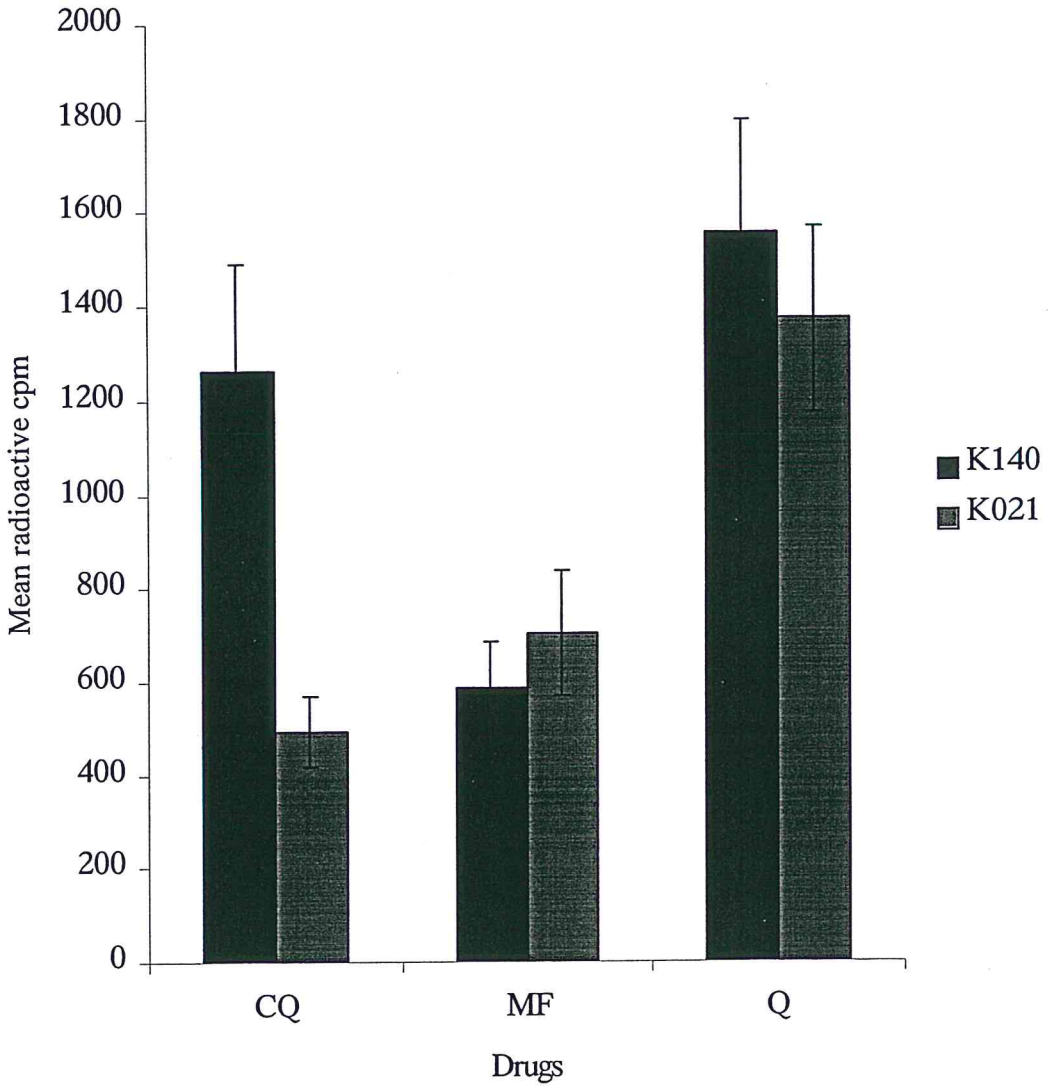
The drug concentration and the mean radioactivity count per minute (cpm) values were used for calculating the IC50s using the modified method of Sixsmith *et al;* (1984). The two field isolates were culture adapted to parasitaemias above 3% and then tested for hypoxanthine uptake using three drugs chloroquine, 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.

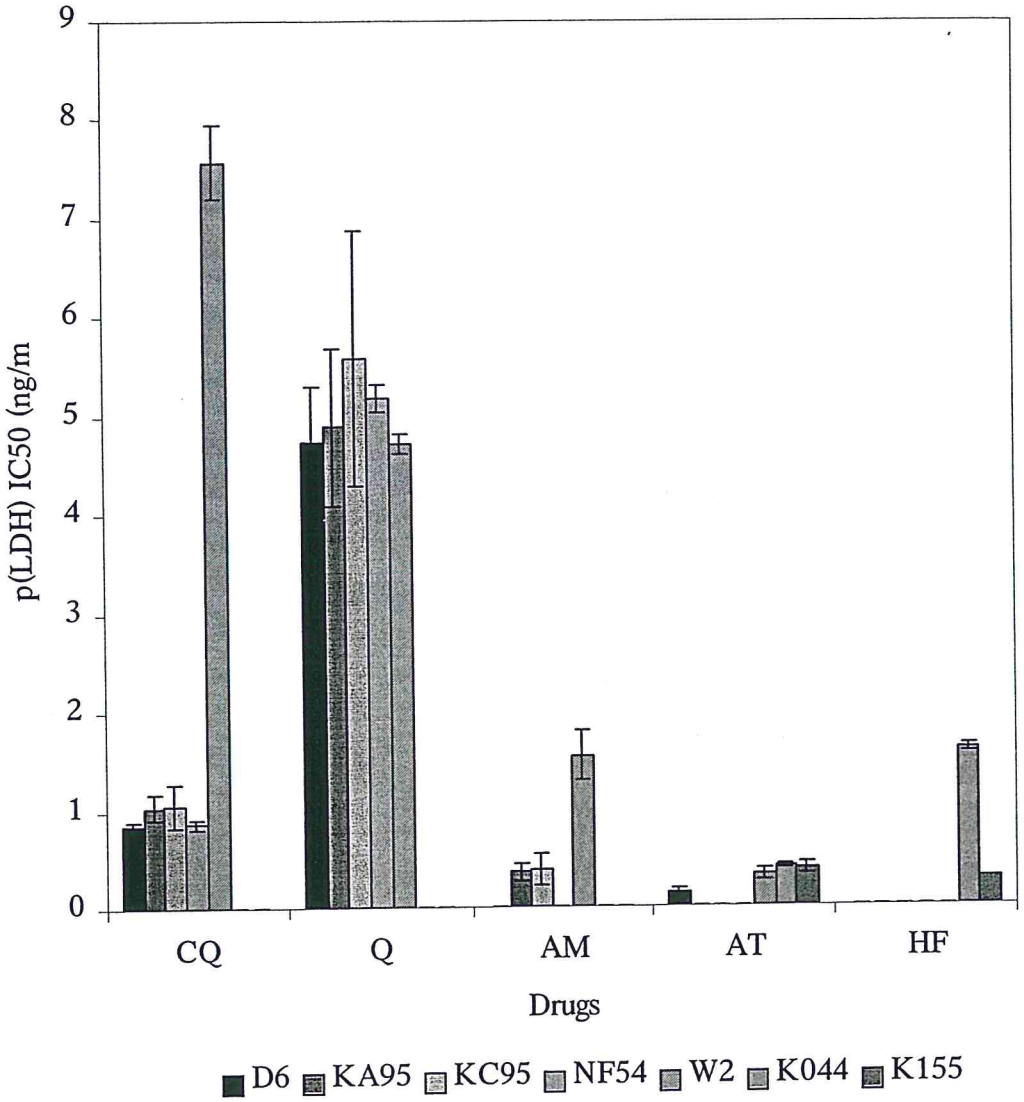
4.4.3 IC50 (in ng/ml) pLDH values for three antimalarial drugs chloroquine (CQ), amodiquine(AM)and quinine(Q),using three laboratory (D6, W2, NF54) and two field (KA95, KC95) adapted malaria parasites on WHO standard plates.

