Addis Ababa University

School of Graduate Studies

Assessing the Efficacy of Chloroquine in the Treatment of *Plasmodium vivax* Malaria in Debre Zeit, Ethiopia

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List of Abbreviations

AHRI	Armauer Hansen Research Institute
ALERT	All Africa Leprosy and Tuberculosis Rehabilitation, Research and Training Center
CQ	Chloroquine
DCQ	Desethylechloroquine
DDT	Dichloro-diphenyl-trichloroethane
EDTA	Ethylene-diamine- tetra-acetic acid
FMOH	Federal Ministry of Health
G6PD	Glucose six phosphate dehydrogenase
HIV	Human immunodeficiency virus
HQC	High quality control samples
LQCs	Lower quality control samples
MEC	Minimal effective concentration
MQCs	Medium quality control samples
PCR	Polymerase chain reaction
P. vivax	*Plasmodium vivax*
P. falciparum	*Plasmodium falciparum*
PvMSP-3α	*Plasmodium vivax* merozoite surface protein-3 alpha
PfCRT	*Plasmodium falciparum* digestive vacuole trans-membrane protein
QD	Quinidine
RSD	Relative standard deviation
RBC	Red blood cells
RFLP	Restriction fragment length polymorphism
WBC	White blood cells
WFP	Whatman filter paper
WHO	World Health Organization

Abstract

Chloroquine (CQ) is the first line drug for treatment of *P. vivax* malaria. However, drug resistance has compromised its use. In Ethiopia, there have been few reports of treatment failure of CQ in treating *P. vivax* malaria. Therefore, this study aimed to assess the emergence of chloroquine resistance to *P. vivax* malaria in Debre Zeit, Ethiopia. For this purpose, an *in vivo* drug efficacy study was conducted in Debre Zeit from June-August, 2006. Eighty-seven patients with microscopically confirmed *P. vivax* malaria, aged between 8 months to 52 years, were recruited and treated under supervision with chloroquine 25 mg/kg over three days. On enrollment, 39.8% had documented fever and 60.2% had a history of fever. Their geometric mean parasite density was 7,045 parasites/µl. Out of the 87 patients recruited for the study, one was lost to follow-up and three were excluded due to *P. falciparum* infection during follow-up. A total of 83 (95%) of the study participants completed the 28 days of follow-up. Among these, four patients had recurrent parasitemia on Day 28 showing treatment failure. The blood chloroquine level of the four patients with recurrent parasitemia was determined using high performance liquid chromatography (HPLC). It was 524.6, 672.1, 868.6 and 1164.0ηg/ml, all above the minimal effective concentration (MEC), 100ηg/ml. Parasites from patients with recurrent parasitemia were genotyped using PCR-RFLP to distinguish the parasite strains at Day0 and Day28. Amplification of Day 0 and Day 28 samples was possible for only one patient, which revealed presence of mixed strains of parasite population, which is an indication of parasite diversity. Based on the results from the determination of CQ-DCQ in patients with recurrent parasitemia, it was concluded that clinically resistant parasites are present in Debre Zeit. To better understand the extent of *P. vivax* resistance to CQ in Ethiopia a multi-center study is recommended.

Key words: Chloroquine resistance, Debre Zeit, Ethiopia, HPLC, *Plasmodium vivax*, PCR-RFLP

1. Introduction

1.1 Parasite Biology

Malaria is caused by protozoan parasites belonging to the family Plasmodidae within the order Coccidiidae, suborder Hemosporidiidae, which comprises more than 120 parasite species found in the blood of reptiles, birds and mammals (Schmidt and Roberts, 2000). Depending on the developmental stages and species, the parasite can be spherical, amoeboid, elongated; ring or crescent shaped and can range in size from 1-20 microns in diameter. Four species of the genus *Plasmodium* cause malarial infections in humans. These are *P. falciparum*, *P. vivax*, *P. ovale,* and *P. malariae*. Among the four species *P. falciparum* and *P. vivax* are the important health concerns in the world. However, some monkey parasites such as *P. knowleis* can also cause infection in human (Singh *et al.,* 2004).

The human malaria parasite has a complex life cycle that includes an invertebrate vector and a vertebrate human host (Fig 1). The infection starts when female *Anopheles* mosquito, which is the vector responsible for transmission, inoculates the parasites into the host's blood stream while taking blood meal. Within 30 minutes of entering the host's bloodstream, the thread-like forms (sporozoites) enter parenchyma cells of the liver where they multiply asexually and develop into exoerythrocyte schizonts, which contain 10,000-30,000 merozoites. In this developmental stage of the parasite, the host remains asymptomatic.

After 9-16 days, hepatocytes rupture to release merozoites that infect red blood cells (RBCs). Within the RBCs, the parasites further multiply asexually, and give rise to mature merozoites. Subsequently, RBCs will burst to release merozoites, which are capable of infecting other RBCs. This cycle continues until the patient is treated. Periodic episodes of fever, which is the major symptom of the disease, correlate with periodic synchronized rupture of RBCs.

Meanwhile, other merozoites will develop into male and female gametocytes. The life cycle completes only when the mosquito takes up gametocytes while feeding. Sexual development of the parasite occurs inside the mosquito's gut. There, a male gamete fertilizes the female to produce a zygote, and then become motile ookinete, which then mature to oocysts. In a period of 10-18 days of multiplication, the oocyst develops into infective sporozoites, which will

penetrate the gut and migrated to the mosquito salivary gland, to be transmitted to another human host while the mosquito takes another blood meal (Schmidt and Roberts, 2000).

In the case of *P. vivax* and *P. ovale,* some of the sporozoites remain in liver cells as a resting stage called hypnozoites that can be activated during malarial relapses weeks or months later. This feature is related with morbidity caused by these two species. The pattern of relapse in *P. vivax* varies with the geographic origin. *P. vivax* in the temperate region relapses at a longer interval (>6 months) than the tropical strains, which usually relapse very frequently, with high probability of relapse within 3 months (Baird and Hoffman, 2004).