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.

REFERENCES

Anthony, R.L; Bangs, M.J; Anthony, J.M; Purnomo, J. (1992). On site diagnosis *of Plasmodium falciparum, P. vivax,* and *P. malariae* by using the quantitative buffy coat system. *Journal of Parasitology,* Dec. **78** (6): 994-998.

Avila, S.L; Leandro, M.C; Oloveira,M.S; Arruk, V.G; Sanchez, M.C; Boulos, M; Ferreira, A.W. (1994). Evaluation of different methods for plasmodia detection, in well defined population groups in an endemic area of Brazil. *Rev Inst. Med.-TROP- Sao Paulo.* **36** (2): 157-162.

Avila ,S.L. and Ferreira, A. W. (1994). Evaluation of the performance of laboratory methods for malaria diagnosis in endemic areas of Brazil: suggestions for CODE. *American Journal for Tropical Medicine and Hygiene,* **39**: 337-342 .

Basco, L.K and LE Bras, J. (1994). Short- term *in vitro* culture of *Plasmodium vivax* and *P.ovale* for drug susceptibility testing. *Parasitology Research* **80**: 262-264.

Basco, L.K; Marquette, F; Makler, M.M and Le- Bras, J. (1995). *Plasmodium falciparum* and *Plasmodium vivax*: lactate dehydrogenase activity and its application for *in vitro* drug susceptibility assay. *Experimental Parasitology* **80** (2): 260-271.

Bennett, G.F. (1962). The haematocrit centrifuge for laboratory diagnosis of haematozoa. *Canadian Journal of Zoology,* **40**: 124 – 125.

Bloland, P.B; Lackritz, E.M; Kazembe, P.N; Were ,J.B.O; Steketee, R; and Campbell, C.C. (1993). Beyond chloroquine; Implications of drug resistance for evaluating malaria therapy efficacy and treatment policy in Africa- *Journal of infectious diseases* **167** :932 - 937.

Boudreau, E.F; Webster, K.H; Povanand, K. and Thosinga, L. (1982). Type II mefloquine resistance in Thailand. *Lancet* 2: 1335.Brabin, B.J. (1993). Analysis of malaria in pregnancy in Africa. *Bulletin of world health organization.* **6**: 1005-1016.

Brabin, B.J. (1993). Analysis of malaria in pregnancy in Africa. Bulletin of World Health Organization. 61: 1005-1016.

Brasco, L.K; Ramiliorisoa, O. and Le Bras, J. (1995). *In vitro* activity of atovaquone against the African isolates and clones *of Plasmodium falciparum. American Journal of Tropical Medicine and Hygiene*; **53**(4): 388-391.

Brasseur, P; Kouamouo, J; Brandicourt, O (1988). Patterns of *in vitro* resistance to chloroquine, quinine, and mefloquine of *Plasmodium falciparum* in Cameroon in 1985-1986. *American Journal of Tropical Medicine and Hygiene*; **39**: 166-172.

Brockelman, C.R; Tan-Ariya, P. and Laovanitch, J. (1985). Observation on complete schizogony of *Plasmodium vivax in vitro.* Journal of Protozoology, **32**: 76-80.

Bruce-Chwart, L.J.(Ed); Black, R.H; Canfield, C.J; Clyde, D.F; Peters,W and Wernsdorfer, W.H. Chemotherapy of malaria 2nd edition. Geneva, *WHO monograph series.* No. **27**. 1981.

Bruce-Chwatt, L.J (1988). History of malaria from prehistory in eradication. In Malaria: *Principles and practice of malariology Volume1. Ed. Wernsdorfer WH and McGregor I, Churchil Livingstone*, London.

Bzik, D.J; Fox, B.A and Gonyer, K. (1993). Expression of *Plasmodium falciparum* lactate dehydrogenase in *Escherichia coli. Molecular and biochemical Parasitology*; **59**: 155-166.

Caraballo, A. and Ache, A. (1996). Evaluation of a Parasight-F dipstick test for *Plasmodium falciparum* in mining area of Venezuela. *American Journal of Tropical Medicine and Hygiene* **55**(5). 482-484.

Carlson, J; Helmby, H; Hill, A.V.S; Brewster, D; Greenwood, B.M and Wahlgren, M. (1989). Human cerebral malaria association with erythrocyte rossetting and lack of anti rossetting antibodies. *Lancet* **336**: 1457 – 1460.

Carter, R. (1978) Studies of enzyme variation in Murine malaria parasites *P. berghei, P. yoelii, P. vinckei* and *P. chabaudi* by starch gel electrophoresis. *Parasitology* **76**: 241-267.