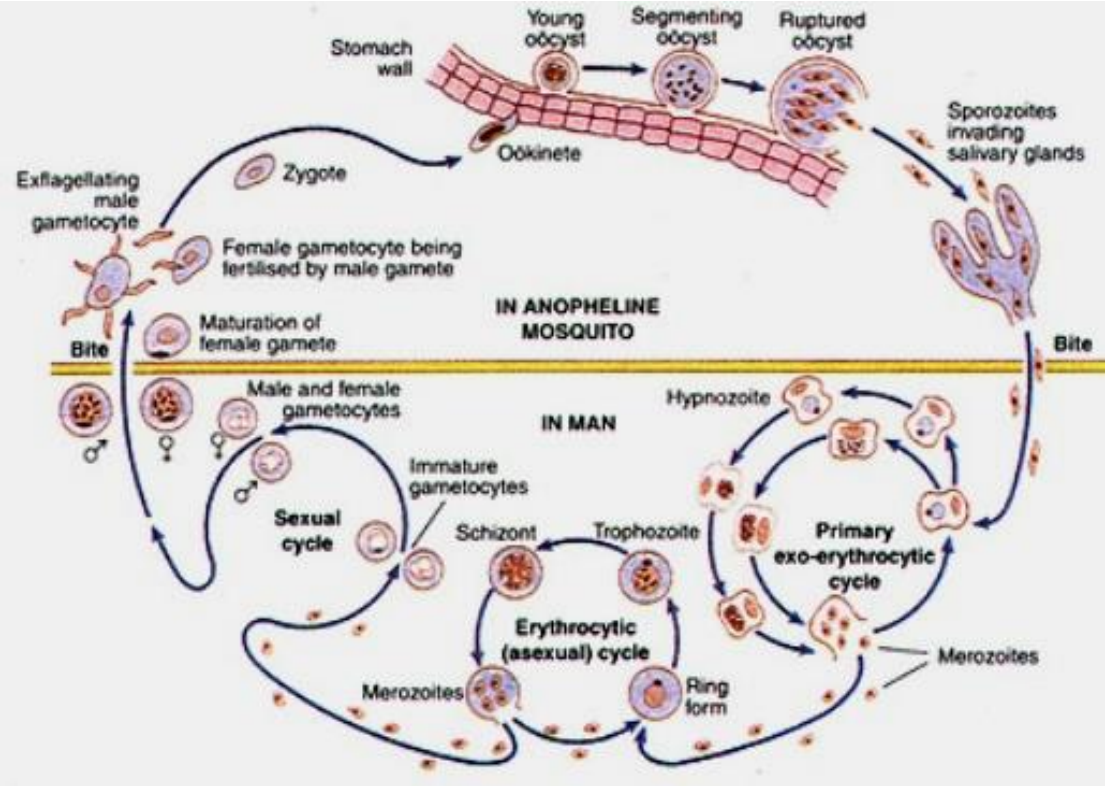


Figure 1. The life cycle of malaria parasites
(source: http://www.malaria.com).

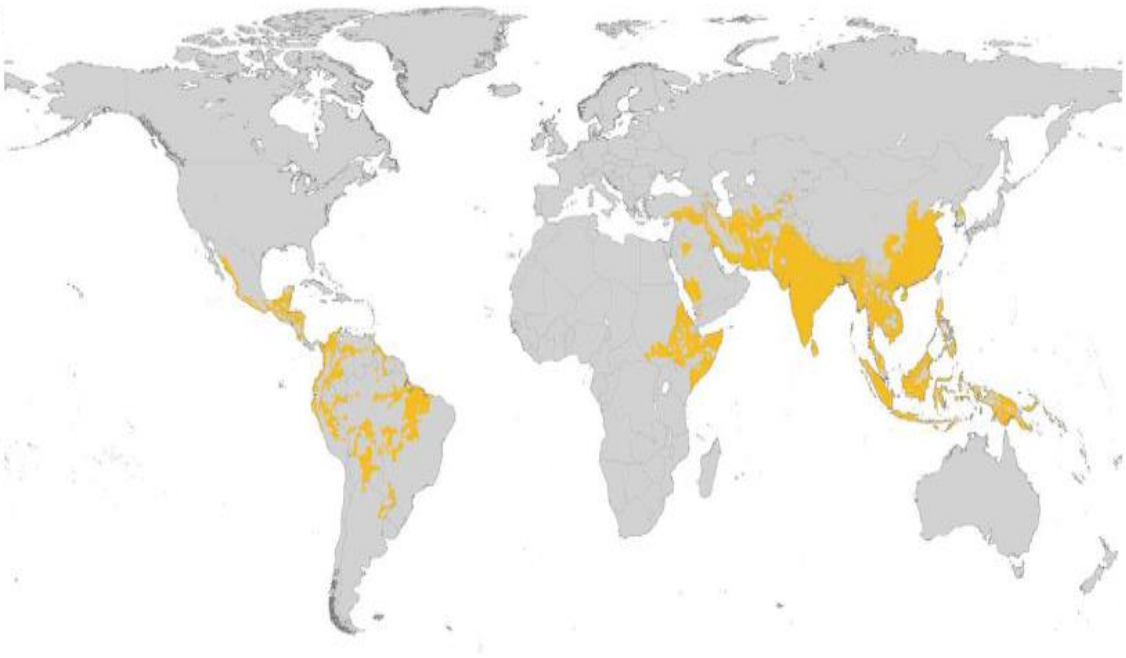
Malaria due to *P. vivax* in non-immune individuals is an acute and agonizing illness. A sudden and dramatic paroxysm characterizes *P. vivax* malaria. Unlike the paroxysms of *P. falciparum* that recur irregularly over 48 hours, *P. vivax* give rise to a well defined and recurring

paroxysmal fever with a regular 48 hour periodicity. Paroxysms start with malaise, abrupt chills and fever that rises to 39 - 41°C. Other symptoms, such as increasing headache, nausea, muscle, joint and back pain, accompany paroxysms. This situation is followed by fall of fever and profuse sweating, which occurs over a period of 2 to 3 hours. These symptoms coincide with the release of merozoites from ruptured RBCs, which results in the release of malarial products that stimulate the host to respond against the parasite. From *in vitro* studies, it has been shown that the host response involves T-cells, monocytes and neutrophils. Cytokines such as tumor necrosis factor-α, interleukin-2 and granulocyte macrophage colony stimulating factor are also involved in the process. However interferon-γ activity associated with immunopathology is absent unlike *P. falciparum* infection (Karunaweera *et al*., 2003). Recently the parasite factors released during the paroxysm were identified as lipids, a phospholipid and cholesterol/triglyceride (Karunaweera *et al*., 2007).

1.2 Epidemiology of *Plasmodium vivax* Malaria

Malaria is present in 107 countries and threatens the lives of 3.2 billion people in the world. An estimate of 300-500 million clinical cases of malaria occurs annually and, among these, 1.1 million individuals die every year. Eighty percent of these deaths occur in Africa mainly among children under five years of age (WHO, 2005). Death is mainly caused by *P. falciparum*. Although *P. vivax* malaria is less virulent when compared to *P. falciparum*, yet, it is a debilitating disease with tremendous impact on health and economic productivity.

P. vivax malaria is widespread and the second most prevalent cause of malaria. Annual estimates of *P. vivax* cases range from 75 to 90 million, with most infections occurring outside Africa (Mendis *et al.,* 2001). It accounts only for 10% of cases in South and East Africa. Recent mapping of *P.vivax* malaria shows that eastern part of Africa is affected by these parasite species (Fig-2). Its very low prevalence in West Africa was explained to be due to the presence of Duffy-negative blood group that lack the Duffy protein receptors on the surface of their RBCs which are necessary for the invasion of red blood cells by merozoites of *P. vivax* (Miller *et al*., 1976).



Distribution of *P. vivax* malaria worldwide

Figure 2. Global distribution of *P. vivax* in 2005
(Source: Guerra *et al.,* 2006).

In Ethiopia, malaria is a major public health problem. It is found in about 75% of the total area of the country, and >65% of the total population is at risk of infection. Annually 5 - 6 million clinical malaria cases and over 600,000 confirmed cases are reported from health facilities (FMOH 2004a). The disease accounted for 17.8 % of the total outpatient visits, 14.1% of total admission and 21.8% of total hospital deaths in 2005/06 (FMOH, 2005/06). Since most of the people affected by the disease do not have access to health services, the number of malaria cases reported by health facilities is only a portion of the actual magnitude.

Malaria transmission in Ethiopia is seasonal and unstable. It usually occurs at altitudes less than 2000m above sea level. The two main seasons for transmission of malaria are September-December, after the main rainy season, and April-May, after short rain season. Epidemics are frequent and widespread in this country. Most of the areas affected by epidemics are highland or highland fringe areas (mainly 1,000-2,000m above sea level), where the population lacks protective immunity to malaria (Kiszewski and Teklehaimanot, 2004). Occasionally,

transmission of malaria occurs in areas previously with no reports of malaria, including areas >2,000 m above sea level, in which the microclimate and weather conditions are not normally favorable for malaria. However, recent reports have indicated that epidemics have expanded to areas up to 2,400 m above sea level, surpassing the general mark of 2,000 m (FMOH, 2007).