Carter, E.H., Suhrbier,A., Beckers, P.J.A and Sinden, R.E (1987). The in vitro density gradients. *Parasitology*. **95**: 25-30.

Davidson, D. (1993). Role of arteether in the treatment of malaria and plans for further development. In:artemisinin meeting: Abstracts and Programme. London, The Welcome Trust, 33-34. *Transaction of The Royal Society of Tropical Medicine and Hygiene*, 1994 supplement.

Desjardin, R.E; Canfield, C.J; Haynes, J.D. and Chulay, J.D. (1979).Quantitative assessment of anti malarial activity *in vitro* by a semi automated micro dilution technique. *Antimicrobial Agents and Chemotherapy.* **16**: 710-718.

Dibyendu, De; Krogstad, F.M; Cogswell, F.B. and Krogstad ,D.J. (1996). Aminoquinolines that circumvent resistance in *Plasmodium falciparum in vitro. American Journal of Tropical Medicine and Hygiene.* **55** (6): 579-583.

Dowling, M.A.C. and Shute; G.T. (1966). A comparative study of thin and thick blood films in the diagnosis of scanty malaria parasitaemia. *Bulletin of the World Health*

Organization . **34** : 249 - 267.

Duggan, A. J. and Hutchson, M.P (1966). *Journal of tropical medicine and Hygiene*. **69**: 124-131.

Earle, W.C and Perez, M.(1932). Enumeration of parasites in the blood of malaria patients. *Journal of laboratory and clinical Medicine*. **17**: 1124-1130.

Fisher, M.J; Gazzard, B.G; Hawkins, D.A and Nelson, M.R. (1994). Atovaquone as prophylaxis against *Pneumocystis carinii* pneumonia. *Journal of infection* **28** ; 103 - 104

Flow Incl.6127 S.W corbett, Portland, O.R 97201 U.S.A.

Fry, M. and Pudney, M. (1992). Site of action of antimalarial hydroxinynaphthoquinone, 2-trans-(4-chlorophenyl cyclohexyl) 3-hydroxy-1,4-naphthoquinone (566C800) . *Biochemical pharmacology*, **43**: 1545-1555.

Galen, R.S. and Gambino, S.R. (1975). Beyond Normality. The predictive value and efficiency of Medical diagnosis. *New York: John Wiley and sons* 115.

Garin, B; Salun, J.J; Peyron, F; Vigier, J.P; Busangu, I; Perone, J. (1992). Rapid *in vivo* detection of chloroquine resistance by quantitative buffy coat malaria diagnosis system. *American Journal of Tropical Medicine and Hygiene*. **47** (4): 446-449.

Gevers, T; and Kustner, H.G.V. (1988). Malaria, diagnostic criteria. *South African Medical Journal 74* (8):427.

Gomez, M.S; Piper, R.C; Hunsaker, L.A; Royer, R.E; Deck, L.M; Makler, M.T; Jagt, DL-vander and Vander, Jagt, D.L. (1997). Substrate and cofactor specificity and selective inhibition of lactate dehydrogenase from the malarial parasite *P. falciparum*. *Molecular and Biochemical Parasitology*. **90** (1): 235-246.

Goodyer, I.D. and Taraschi, T.F. (1997). *Plasmodium falciparum*: A simple rapid method for detecting parasite clones in microtiter plates. *Experimental Parasitology* **86**; 158-160.

Goyal, R; Mahajan, R.C; Ganguly, N.K; Sehgal, R; Gorowara, S and Singh, K. (1993). Macrophage-mediated enterocyte damage in BALB/c mice infected with different strains of *Giardia lamblia*. *Scandinavian Journal of Gastroenterology* **28** (9): 845-848.

Gray, J.S. (1983) The chemotherapy of *Babesia divergens* in the gerbil, *Meriones unguiculatus*. *Research in veterinary Science* **35**: 318 - 324.

Greenberg, R.S; Daniels, S.R; Flanders, W.D; Eley, J.W and Boving, J.R. (1993). Diagnostic testing: Medical epidemiology. *Appletion and Lange, Norwalk, Connecticut* 58-67.

Gyorkos, T.W; Svenson, J.E; Maclean, J.D; Mohamed, N; Remondin, N.H. and Franco, E.D. (1995). Compliance with antimalarial chemoprophylaxis and the subsequent development of malaria a matched case control study. *American Journal of Tropical Medicine and Hygiene* **53** : 511 – 517.

Hagos, B; Khan, B; Ofulla, A.V.O; Kariuki, D. and Martin, S.K. (1993). Response of *falciparum* malaria to chloroquine and three second line antimalarial drugs in a Kenyan coastal school age population. *East African Medical Journal* **70** (10) : 620-623.

Handunnetti, S.M; David, P.H; Perera, KLRL. and Mendis, K.N. (1989). Uninfected erythrocytes form rosette around *Plasmodium falciparum* infected erythrocytes. *American journal of Tropical Medicine and Hygiene* **40**: 115 –118.

Harinasuta, T. and Bunnag D. (1988). The clinical features of malaria. Wernsdorfer, W.H,Mc George I. eds . malaria. *Principles and Practice* of malariology. *Edinburgh: Churchill Livingstone*, 709 - 734.

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

Hudson, A.T. (1993). Atovaquone-A novel broad spectrum anti-infective drug. *Parasitology Today* 9:66-68.

Hudson, A. T; Dickins, M; Ginger, C.D; Gutteridge,W.E; Holdich, T; Hitchinson, B.A; Pudney, M; Rendal, A.W. and Latter, V.S. (1991). 566C80: A broad spectrum anti infective agent with activity against malaria and opportunistic infections in AIDS patients. *Drugs in experimental and clinical research* **17**: 427 – 435.

Hughes, W.T; Kennedy, W.T; Shenep, J.L; Flynn, P.M; Hetherington, S.V; Fullen, G; Lancaster, D.J; Stein, D.S; Palter, S; Rosenbaum,D; Liaos, S.H.T; Blum, M.R; Rogers, M.D. (1991). Safety and pharmacokinetics of 566C80, a hydroxynaphthoquinone with anti-*pneumocystis carinii* activity: phase 1 study in human immunodeficiency virus (HIV)- infected men. *Journal of infectious diseases*, **163**: 843-848.