P. falciparum and *P. vivax* are the dominant human malaria parasites in Ethiopia accounting for about 60% and 40% of cases, respectively (FMOH, 2004b). This proportion varies from place to place and from season to season. Exceptional from other African countries, Ethiopia has the highest proportion of *P. vivax* malaria on the continent (Fig 2.). This might be attributed to the fact that the majority of the population is Duffy positive (Mathews and Armstrong, 1981).

Anopheles arabiansis has been incriminated as the major mosquito vector responsible for transmission of malaria whereas *An. pharoensis*, *An. funestus* and *An. nili* are considered as secondary vectors (FMOH, 2005b).

1.2.1 Public Health Burden of *Plasmodium vivax* Malaria

In humans, the fact that *P. vivax* invades only young RBCs (reticulocytes) limits its total parasite load and disease severity. The most common clinical consequence is anemia. However, contrary to the belief that *P. vivax* does not cause severe malaria, there are increasing evidences that it leads to severe and complicated cases more frequently than formerly reported. Case reports of severe and fatal complications that are associated with pure *P. vivax* malaria include lung injury (Torres *et al.*, 1997), splenic rupture (Zingman, 1993), retinal hemorrhage (Choi *et al.*, 2004) and cerebral complications (Ozen *et al.*, 2006, Kochar *et al.*, 2005). Severe symptoms commonly associated with *P. falciparum* infections such as severe thrombocytopenia (Makkar *et al.*, 2002), renal failure, circulatory collapse, severe anemia, haemoglobinuria, abnormal bleeding, acute respiratory disorder syndrome and jaundice (Kochar *et al.*, 2005) has been reported in *P. vivax* malaria from different geographical regions.

Long-term effects of recurrent malaria due to *P. vivax* infection in children can include significant impairment of educational attainment which can also result in permanent loss of cognitive ability, leading to diminished economic productivity during later working life (Mendis *et al.,* 2001). Even though there are relatively few reports describing the effect of

P. vivax malaria on pregnancy, some investigators linked it with low birth weight and mild maternal anemia (Nosten *et al*., 1999). Recently, in Venezuela, *P. vivax* infection has been implicated in miscarriage, premature delivery, thrombocytopenia, bleeding and severe anemia (Rodriguez-Morales *et al.,* 2006).

As malaria and HIV overlap in the same geographical areas, their interaction is expected to causes tremendous burden. Although there are a few studies on *P. vivax* malaria and HIV co-infection, their interaction has not been addressed. Evidence from studies in *P. falciparum* has indicated the impact of the interaction in places where these two diseases overlap. Studies from Uganda, with a stable malaria transmission has shown that HIV infected adults have higher parasitemia episodes compared to those with out HIV infection. The effect is more pronounced in patients with low CD4 count (<200) (Girmwade *et al.,* 2004). Studies in Kenya on pregnant women with HIV and malaria have also shown that they are at higher risk of adverse birth outcomes, including intera-uterine growth retardation and preterm delivery compared with pregnant women with malaria only (ter Kuile *et al.,* 2004). Other studies have also shown that women with HIV and malaria are at higher risk of developing anemia and malarial infection of placenta. As a result, children born to these women have low birth weight and are more likely to die during infancy. In addition, HIV increases the risk of developing severe malaria in all pregnancies including in multigravidae, those with partial immunity (van Eijk *et al*, 2003).

In addition to the morbidity and mortality, co-infection can affect the treatment of malaria. Evidence from a study in Ethiopia indicates that antimalarial drugs may be less efficacious in people living with HIV; showing an increased parasite and fever clearance time following arthemisinin treatment for uncomplicated malaria in HIV infected adults (WHO, 2004)

1.2.2 Economic Burden of Malaria

The economic impact of malaria is unquestionably large but how large it is, is not well known. It is difficult to estimate due to the complexity of the disease, which has a complex epidemiology, affecting different geographic regions, and communities with different levels of transmission. However, several researchers have made an effort to measure the economic cost

of malaria in two ways. These are macro and microeconomic levels (Kenneth *et al*., 2004).

1. *Macroeconomic level* measures economic effect of malaria in a nation or region. It is a top-to-down approach which considers how the development of a country is affected through the heavy mortality in children, the consequence of the reduction in developmental investments, tourism and trade in endemic areas.

The annual economic burden of malaria in 1995 was estimated at US$ 8 billion, only for Africa (Foster and Phillips, 1998), which has recently been further increased to an estimate of US$12 billion (Gallup and Sachs, 2001).

In another study on cross-country regressions across Africa for the 1965–1990, in countries with intensive malaria, the economy grew 1.3% less per person per year. It was estimated that a 10% reduction in malaria was associated with 0.3% higher growth. In this study, factors such as initial poverty, economic policy, tropical location, and life expectancy were taken into account (Gallup and Sachs, 2001).

2. *Micro economic level* measures the effect on the personal level by calculating the direct cost (money spent on preventing and treating malaria) and the indirect costs of lost wages, time home from school, and time spent caring for sick children.

Studies carried out in four African countries, Burkina Faso, Chad, Rwanda and Congo have shown that three to seven days of production were lost per case of malaria. Extrapolating to the whole of Africa, an average of 2.1 days of output per person or $1.70 per capita per year was lost because of malaria (Shepard *et al.,* 1991).

In Ethiopia, malaria epidemics strike during planting and harvesting seasons, at a time when there is the greatest need for agricultural work, thus impeding the socio-economic development. The disease is also associated with loss of earnings, low school attendance, and high treatment cost. Generally, it accounts for 30% of the disease burden disability adjusted life years in all age groups for the country (FMOH, 2005b).

1.3 Control Strategies for *Plasmodium vivax* Malaria

Malaria is a complex disease with no single control solution, which can be applicable to all epidemiological and geographical settings. It was a target for eradication in the 1950s and early 1960s and as a result of which it had receded from some countries such as parts of Europe and North America. However, due to several reasons, it failed to show a similar success in Africa. Among the reasons for the failure of the eradication program are the emergence of antimalarial resistant parasites, selection of exo-philic mosquitoes, Dichloro-diphenyl-trichloroethane (DDT) resistant mosquitoes and lack of organized health infrastructure are the major ones (Talisuna *et al*., 2004). When eradication of malaria was realized as too ambitious, its control through reduction of morbidity and prevention of mortality using early diagnosis and prompt treatment with affordable antimalarials has been adopted in 1992 (WHO, 1993). However this strategy did not solve the problem of controlling of malaria then WHO came up with a roll back malaria movement in 1998 with a goal to halve malaria deaths by 2010. This program has three core intervention strategies; these are ensuring high insecticide treated bed nets (ITN) coverage, intermittent preventive treatment during pregnancy and early and effective treatment of clinical cases (http://www.rbm.who.int/docs/RBM-EPI-EN.pdf). In addition WHO has been advocating the reintroduction of DDT use in control of malaria. In 2005 it was incorporated as one of the control in preventing this disease as indoor residual spraying in Africa (http://www.afro.who.int /malaria/publications/statement final website version.pdf).

Especially control of *P. vivax* malaria has been difficult, as most of the time malaria control strategies have been initiated based on studies made on *P. falciparum*. This mainly causes a problem in areas where *P. falciparum* and *P. vivax* exist together. *P. vivax* has a different course of development, such as early gametocytogenesis. This is used as a strategy for transmission that makes them less likely to be controlled by mass treatment that has been effective to control *P. falciparum* malaria. On the other hand, the development of hypnozoites in the liver makes the disease relapse after effective treatment with blood schizontocide so a patient needs to be treated with another drug for the liver stage of the parasite (Jetsumon *et al*., 2003).