Hughes, W.T; Gray,V.L; Gutteridge, W.E; Lutter, V.S. and Pudney, M. (1990). Efficacy of a hydroxynaphthoquinone, 566C80,in experimental *Pneumocystis carinii* pneumonitis. *Antimicrobial Agents and Chemotherapy* **34**: 225 – 228.

Hurwitz, E. S; Johnson, D. and Campbell, C. (1981). Resistance of *P. falciparum* malaria to sulfadoxine- pyrimethamine in a refugee camp in Thailand. *Lancet* 1: 1068.

Jamjoom, G.A (1983). Dark- field microscopy for the detection of malaria in unstained

blood films. *Journal of clinical microbiology* **17**; 717 - 721.

Jelinek,T; Kilian, A.H.O; Henk, M; Mughusu, E.B; Northdurfr, H.D; Loscher, T; Knobloch, J. and Sonnenburg, F.V. (1996a). Parasite specific lactate dehydrogenase for the diagnosis of *Plasmodium falciparum* in an endemic area in Western Uganda. *Tropical Medicine and International Health.* **I** (2). 227-230.

Jelinek, T; Proll, S; Hess, F; Kamagambe, G; Von Sonnenburg, F; Loscher, T. and Kilian, A.H.D. (1996b). Geographic differences in the sensitivity of polymerase chain reaction for the detection of *Plasmodium falciparum* infections. *American Journal of Tropical Medicine and Hygiene*. **55** (6): 647-651.

Kager, P. (1993). Safety, tolerance and pharmacokinetics of arteether in man. In: Artemisinin meeting: Abstracts and programme. London, The Welcome Trust 31-32,. *Transaction of The Royal Society of Tropical Medicine and Hygiene*, 1994 supplement.

Kamau, L; Lettman, T; Hawley, W:A; Orago, A:S:S and Collins F:H (1998). Microgeographic genetic differentiation of *Anopheles gambiae* mosquitoes from Asembo Bay, Western Kenya: A comparison with Kilifi in coastal Kenya. *American Journal of Tropical Medicine and Hygiene.* **58**: (1) 64-69.

Kanaani, J. and Ginsburg, H. (1991). Transport of lactate in *Plasmodium falciparum*-infected human erythrocytes. *Journal of cellular physiology* **149**: 469-476.

Kariuki, M.M; Kiaira,J.K; Mulaa,F.K; Mwangi,J.K; Wasunna,M.K and Martin,S.K. (1998). *Plasmodium falciparum*: Purification of the various gametocyte developmental stages from in vitro- cultivated parasites. *American Journal of Tropical Medicine and Hygiene* **59** (4): 505-508.

Kaushal, D.C; Srivastava, K.K; Puri, S.K; Kaushal, N.A. and Dutta, G.P. (1985). Studies on enzyme variation in simian malaria parasites by polyacrylamide gel electrophoresis. *Indian Journal of Parasitology* **9** : 293-296.

Kaushal, D.C; Watts, R; Haider, S; Singh, N; Kaushal, N.A. and Dutta, G.P. (1988). Antibodies to lactate dehydrogenase of *Plasmodium knowlesi* are specific to *Plasmodium* species. *Immunol. Inves.* **17** : 507 - 516.

Kaushal, D.C; Kaushal, N. A. and Kaslow, D.C. (1993). Amino acid sequence of the NH_2-terminus of *Plasmodium knowlesi* lactate dehydrogenase. *Molecular and Biochemical Parasitology* **59**: 167-170.

Kaushal, D.C; Kaushal, N.A; Chandra, D and Palni, R (1995). Immunodiagnosis of malaria based on detection of parasite enzyme. *Journal of Parasitic Diseases* **19** (1): 21-24.

Kirkman, L.A; Su, X.Z. and Wellems T.E. (1996). *Plasmodium falciparum*: Isolation of large numbers of parasite clones from infected blood samples. *Experimental Parasitology* **83**: 147-149.

Kitchen, S.F (1949). Symptomatology: general considerations and *falciparum* malaria. In Boyd M.F. (Ed). *Malariology, vol. 2. Philadelphia: W. B Sauners,*: 996-1017.

Knobloch, J. and Henk, M. (1995). Screening of malaria by determination of parasite-specific lactate dehydrogenase. *Transactions of the Royal society of Tropical Medicine and Hygiene* **89**: 269-270.

Klayman, D.L.(1985). Qinghaosu (artemisinin). An antimalarial drug from China. *Science* **228**: 1049-1055.

Kremsner, P.G; Zotter, G.M; Fielmeier, H. (1988). A comparative trial of three regimens for treating uncomplicated *falciparum* malaria in Acre, Brazil. *Journal of Infectious Diseases 158* : 1368-1371.

Kumar, B.K; al Fadeel, M; and Sehgal, S.C.(1993). Efficacy and limitations of QBC acridine orange staining as a routine diagnostic technique for malaria in developing countries. *Journal of Tropical Medicine and Hygiene* **96** (4): 245-248.

Kumar, N and Zheng, H. (1990). Stage- specific gametocytocidal activity effect in vitro of the antimalarial drug qinghaosu on Plasmodium falciparum. Parasitological Research, 76:214-218.

Kwiatkowski, D; Hill, A.V.S; Sambou, I. (1990). TNF concentrations in fatal cerebral, non-fatal cerebral and uncomplicated *Plasmodium falciparum* malaria. *Lancet* **336**:1201-1204.

Lambros, C. and Vanderberg, J.P. (1979). Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *Journal of Parasitology*, **65**: 418-420.

Lang-Unnasch, N. (1992). Purification and properties of *Plasmodium falciparum* malate dehydrogenase. *Molecular and biochemical Parasitology*, **50**: 17-26.

Lee, I.S; and Hufford, C.D. (1990). Metabolism of antimalarial sequiterpene lactones. *Pharmacological Therapeutics* **48**: 345-355.

Lelijveld, J and Kortmann (1970) .The oesin colour test of Dii and Glazko: a sample field test to detect chloroquine in urine. *Bulletin of World Health Organisation* **42**: 477.

Logt, J.T.M-van-der; Kissing, J; Melchers, W.J.G. and Van-der-Loft, J.T.M. (1994). Enzymatic amplication of lactate dehydrogenase- elevating virus. *Journal of Clinical Microbiology* **32** (8): 2003-2006.