In addition, a study has shown that mosquitoes infected with *P. falciparum* and *P. vivax* show a different pattern of biting behavior. *P. vivax* infected mosquitoes feed earlier before people rest

in bed covered with ITN, which in turn affects the control of the disease using ITN (Bockarie *et al.,* 2006). In this study, a decrease of *P. falciparum* sporozoite infected mosquitoes from 2% to 1% was observed and a slight increase in *P. vivax* infected mosquitoes from 1% to 1.3% after the introduction of ITN occurred. In addition, the lag of research on *P. vivax* (in contrast to that of *P. falciparum*) contributes to the slow progress in controlling *P. vivax* malaria.

Therefore, to control *P. vivax* malaria, integrated approaches are proposed comprising prevention of transmission and treatment with effective antimalarials, and strategies should be developed based on studies on *P. vivax*.

1.4 *Plasmodium vivax* Chemotherapy

Malaria is one of the oldest infectious diseases that affect humans and as a remedy for its fever ancient people have used limb bloodletting, emesis, skull operations and consuming opium-laced beer. Even the help of astrology was sought as the periodicity of malarial fevers suggested a connection with astronomical phenomena. The turning point in the therapy of malaria was in the 1632 in Europe using the cinchona bark from Peru which was brought by the Spanish Jesuits. Later, quinine was extracted from the bark of the cinchona tree (http://www.malariasite.com).

Quinine was used until the 19th century, since it was the only known anti-malarial agent. Then primaquine and quinacrine were produced after the First World War. Shortly thereafter in 1934 chloroquine (CQ) was produced by the Germans. However it was considered too toxic for human use. Around 1946 the Americans in Algeria produced a less toxic and well tolerable and effective CQ, which was then designated as the wonder drug for treatment of all forms of malaria until resistance was developed by *P. falciparum* (Talisuna *et al.* 2004). However, to date CQ is a drug of choice for *P. vivax* malaria in most parts of the world where the parasite is susceptible.

CQ is a 4-amino-quinoline, a weak base that has a capacity to concentrate itself from nano-molar levels outside the parasite to milli-molar levels in the digestive vacuole of the intra-

erythrocytic trophozoite. It is a blood-schizontocide, and has an effect on gametocytes of *P. vivax* malaria (Powell and Tigertt, 1968).

CQ acts on the parasite by inhibiting polymerization of heme, which is the byproduct of hemoglobin digestion in the parasite food vacuole. The parasite cannot metabolize heme; instead it accumulates it in the form of an inert polymer known as the malaria pigment (hemozoin) (Francis *et al.,* 1997). Hemozoin formation is a process by which the parasite detoxifies toxic heme. Accumulation of heme causes parasite death as a consequence of increased membrane permeability and lipid peroxidation (Bray *et al*., 1999).

Since *P. vivax* can relapse weeks or months after first infection, it should be treated with the tissue schizontocide, primaquine (PQ), to ensure radical cure. PQ is an 8-amino quinoline that destroys the liver form of the parasite. However, in areas of high transmission where there is a high re-infection rate and where true relapse cannot be distinguished from re-infection, giving PQ is not recommended. This could increase the risk of widespread deployment of primaquine which will increase the drug pressure hence development of drug resistance. These outweigh its benefits in high transmission setup (WHO, 2006). In addition, determination of glucose six-phosphate dehydrogenase (G6PD) activity is important, in the identification of susceptible individuals to hemolysis of RBCs caused by ingestion of PQ (Frischer *et al*., 1973). Screening for G6PD is unavailable for most individuals (WHO, 2006), thus hinders the administration of PQ.

Since CQ resistance in *P. vivax* malaria is a recent phenomenon, there are few data on the treatment response of chloroquine resistant *P. vivax* malaria. Studies from Indonesia indicated that CQ plus PQ and halofantrin are effective with only 5% and no treatment failure respectively compared with 44% of failure for CQ alone (Baird *et al*., 1995). Others have shown that mefloquine is efficacious in treating resistant *P. vivax* malaria with 99.6% efficacy in Papua, Indonesia, where CQ resistance has been documented (Maguire *et al*., 2006). Recently, it showed that a combination of arthemisinin, dihydroartemisinin-piperaquine is efficacious against resistant *P. vivax* malaria (Hasugian *et al.* 2007). There is also some evidence that amodiaquine and quinine could be used for treatment of resistant *P. vivax* malaria (WHO, 2006).

Studies from Vietnam and Thailand, where there are few case reports or no report on drug resistance, have shown the effectiveness of arthemisinin and arthemisinin-lumefantrine in treating *P. vivax* malaria. In Vietnam, it was shown that the effectiveness of arthemisinin, is the same as that of chloroquine (Giao *et al.,* 2002) and in Thailand the effectiveness of both CQ and arthemisinin-lumefantrine was 100% and 97% respectively (Krudsood, 2007).

In the revised guideline for the treatment of malaria, WHO recommended amodiaquine combined with primaquine for the treatment of resistant *P. vivax* malaria (WHO, 2006).

1.5 *Plasmodium vivax* Resistance to Chloroquine

Anti-malarial drug resistance has been defined as "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject" and the drug must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" (Bloland, 2001).

Even though resistance to CQ by *P. falciparum* has been first reported in 1950, CQ continued to be fully effective in eliminating *P. vivax* blood stages. Resistance in *P. vivax* was first reported from Papua New Guinea in two Australian soldiers in 1989 (Rieckmann *et al.*, 1989). Since then, many cases of prophylactic and therapeutic failures have been reported for *P. vivax* from several places in the world (Fig. 3).

In 1995, a study in Irian Jaya, Indonesia, showed resistance in 64% of the *P. vivax* patients during 28 day follow-up of patients treated with chloroquine (CQ) (Braid *et al.* 1997a). Three years later, 17% of infections caused by resistant parasites was reported from Kalimantan, Indonesia (Fryauff *et al.* 1998). The therapeutic failure has reached to 84%, which shows the complete ineffectiveness of CQ in the northeastern coast part of Indonesian Papua (Sumawinata *et al.*, 2003). In Myanmar, 14% of 53 patients had resistant *P. vivax* infection (Marala *et al.*, 1995). The distribution of CQ-resistant *P. vivax* has now extended to India where a 13-year-old girl failed to respond to two cycles of standard CQ therapy; the minimal concentration of CQ was 216 and 106ηg/ml in whole blood, which is higher than the lethal

concentration for the parasites (Dua *et al.*, 1996). Reports from Vietnam showed 7% of treatment failure among the 113 patients treated with CQ during 28 days follow-up (Phan *et al.*, 2002). And treatment failure has extended to Europe, studies in two sites conducted in Turkey showed that 14.7% of the patients from Karacadag and 10.3% of those from Sekerli showed apparent treatment failure between day 3 and day 28 (Kurcer *et al.,* 2004).

P. vivax resistance to CQ has also been observed in the western hemisphere in South America. In 1996, in Guyana, three cases were reported in which 25 mg/kg of CQ failed to eliminate parasitemia despite adequate therapeutic blood levels of CQ (Phillips *et al.,* 1996). In the Brazilian Amazon region, Alecrim reported a 12-year-old girl with resistant *P. vivax* malaria who continued to have parasitemia after receiving a supervised course of 25mg/kg of CQ (Alecrim *et al.,* 1999). Soto and his colleagues have also reported 3 cases of treatment failure of CQ for *P. vivax* in Colombia (Soto *et al.,* 2001). In Peru, out of 177 patients, two were demonstrated to have resistant parasites in their blood with recurrence on day 21 of their follow-up (Ruebush *et al.,* 2003).