Long,G.W; Rickman, L.S; Cross, J.H. (1990). Rapid diagnosis of *Brugia malayi* and *Wuchereria bancrofti* filariasis by an acridine orange / hematocrit tube technique. *Journal of Parasitology*. **76** (2): 278-281.

Li, G.Q; Guo, X.B. and Jiang, R. (1992). Clinical studies on treatment of cerebral malaria with Qinghaosu and its derivatives. *Journal of traditional China Medicine* **2**: 124-130.

MacPherson, G.G; Warrell, M.J; White, N.J; Looaneesuwan, S. and Warrell, D.A. (1985) Human cerebral malaria: a quantitative ultrastructural analysis of parasitized erythrocyte sequestration . *American journal of Pathology* **119**: 385 - 401

Mak, J.W; Normaznah,Y; and Ciang, G.L. (1992). Comparison of quantitative buffy coat technique with the conventional thick blood film technique for malaria case detection in the field . *Singapore Medical Journal* **33** (5): 452-454.

Makler, M.T. and Levine, R.A. (1989). Haematoparasites and QBC analysis. *Parasitology Today* **10** *(5)*: 325-326.

Makler, M.T. and Gibbins-B. (1991). Laboratory diagnosis of malaria. *Clinical Laboratory Medicine* **11**(4): 941-956.

Makler, M.T and Hinrichs, D.J. (1993). Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. *American Journal of Medicine and Hygiene* **48** (2): 205-210.

Makler, M.T; Ries, J.M; Williams, J.A; Bencroft, J.E; Piper, R.C; Gibbins, B.L. and Hinrichs, D.J. (1993). Cultivation of *Plasmodium falciparum* for drug sensitivity. *American Journal of Tropical Medicine and Hygiene*. **48**(6): 739-741.

Makobongo, M; Weiss, W.R; Duffy, P.E; Okello, R:O. and Orago, A:S:S. (1997). A longitudinal study of natural killer (NK) cells and T. lymphocyte levels in adult residentsof a *Plasmodium falciparum* malaria holoendemic area of Western Kenya. Abstact 3rd Federation of African Immunological Societies congress (FAIs). Arthurs street Hotel, Cape Town, South Africa, March, 9-13.

Mc.Gregor, I.A. (1984). Epidemiology, malaria and pregnancy. *American Journal of Tropical Medicine and Hygiene* **33**: 517-525.

Mead, R and Curnow, (1986). Statistical Methods in Agriculture and Experimental Biology. Chapman and Hall, London. NewYork. 267-273.

Mehra, N and Bhasin, V.K (1993). *In Vitro* gametocytocidal activity of artemisinin and its derivatives on *Plasmodium falciparum*. *Japanese Journal of Medical Sciences and Biology*; 46: 37-43.

Milhous, W.K; Weatherly, N.F; Bowdre, J.H; Desjardins, R.E. (1985). *In vitro* activities of and mechanisms of Resistance to antifol antimalarial drugs. *Antimicrobial agents and chemotherapy* **27**(4): 525-530.

Miller, L.H. and Warrell, E.A. (1990). Malaria. Warren K.S, Mahmoud AAF, eds. *Tropical and Geographical Medicine*. Second edition. New York: McGraw-Hill, Inc., 245-264.

Nevill, C.G; Munafu, C.G; Vehof, F; Ten Hove, R. and Were, J.B (1990). Is there still a role in amodiaquine in the therapy of *P. falciparum* malaria in Eastern Africa. The eleventh annual Medical Scientific Conference of Kenya Medical Research Institute (KEMRI) and Kenya Trypanosomiasis Research Institute (KETRI) 5 - 9 February.

Nevill, C.G; Verhoeff, F.H; Munafu, C.G; Ten Hove, W.R; Van Der Kaay, H.J. and Were, J.B.O. (1994). A comparison of amodiaquine and chloroquine in the treatment therapy of malaria in Kenya. *East African Medical Journal.* **71** (3): 167-170.

Nguyen-Dinh, P; Makler, M.T; Wentworth, L. K. and Collins, W.E. (1994). Evaluation of parasite lactate dehydrogenase as a diagnostic tool for the detection of malaria parasites. *American Journal of Tropical Medicine and Hygiene*; **51**:264.

Ofulla, A.V.O (1994). Serum-free culture media for in- vitro studies of *Plasmodium falciparum*. A Ph.D.thesis submitted in the Department of Zoology, Kenyatta University, Nairobi, Kenya.

Ofulla, A.V.O; Okoye, V.C.N; Khan, B; Githure, J.I; Robert, C. R; Johnson, A.J. and Martin, S. K. (1994). Determination of fifty per cent inhibitory concentrations (IC_{50}) of antimalarial drugs against *Plasmodium falciparum* in a serum-free medium. *American Journal of Tropical Medicine and Hygiene*. **51** (2): 214-218.

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

OMS (1990). Practical chemotherapy malaria *Technical report* 805.

Orago, A.S.S and Solomon, J.B (1986). Antibody- dependent and independent cytotoxic activity of spleen cells for *Plasmodium berghei* from susceptible and resistant rats. *Immunology*, **59:** 283-288.

Orago, A.S.S. and Facer, C.A. (1991).Cytotoxicity of human natural (NK)cell subsets for *Plasmodium falciparum* erythrocyte schizonts: Stimulation by cytokines and inhibition by neomycin. *Clinical and experimental immunology*. **86**: 22-29.

Othoro, C (1997). Cytokine expression during acute Plasmodium falciparum infection of children resident in an endemic region of Western Kenya. MSC.Thesis- Kenyatta University.

Othoro, C., Lal, A.A., Nahlen, B., Koech, D., Orago, A.S and Udhayakumar,V. (1999). A low interleukin-10 tumour necrosis factor- alpha ratio is associated with malaria anaemia in children residing in a holoendemic malaria region in Western Kenya. *Journal of Infectious Diseases* **79** (1) 279-282.

Oppenheimer, S.J; Gibson, F.D.; Mcfarland, S.B. (1986). Iron supplimentation increases prevalence and effects of malaria: a report on clinical studies in Papua New Guinea. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **80**:603-612.

Pampana, E.J. (1969). A textbook of malaria eradication, 2nd edition. *London. Oxford University Press*.

Parra, M.A; Evans, C.B. and Taylor, D.W. (1991). Identification of *Plasmodium falciparum* histidine-rich protein-2 in the plasma of humans with malaria. *Journal of Clinical Microbiology* **29**: 1629-1634.

Patnaik, J.K; Sanar Das, B; Mishra, S.K. and Mohonty, D. (1994). Vascular clogging, Mononuclear cell margination and enhanced vascular permeability in the pathogenesis of human cerebral malaria. *American Journal of Tropical Medicine and Hygiene*. **51**(5) 642-647.

Payne, D. (1988). Use and limitations of light microscopy for diagnosing malaria at the primary health care level. *Bulletin of the World Health Organization* **66**(5): 621-626.

Peel, S.A; Bright, P; Yount, B; Handy, J. and Baric, R.S. (1994). A strong association between mefloquine and halofantrine resistance and amplification, overexpression and mutation in the P- Glycoprotein Gene homolog (pfmdr) of *Plasmodium falciparum in vitro*. *American Journal of Tropical Medicine and Hygiene* **51**(5): 648-658.

Peters, W. (1985). The two problems of drug resistance in malaria. *Parasitology* **90**: 705 – 715.

Petterson, T. Kyoronsappa, H and Pitkanen, T (1981) Chloroquine resistant *falciparum* malaria from East Africa. *Transactions of Royal Society of tropical medicine and Hygiene* **75**:112

PettyJohn, F.S. (1975). Centrifuge techniques in the preparation of malaria thin blood smears. *Military medicine* **140**: 535 - 537.

Pfaller, M.A; Krogstad, D. and Parquette, A. (1982). *Plasmodium falciparum*: stage-specific lactate production in synchronized cultures. *Experimental Parasitology* **54**: 391-396.

Playfair, J.H.L; Taverne, J; Bate, C.A.W. and de Souza J.B. (1990). The malaria vaccine: antiparasite or anti-disease immunity. *Immunology Today* **11**: 25-27.

Pongponratn, E; Riganti, M; Punpoowong, B. and Aikawa, M. (1991). Microvascular sequestration of parasitized erythrocytes in human *falciparum* malaria- a pathological study. *American journal of Tropical Medicine and Hygiene* **44**: 168-175.

Premji, Z; Minjas, J.N. and Shift, C.J. (1994). Laboratory diagnosis of malaria by village health workers using the rapid manual Parasight-F tet. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88**: 418.

Price, R.N; Nosten, F; Luxemberger, C. (1996). Effect of artemisinin derivatives on malaria transmissibility. *Lancet*, **347**: 1654-1658.

Price, R.N; Nosten,F; Luxemberger, C (1997). Artesunate- mefloquine treatment of 1967 patients with multidrug resistant *falciparum* malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **91**: 574-577.

Pudney, M. and Gray, J.S. (1997). Therapeutic efficacy of atovaquone against the Bovine intraerythrocytic parasite, *Babesia divergens*. *Journal of Parasitology* **83** (2): 307-310.

Quintana, M; Piper, R; Boling, H-L; Makler, M; Sherman, C; Gill, E; Fernandez, E and Martin, S.K (1998). Malaria diagnosis by dipstick assay in a Honduran population with coendemic *Plasmodium falciparum* and *Plasmodium vivax*. *American Journal of Tropical Medicine and Hygiene* 59 (6): 868-871.

Raghavan, K. (1966). Statistical consideration in the microscopial diagnosis of malaria with special reference to the role of cross checking. *Bulletin of the World Health Organisation*. **34**: 788 –791.

Republic of Kenya, Ministry of Health, (1997). National guidelines for diagnosis, treatment and prevention of malaria for health workers.

Republic of Kenya (1990). Ministry of Health: *Guidelines for the management of malaria in Kenya. Division of vector borne disease, Nairobi, Kenya.*

Riandey, M.F; Sannier, C; Peltre, G; Monteny, N; and Covaleyra, M. (1996). Lactate dehydrogenase as a marker of *Plasmodium* infections in malaria vector anopheles. *Journal of American Mosquito Control Association* **12**(2): 194-198.

Rickman, L.S; Long, G.W and Oberst, R. (1989). Rapid diagnosis of malaria by acridine orange staining of centrifuged parasites. *Lancet* **I**: 68-71.

Rieckmann, K.H; Mc Namara, J.V; Frischer, H; Stockert, T.A; Carlson, P.E. and Powel, R.D. (1968). Effects of chloroquine, quinine and cycloguanil upon the maturation of asexual erythrocytic forms of two stains of *Plasmodium falciparum in vitro*. *American Journal of Tropical Medicine and Hygiene* **17**: 661-671.

Rieckmann, K.H; Sax, L.J; Campbell, G.H. and Mrema, J.F. (1978). Drug sensitivity of *Plasmodium falciparum*: An *in vitro* microtest. *Lancet* **1**: 22-23.

Riley, E.M., Anderson, L and Otoo, N (1988). Cellular immune responses to Plasmodium falciparum antigen in Gambian children during and after an acute attack of Plasmodium falciparum malaria

Rock, E.P; Marsh, K; Saul, A.J; Wellems, T.E; Taylor, D.W; Maloy, W.L and Howard, R.J. (1987). Comparative analysis of the *Plasmodium falciparum* histidine-rich proteins HRP-I, HRP-II and HRP-III in malaria parasites of diverse origin . *Parasitology* **95**: 209-227.

Ross, R. (1897). On some pecular cells found in two mosquitos fed on malarial blood . *British Medical Journal* 1879i: 1786 - 1788.

Ross, R. (1903). An improved method for the microscopical diagnosis of intermittent fever. Lancet **1**: 86.

Roth, E.F; Jr Calvin, M.C; Max-Audit, I; Rosa, J. and Rosa, R. (1988). The enzyme of the glycolytic pathway in erythrocytes infected with *Plasmodium falciparum* malaria parasites. *Blood* **72**: 1922-1925.

Royer, R.E; Deck, L.M; Campos, N.M; Hunsaker, L.A; Jagt, D.L-van-der; andVan-der-Jagt, D.L. (1986). Biologically active derivatives of gossypol: synthesis and antimlarial activities of peri-acylated gossylic nitriles. *Journal of Medicinal Chemistry* **29**(9): 1799-1801.