There have been some reports of *P. vivax* malaria CQ treatment failure in Ethiopia (personal communication Mr Gezahegn Tesfaye, 2006, MOH). However, only three study reports exist. The first report of *P. vivax* CQ resistance was from Debre Zeit in which 2% of 225 patients who were followed for 7 days failed to respond to treatment (Tulu *et al.*, 1996). The subsequent two studies were conducted by MOH in collaboration with WHO, showing a treatment failure rate of 2.1% (n=145) in 2003 in Nazareth and 7.0% (n=57) in 2005 in Debre Zeit (Personal communication Mr. Ambachew Medhin, 2006, WHO). Samples from both studies were PCR verified. In the samples from Debre Zeit, 50% of the resistant cases were genetically different from the sample collected on the day of enrollment, suggesting new infection.

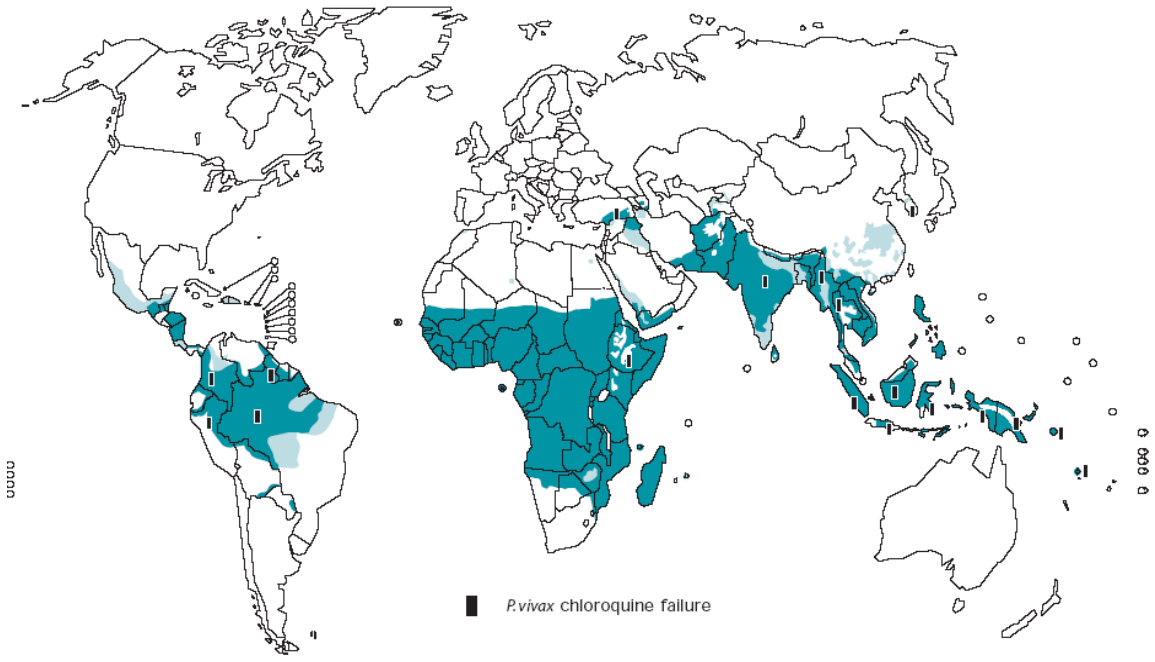


Figure 3. Chloroquine prophylactic and treatment failure reported, 2004
(Source: http://www.who.int/malaria/rbm/Attachment/20041108/chloroquineResistances.pdf)

1.5.1 Mechanism of Chloroquine Resistance in *Plasmodium vivax*.

Resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs, provided the mutations are not deleterious to the survival or reproduction of the parasite.

Mutation in the digestive vacuole trans-membrane protein (PFCRT) at K76-T position was found to be the main mutation associated with resistance to CQ in *P. falciparum* (Lakshmanan *et al.,* 2005). Other nine mutations have also been identified on the same gene that encodes the PFCRT; however these mutations were suggested to modulate the mutation at K76-T. The physiological impacts of this mutation are reduced chloroquine accumulation in the digestive vacuole and increased efflux (Newton and White, 1999). This has been supported by an *in vivo* test; where this efflux can be reversed by a diverse array of compounds including calcium-channel blockers such as verapamil, and other compounds such as tricyclic antidepressants, antihistamines and phenothiazines (Newton and White, 1999). Lowering of pH in the food

vacuole has also been implicated in the inhibition of CQ attachment to heme leading to parasite survival (Bennett *et al*., 2004).

However, a study on a mutation on the homologous gene for *P. vivax* chloroquine resistant trans-membrane protein gene did not show any association with resistance to CQ (Nomura *et al.,* 2001). This shows that these two species have different molecular mechanisms of resistance to CQ. Despite the same exposure to a comparable selection pressure from CQ on *P. vivax* and *P. falciparum,* the late occurrence of CQ resistance in *P. vivax* compared with *P. falciparum in vivo* also suggests the possibility of different resistance mechanisms between these two species.

1.5.2 Factors Responsible for Emergence and Spread of Drug Resistance

The emergence of drug resistance is through a rare genetic occurrence that produces a resistant mutant parasite, which subsequently will be selected by drug pressure that leads to preferential transmission and spread. There are several factors that play a key role in the development of resistance. These factors include: -

1. Long term use of drug: - in studies carried out in Uganda, prevalence of high CQ resistant *P. falciparum* malaria has been associated with areas with high frequency of CQ use in the sites investigated (Talisuna *et al*, 2002).
2. Drug elimination half-life: - drugs with longer elimination half-life can exert undesirable drug selection pressure, which will accelerate the evolution of mutant parasites and favor their transmission to the mosquito vector (Talisuna *et al.,* 2004).
3. Transmission intensity of malaria: - there are three different propositions on the effect of level of transmission on the emergence of drug resistant malaria (Talisuna *et al.,* 2004).
    - The first is, in high transmission area resistant parasites spread faster due to the crowding effect in the host, which benefits resistant parasite selection due to drug selection following chemotherapy.
    - The second is, in areas with low transmission, the majority of malaria infection is symptomatic with high parasitemia. This high parasite biomass thus increases the

drug parasite interaction and drug selection; thus leading to the emergence of parasite resistance. In addition, the low level of acquired immunity of the people who live in such an area also helps the drug resistant parasites to be selected.

- The third hypothesis is that transmission intensity doesn't have any effect on the spread of resistant parasites.

The effect of transmission on the emergence and spread of drug resistance seems complex in which sexual recombinant, intra-host dynamic and the number of genes involved plays a role in determining the net result.

4. Immune status of the individual: - individuals with partial immunity have lesser parasitemia than non-immune individuals and tend to clear parasitemia independent of the drug. In addition, non-immune individuals have higher degrees of parasitemia and are severely affected. Statistically, it was evidenced that mutant parasites are more likely to occur with high parasitemia than low parasitemia.
5. In addition, other factors such as incorrect dosing, non-compliance with duration of dosing regimen, poor or erratic absorption, and misdiagnosis will cause treatment failure in the individual. This may also contribute to the development and intensification of true drug resistance through increasing the likelihood of exposure of parasites to suboptimal drug levels (White, 1997).