Sanderson, A; Walliker, D. and Molez, J.F. (1981). Enzyme typing of *Plasmodium falciparum* from African and some other old world coutries. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **75**: 263-267.

Schapira, A; Beales, P.F. and Halloran M.E. (1993). Malaria: living with drug resistance. *Parasitology Today* **9**: 168 - 173.

Scheibel, L.W., Ashton, S.H and Tragger W (1979). *Plasmodium falciparum*: Microaerophilic requirements in human and red blood cells. *Experimental Parasitology* **47**: 410-418.

Shakespeare, P.G; Trigg P.I., Kyd, S.I and Tappenden L (1979). Glucose metabolism in the simian malaria parasite *Plasmodium knowlesi*. Activities of the glycolytic and pentose phosphate pathways during the intraerythrocytic cycle. *Annals of Tropical Medicine and Parasitology* **73**: 407-415.

Sherman , I. (1961). Molecular heterogeneity of lactic dehydrogenase in avian malaria (*Plasmodium lophurae*) . *Journal of experimental medicine* ,**114**: 1049-1062.

Sherman , I. W. (1979) . Biochemistry of P*lasmodium* (malarial parasite*). Microbiological review* 43: 453- 495.

Sherman , I.W. (1983) . Biochemistry of *Plasmodium*. 5th meeting of Scientific Working Group on the Chemotherapy of malaria in : Modern design of antimalarial drugs 17-34. *UNDP / World Bank / WHO, Geneva.*

Sherman, I.W (1984). Metabolism. In: Antimalarial drugs. I. Biological background, experimental methods and drug resistance (Peters, W and Richards, W.H.G., eds) 31-81. Springer verlag Heidelberg.

Sherman, I.W. (1991). The biochemistry of malaria: An overview. In "Biochemical protozoology"(G.H. Coombs and M.J. North, Eds.) 6-34

Shiff, C.J., Premji, Z. Minjas J.N (1993). The rapid manual Parasight®-F test. A new diagnostic tool for *Plasmodium falciparum* infection. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **87**: 646-648.

Shiff, C.J; Premji, Z. and Minjas, J.N. (1994). The Parasight-F test. A simple rapid manual dipstick test to detect *Plasmodium falciparum* infection . *Parasitology Today* **10**: (12) 494 - 495.

Shililu, J.I; Maier, W.A; Seitz, H.M. and Orago, A:S:S. (1996). Seasonal patterns of malaria morbidity in two high altitude sites in Kakamega District, Western Kenya. *Journal of protozoology Research*. **6**: 37-45

Shililu, J.I; Maier, W.A; Seitz, H.M. and Orago, A.S.S. (1998). Seasonal density, sporozite rates and entomological innoculation rates of *Anopheles gambiae* and *Anopheles funestus* in a high altitude sugarcane growing zone in Western Kenya. *Tropical Medicine and international health*. Accepted.

Shute, G.T. and Sodeman, T.M. (1973). Identification of malaria parasites by fluorescence microscopy and acridine orange staining . *Bulletin of the World Health Organisation*, **48**: 591 - 596.

Silamut, K. and White, N.J. (1993). The relationship of stage of parasite development to prognosis in *falciparum* malaria. *Transactions of the Royal society of Tropical Medicine and Hygiene* **87**: 436-443

Sixsmith, D.G; Watkins, W.M; Chulay, J.D. and Spenser, H.C. (1984). *In vitro* antimalarial activity of tetrahydrofolate dehydrogenase inhibitors. *American Journal of Tropical Medicine and Hygiene.* **33** (5): 772-776.

Snounou,G; Viriyakosol, S; Zhu, X.P; Jarra, W; Pinhriro, L; Rosario, V.E; Thaithong, S and Brown, K.N. (1993a). High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Molecular Biochemical Parasitology* **61**: 315-320.

Snounou,G; Viriyakosol, S; Jarra, W; Thaithong, S and Brown, K.N. (1993b). Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and the detection of high prevalence of mixed infections. *Molecular and Biochemical Parasitology* 58: 283-292.

Spencer, H.C; Kariuki, D.M. and Koech, D.K. (1983). Chloroquine resistance in *Plasmodium falciparum* from Kenyan infants. *American Journal of tropical Medicine and Hygiene* **32**: 922.

Spielman, A; Perrone, J.B; Teklehaimanot, A; Balcha, F; Wardlaw, S.C. and Lavine, R. A. (1988). Malaria diagnosis by direct observation of centrifiged samples of blood. *American Journal of Tropical Medicine and Hygiene* **39** : 337 - 342.

Svenson, J.E; Gyrkos,T.W. and Maclean, J.D. (1995). Diagnosis of malaria in the febrile traveller. *American Journal of Tropical Medicine and Hygiene* **53**(5): 518-521.

Taylor, N (1945). Cinchona in Java: The story of quinine. Greenberg, New York. T.D.R (1990). Tenth programme Report, W.H.O. Geneva.

Taylor, D.W. and Voller, A. (1993). The development and validation of a simple antigen detection ELISA for *Plasmodium falciparum* malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **87**:29-31.

Taylor, P. and Mtambu, S.L. (1986). A review of the malaria situation in Zimbabwe with special reference to the period 1972 -1981 . *Transactions of the Royal Society of Tropical Medicines and Hygiene* **80**: 12 – 19.

Taylor, P. and Taputaria, A. (1988). Forty years of malaria control in Zimbabwe. *Proceedings of the First National Symposium on Science and Technology, Harare*, 4-6 January.

.ter Kuile, F; White, N.J. and Holloway, P. (1993). *Plasmodium falciparum*: *In vitro* studies of the pharmacodynamic properties of drugs used in the treatment of severe malaria. *Experimental Parasitology* **76**:85-95.

Trager, W. and Jensen. (1976). Human malaria parasites in continuous culture. *Science* **193**: 673-675.

Udomsangpetch, R.J; Todd, J; Carlson, N; and Greenwood, B.M, (1993). The effect of haemoglobin genotype and the ABO blood on the formation of rosettes by *Plasmodium falciparum* infected red blood cells. *American Journal of Tropical Medicine and Hygiene.In Press.*

UNDP/ World Bank/ WHO (1994). Special Programme for Research and Training in Tropical Diseases, WHO, Geneva. Malaria Unit, Division of control of Tropical Diseases. Division of drug management policy.