1.5.3 Impact of Drug Resistance

Treatment using antimalarial drugs is one of the strategies in malaria control. However, drug resistance impaired this strategy. Resistance to chloroquine in *P. falciparum* is the major factor responsible for the doubling of mortality rate in sub-Saharan Africa in the past decade (Trape *et al.,* 2002). In Senegal an increase in child mortality was observed following CQ resistance.

In addition, ineffective treatment with failing drug leads to increased morbidity. Patients whose treatment fails are often re-treated with the same antimalarial; hence they enter a downward spiral of chronic morbidity. In children, this will cause anemia, which in turn increases the risk of HIV infection through blood transfusions (Hedberg *et al.* 1993).
Emergence of resistance may lead to epidemics of malaria by increasing the infective reservoirs in the community, thus increasing transmission. Drug resistance has been implicated as one of

the factors to the occurrence of epidemics in the highlands of eastern Africa (Hay *et al.,* 2002).

In addition to its impact on health, the effect on the individual and country's economy is enormous. It increases the cost of treatment and loss of working hours, while ill or caring for the sick family members. At a country level, it increases costs for control, increasing the expenditure in health sector, which will in turn affect the development of the country (Talisuna *et al.,* 2004).

1.6 Monitoring Antimalarial Drug Resistance

Monitoring antimalarial drug resistance is necessary in order to understand the efficacy of the antimalarial treatment being delivered, to ensure prompt access to effective treatment and to assess if there is a need for treatment policy change.

Variety of methods has been used to assess resistance to antimalarial drugs. In general, four basic methods have been routinely used. These are *in vivo, in vitro*, animal model studies, and molecular characterization (WHO, 2001).

1.6.1 *In vitro* Test

It is the most frequently used procedure. This technique uses parasites obtained from blood samples that are exposed in microtitre plates to precisely known quantities of drug. Then inhibition of maturation into schizonts will be observed. Since it is not confounded by host factors, this method reflects the true antimalarial drug resistance. However, the results from *in vitro* response do not show clear or consistent correlation with clinical response in patients. The correlation appears to depend on the level of acquired immunity within the population being tested.

Standard diagnosis of *P. falciparum* drug resistance using *in vitro* procedures has been in use since 1970s. Although *P. vivax* has been cultivated continuously, the need of a high amount of reticulocytes to enrich the media limits this method (Golenda *et al*., 1997). Yet, short-term culture was possible, which is sufficient for assessing drug resistance *in vitro*. However there are no standard criteria for classifying *in vitro* responses as sensitive or resistant (Baird, 2004). In Thailand, where the parasite strains are still sensitive to CQ, it was attempted to characterize

the sensitivity of strains to CQ. This study provided a baseline for *in vitro* sensitivity;~50ηg/ml (IC50) consistently inhibits development by 50% (Tasanor *et al.,* 2002). A study by Russell and his colleagues has also shown the IC50 as 49.8ηg/ml, which was similar with the pervious study (Russell *et al*., 2003).

1.6.2 Animal Model Studies

This type of test is an *in vivo* test conducted in a non-human animal model. It is also influenced by host immunity as the *in vivo* test is in humans. However, the influence of host immunity can be minimized by using laboratory-reared animals or animal-parasite combinations unlikely to occur in nature; although other host factors that could not be modified would still be present. These methods allow for the testing of parasites, which cannot be adapted to the *in vitro* environment. It also allows the testing of experimental drugs, which are not yet approved for use in humans. A significant disadvantage is that only parasites that are adaptable to animals can be investigated.

In the development of this method for *P. vivax* malaria, Collins and his colleagues used pooled blood samples taken from patients with resistant parasites. They have shown that these strains could not be cleared with 30mg of chloroquine in two monkey models, splenectomized *Saimirian* and *Aotus* monkeys (Collins *et al*., 2000). Known human strains such as Chesson, CDC-I, Achiote and Palo Alto strains were found to have a variation in the minimum therapeutic dose of CQ, 9mg, 15mg, 10mg and 18mg, respectively, for the strains. The characterization of therapeutic profiles of CQ among well-characterized strains in humans will provide a basis for the classification of wild isolates as sensitive or resistant in animal models in the future (Baird 2004).

1.6.3 Molecular Techniques

Molecular tests use the polymerase chain reaction (PCR) to detect the presence of mutations encoding biological resistance to antimalarial drugs. This method requires a very small DNA sample and allows a very large number of samples to be processed in a relatively short time. However it requires sophisticated equipment and trained manpower.

In addition, confirmation of the association between gene mutation and resistance is also a difficult task. The gene mutations that confer antimalarial drug resistance currently known are for only a limited number of drugs. E.g. molecular markers such as Pfcrt, Pfmdr, Pfdhfr and Pfdhps have been reported to confer resistance to CQ, sulfadoxin and pyrimethamine in *P. falciparum*. The homologous genes Pvdhps and Pvdhfr have also been shown to correlate with resistance to sulfadoxin and pyrimethamine in *P. vivax* malaria. However, correlates of CQ resistance genes were not identified in *P. vivax* (Boger and Shapiro, 2004).

1.6.4 *In vivo* Test

An *in vivo* test consists of the treatment of a group of symptomatic and parasitemic individuals with supervised doses of drug and the subsequent monitoring of the parasitological and/or clinical response over time. This method reflects the actual therapeutic response of a circulating parasite affecting a population at a time. However, this method will not tell us the true value of resistance due to host factors that play a part in the clearance of parasitemia. For example, the immunity of an individual might disguise the efficacy of a drug by clearing parasitemia independently of the drug, therefore making poorly effective drugs appear good (Djimdé *et al.*, 2003).

The first *in vivo* test protocol was developed in 1962 for uncomplicated *P. falciparum* malaria. Since then, the protocol has been modified several times. However, this protocol did not include an *in vivo* test for *P. vivax* malaria. The latest modified protocol for *P. vivax* malaria was introduced as a draft by WHO in 2002. In this protocol definition of resistance requires, demonstration of recurrent parasitemia within 28 days in a patient who has received an observed treatment dose of an antimalarial drug. Simultaneous demonstration of adequate blood level of drug and parasite genotyping at a time of recurrent parasitemia would be beneficial to support the clinical treatment outcome classification. The latter methods are useful to rule out possible confounding factors such as recurrent parasitemia due to inadequate drug levels in the blood as well as for differentiation of new infection from relapse/recrudescence cases (WHO, 2002).

Lack of molecular markers that correlate with CQ resistance, difficulty in culturing *P. vivax* in the laboratory and the lack of standard in classify out come in animal models makes

monitoring CQ resistance in *P. vivax* has been difficult therefore, an *in vivo* efficacy testing method has been used frequently.

1.7 Significance of the Study

For over 40 years, chloroquine has been used as a first line drug for uncomplicated *P. falciparum* and *P. vivax* malaria in Ethiopia. Due to the emergence of drug resistance by *P. falciparum* in 1997/98 it was replaced by sulfadoxin pyrimethamine and by artemether/lumefantrine in 2004. Unlike *P. falciparum,* CQ remains in use as a first line drug for *P. vivax* malaria. However, its use has been compromised by the emergence of drug resistance. In Ethiopia, there are few reports of treatment failures. However, these reports were not substantiated with evidence. Therefore, in this study, we aimed to determine the level of CQ resistance in *P. vivax* malaria in Debre Zeit, Ethiopia.

Since effective case management remains the mainstay of malaria control in Ethiopia. Monitoring drug resistance to currently available drugs such as CQ is an important tool to ensure proper management of clinical cases and to help in the early detection of changing patterns of resistance. Understanding of the change in the resistance pattern will help in alerting responsible authorities to assess the efficacy of the available treatment options and make changes in the treatment policy based on evidences. Therefore, the findings of this study will provide baseline data for further investigation on the response of *P. vivax* malaria to CQ.

2. Hypothesis

The reported therapeutic failure of chloroquine for the treatment of *P. vivax* malaria in Debre Zeit is due to emergence of chloroquine resistance.

3. Objectives

3.1 General Objective

To assess the efficacy of chloroquine in the treatment of *P. vivax* malaria in Debre Zeit, Ethiopia.

3.2 Specific Objectives

1. To determine the rate of failure of CQ treatment against *P. vivax* malaria during 28 days of *in vivo* follow-up.
2. To determine blood levels of CQ and DCQ at a time of recurrent infections during the follow-up.
3. To determine whether recurrent infection arises from recrudescence/relapse of primary infection or new infection by using PCR/RFLP technique.

4. Materials and Methods

4.1 Study Design

The study is a cross-sectional study, which was conducted during low *P. vivax* malaria transmission season at Debre Zeit, Ethiopia.

4.2 Study Area

The study was done in Debre Zeit (in Adea district) (8.75 N, 38.99 E) (Fig 4.), which covers an area of 1,635 km^2 and is found in East Showa Zone, central Ethiopia. It lies at 44 km south of Addis Ababa. According to the Central Statistics Agency an estimated population of 367, 534 live in this area (Central Statistics Agency, 2006).

It is a mid-altitude area in which the altitude ranges between 1,500-2,200m. It has similar rainfall pattern as other Ethiopian *woina dega* regions, receiving *belg* (short rainy season) from March to April and *meher* (main rainy season) from June to September. The average rainfall recorded for the 35 years (1967-2002 G.C) is 898.8 mm. The average annual maximum and minimum temperature in 2005 was 27.5 °C and 10.0 °C respectively.

Malaria is one of the frequently diagnosed acute illnesses and a principal cause of morbidity affecting all age groups. Malaria transmission occurs throughout the year where *P. vivax* is the dominant species over *P. falciparum* (prevalence of 70% to 30% respectively at this site) (Table 1). Malaria transmission is generally more intense following the main rainy season (between September to December), (Fig 5).

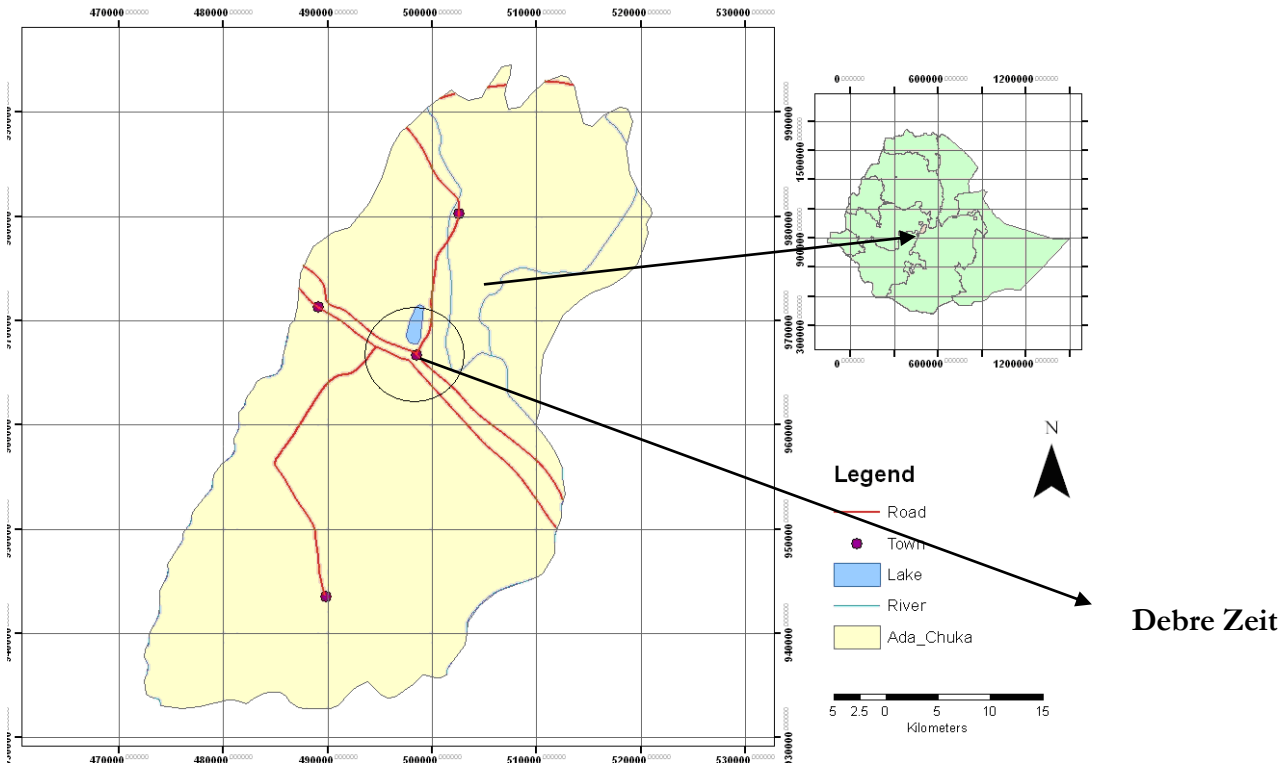


Figure 4. Location of the study area, Debre Zeit (8.75 N, 38.99 E; 1500-2200m)

Table 1. Number of reported malaria cases in Debre Zeit Malaria Sector from 2002-2006.

Year	Examined	Positive	*P. falciparum* (%)	*P. vivax* (%)
2002/2003	30,192	13,178	5,884 (44.7)	7,310 (55.3)
2003/2004	22,669	7,059	4,038(57.2)	3,017 (42.8)
2004/2005	15,363	2,629	728(27.6)	1,890 (72.4)
*2005/2006	18,789	5,244	1,515(28.9)	3,619(71.1)

* Data for one month are missing (May, 2005/2006).

Source: Ministry of Health, Oromia Regional Health Bureau and Debre Zeit Malaria Sector

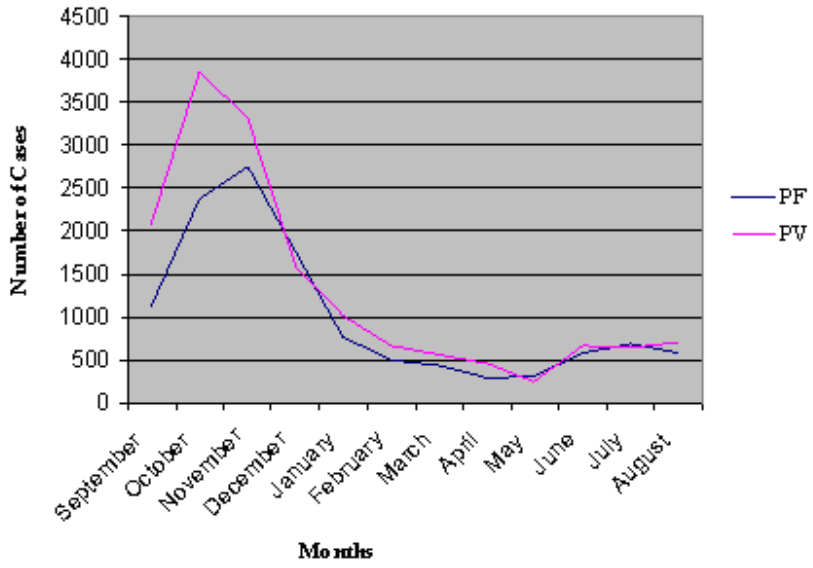


Figure 5. Seasonal distribution of malaria due to *P. vivax* and *P. falciparum* in Debre Zeit, 2002-2006.
(Source: Ministry of Health, Oromia Regional Health Bureau and Debre Zeit Malaria Sector)

4.3 Study Participants

4.3.1 Sample Size Determination

The sample size is calculated based on the expected proportion of *P. vivax* treatment failures with CQ in the study population. Assuming a maximum of 5% treatment failures in a population of infinite size, a precision of ±5%, and a 5% significance level, 73 patients are needed in the trial using the following formula.

$$N = (Z/d)^2 P(1-P)$$
$$= (1.96/0.05)^2 0.05(1-0.05)$$
$$= 73$$

Where N – number of samples Z – confidence interval (95%)
P – proportion (5%) d – the level of confidence is 5%

Assuming a loss-to-follow-up rate of 20% over a 28-day study, a total of 87 subjects were enrolled in the study.