Unnasch-Lang, N. (1992). Purification and properties of Plasmodium falciparum malate dehydrogenase. *Molecular and Biochemical parasitology* **50**: 17-26.

Vandet Jagt, D.L; Hansaker, L.A. and Heidrich, J.E. (1981). Partial purification and characterisation of lactate dehydrogenase from *Plasmodium falciparum* . *Molecular and Biochemical Parasitology* **4**: 255 - 264.

Vennerstrom, J.L; Makler, M.T; Angerhofer, C.K. and Williams, J.A. (1995). Antimalarial dyes revisited: xanthenes, azines, oxazines, and thiazines. *Antimicrobial agents and Chemotherapy* **39** (12): 2671-2677.

Wang-FuYong; Cheng-YiLiang; Chen-XiXin; Wang-HuiDong; Liu-LunHua; Yu-ZhenHua; Kou-JingXuan; Wang-FY; Cheng- YL; Chen-XX; Wang-HD; Liu-LH; Yu-ZH; and Kou- JX (1996). Studies on the detection of specific lactate dehydrogense in the blood of patients with malaria. *Chinese Journal of Parasitic Disease Control* **9** (1); 13-15.

Warsame, M; Wernsdorfer, W.H, Payne, D. and Bjorkman, A.(1991). Susceptibility of *Plasmodium falciparum in vitro* to chloroquine, mefloquine and sulfadoxine /pyrimethamine in Somalia: relationship between the responses to different drugs. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **85**:565-569.

Wasonga, W (1997). A longitudinal follow-up of natural immune response to *Plasmodium falciparum* infection in children living in an endemic area of Western Kenya. MSc. Thesis-Kenyatta University.

Watkins, W.M, Parcy, M., Crampton, J.M. (1988). The changing responses *of Plasmodium falciparum* to anti malarial drugs in East Africa. *Transaction of the Royal Society of Tropical Medicine and Hygiene* **82**: 21-26.

Watts, R; Kaushal, D.C; Halder, S; Singh, N; Kaushal, N.A; Puri, S. K. and Dutta, G,P. (1987). Detection of antibodies to lactate dehydrogenase of *Plasmodium Knowlesi* by immunodot enzyme staining . *Biol. Memb.* **13**:161 –164.

Weiss, W.R; Oloo, A.J; Johnson, A. (1995). Daily primaquine is effective for prophylaxis against *falciparum* malaria in Kenya: Comparison with mefloquine, doxycycline, and chloroquine plus proguanil. *Journal of Infectious Diseases* 171: 1559-1575.

Wernsdorfter, W.H. and Pyne, D. 1991. The dynamics of drug resistance in *Plasmodium falciparum. Pharmacologicl Therapeutics* 50: 95-21.

White, N.J. (1992). Antimalarial drug resistance: the pace quickens. *Journal of antimicrobial chemotherapy* **30**: 571-585.

White, N.J. (1996). Malaria: In "Mansons tropical diseases". Edited by Gordon, C. Cook. Chapter 61: *W.B. Saunders company limited.* 20th Edition.

White, N.J. and Ho, M. (1992). Pathophysiology of malaria. *Advanced Parasitology* **31**: 34-173.

White , N. J.; Miller, K. D. and Marsh, K. (1987). Hypoglycaemia in African children with severe malaria. *Lancet* **I**: 708-711.

White, N.J; Warrell, D.A. and Looareesuwan, S. (1985). Pathophysiological and prognostic significance of cerebospinal-fluid lactate in cerebral malaria. *Lancet* **I**:776-778.

Wongsrichanalai-C; Chuanak-N; Webster, H.K, Prasittisuk-M. (1992). Practical uses of acridine orange fluorescence microscopy of centrifuged blood (QBC malaria test) and the Q.B. C. 11 haematology system in patients attending malaria clinics in Thailand. *South East Asian Journal of Tropical Medicine and Public Health* **23**(3): 406-413.

World Health Organization. *Technical report series* No. 712, 1984 (Malaria control as part of primary health care: Report of a WHO study group).

World Health Organization. Malaria action programme . Severe and complicated malaria (1986a). *Transactions of the Royal society of tropical medicine and Hygiene* **8** (supplement): 1 – 50.

World Health Organization. *Technical report series* No. 735, 1986b (WHO expert committee on malaria : eighteenth report).

World Health Organization (1988). Malaria diagnosis: Memorandum from a WHO meeting. *Bulletin of the World Health Organization* **66**(5) 575-594.

World Health Organization. Practical Chemotherapy of malaria. *WHO technical report series*, 1990a:805.

World Health Organization. Division of control of tropical diseases: severe and complicated malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 1990b. **84** (supplement 2): 1-65.

World Health Organization (1992). Ministerial Conference on Malaria . Amsterdam, The Netherlands, 26-27 October,. Geneva *: World Health Organisation , Mimeographed document CTD /MEM/92.3.*

World Health Organization (1994) World malaria situation in 1993. *Weekly epidemiological record* **71**: 17 - 22.

World Health Organization WHO/ MAL/94/1067. Report of an informal consultation convened by WHO in Geneva 27-29 September1993.

World Health Organization (1997a) World malaria situation in 1993. *TDR News* 54: Geneva, Switzerland.

World Health Organization (1997b) World malaria situation in 1993. *Weekly epidemiological record* **72**: 269-276.

Worth, R.M. (1964). The heparinized capillary tubes as an epidemiological tool II. Concentration of blood parasites by centrifugation *American journal of hygiene*. **80**: 70-74.

World Health Organization (1994) World malaria situation in 1993. *Weekly epidemiological record* **71**: 17 - 22.

Wyler, D.J. (1990). *Plasmodium* species (malaria). Mandell G.L, Douglas, R.G, Bennet, J.E, eds. *Principles and Practice of Infectious Diseases*. Third edition. New York: Churchill Livingstone, 2056-2066.

Zucker, J.R; Lackritz, E.M; Reubush II, T.K; Hightower, A.W; Adungosi, J.E; Were, J.B.O.; Metchock, B.M; Patrick, E. and Campbell, C.C. (1996). Childhood mortality during and after hospitalization in Western Kenya: Effect of malaria treatment regimens. *American Journal of Tropical Medicine and Hygiene* **55**(6): 655-659.

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.