Recruitment of study participants was from patients who came to a malaria laboratory at

Debre Zeit Malaria Sector from June to August 2006, with complaints of signs and symptoms suggestive of malaria. Enrollment criteria were as per WHO standard for *in vivo* anti malarial drug efficacy testing (WHO, 2003), briefly described below:

4.3.2 Inclusion Criteria

- Patients age > 6 months
- Positive for *P. vivax* mono-infection with parasite density above 250 parasites/μl
- Temperature ≥37.5 °C or history of fever 48hrs prior to time of recruitment
- Ability and willingness to participate in the study based on information given to patients or parent / guardian in case of children (below 18 years of age) and easy access to a health facility
- Availability for follow up at least for 30 days after enrollment
- Informed consent from patients or parents/guardians in case of children and assent from children of 12-17 years old.

4.3.3 Exclusion Criteria

- Presence of clinical condition requiring hospitalization
- Presence of severe malnutrition
- Pregnancy
- Significant concomitant febrile illness that would interfere with follow-up
- Clinically evident chronic infectious diseases other than malaria (e.g. tuberculosis)
- History of allergy and/or intolerance to chloroquine

4.3.4 Patient Follow-up

Eligible study participants were clinically followed using the WHO guideline for *in vivo* drug test for 28 days. Participants were asked to return on Days 1, 2, 3, 7, 14, 21, and 28 when clinical and parasitological assessment was done. Participants and parents/ guardians (in the case of children) were encouraged to return to the clinical team for further assessment and/or treatment at any time point during follow-up when the participant perceived himself/herself (or was perceived by parents or guardians) to be ill. The study participants were treated by the

project according to the guideline of the Ministry of Health (FMOH, 2004a). Patients who showed recurrent parasitemia during the follow-up period were treated with quinine plus doxycycline according to WHO recommendation (2002). The standard WHO definition of therapeutic response was used to classify treatment response to CQ (Annex 3).

4.3.5 Antimalarial Therapy

CQ doses of 25 mg base/kg body weight divided into single daily doses over 3 days: (10 mg/kg on the first day; 10 mg/kg on the second day; and 5 mg/kg on the third day) was administered under supervision by the study team. Participants were observed for 30 minutes after treatment for adverse reactions or vomiting. Study participants who vomited during this period were treated again with the same dose of CQ and observed for an additional 30-minute period. Study participants who vomited a second time were treated with quinine and were excluded from the study. Paracetamol was given to patients with axillary temperature greater than 38.0^{o} C.

4.4 Laboratory Methods

4.4.1 Drug Quality Assay

Four tests, disintegration, dissolution, identification and test to measure the content in the tablets were done to confirm the quality of the drug, (Chloroquine phosphate 250mg containing 150mg of chloroquine base, Batch Number 0073, Adigrat Pharmaceutical Factory) by using the standard procedures recommended by British Pharmacopoeia (2004).

Disintegration Test

Six tablets of chloroquine phosphate were put in the basket of the disintegration apparatus in a medium of water and the time it took to be dissolved was recorded.

Dissolution Test

Six tablets of chloroquine phosphate were placed in a basket of the dissolution apparatus and adjusted at 100 revolutions per min for 45 min in a 900ml medium of 0.1M HCl to be rotated.

Then 10ml of the sample and the medium was withdrawn and diluted to measure the absorbance at 344nm using spectrophotometer.

Identification Test

Powdered chloroquine phosphate (0.1g) was dissolved in 10ml of water and 2ml of 2M NaOH. Then chloroquine base was extracted using chloroform. The extract was then washed using water and dried using anhydrous sodium sulfate in water bath. The residue was dissolved in 2ml of chloroform. The final solution was used to determine the spectrum using infrared spectroscopy. The test was done in duplicate.

Test for chloroquine concentration in chloroquine phosphate tablets

Chloroquine phosphate (0.5g) was dissolved in 20ml of 1M NaOH. Chloroquine base was extracted using 4x 25ml of chloroform. The final extract was evaporated on water bath to a volume of 10ml then 40ml of anhydrous acetic acid was added to determine the end point potentiometrically using automatic titrater. This test was done in triplicate.

4.4.2 Pregnancy Test

All female study participants aged 12 years or older who met the inclusion criteria, and who were not obviously pregnant, were tested for pregnancy upon informed consent (parent/guardian consent and child assent for girls between 12-18 years of age) using urine human chorionic gonadotropin (HCG) dipstick test (ACON laboratories, INC. CA, USA).

4.4.3 Blood Sample Collection

Blood Sample for PCR-RFLP

Blood sample specimens were collected on Whatman filter paper before treatment was given to the patients on Day 0 and on any day between Day 3 and Day 28 when parasitemia was noted to have recurred. In each case, approximately 50µl of blood sample was spotted on labeled Whatman filter paper (WFP), air-dried and placed in a plastic bag with same label.

Blood Sample for Giemsa Staining

During the follow-up period, on the dates when patients are examined again (days 1, 2, 3, 7, 14, 21, 28) and any day if the patients develops fever, thick and thin blood smears were prepared side by side on the same slide in duplicate and allowed to air dry.

Blood Sample for Determination of CQ levels

Approximately 50µl blood samples were obtained from study participants from finger pricks before administration of the last dose of CQ (day 2) and on any other day between day 3 and 28 when recurrent parasitemia was detected. The samples were spotted on to WFP, then allowed to air dry and were put into plastic bag and stored until analyzed. To avoid contamination of WFP with CQ particles, samples were collected in a separate room away from the rooms where drug was dispensed.

4.4.4 Giemsa Staining

Blood film slides were stained with Giemsa according to the following protocols (Lebbad, 2004). The air-dried thin film slides were fixed with methanol for about 30 seconds with care not to fix the thick smear. Then a freshly prepared 10% Giemsa solution was poured onto the slides and were put on the staining rack and left for 20 minutes to stain. The stained slides were rinsed with tap water and kept in upright position to air dry.

4.4.5 Handling and Transportation of Samples

Two stained thick and thin blood film slides from each patient were kept into slide boxes. The air-dried WFP spotted blood samples were transferred into a re-sealable plastic bag carrying the same label as the papers. Then the samples were transported to the AHRI/ALERT laboratory in Addis Ababa (Slide reading and PCR), and the Kenya Medical Research Institute laboratory, Kilifi, Kenya (CQ-DCQ levels), where laboratory work was done.

4.4.6 Microscopic Examination

Giemsa stained thin and thick films were observed under 100x light microscope using oil immersion by an experienced laboratory technician and 10% of the slides were cross checked by a second experienced laboratory technician for quality control. Parasitemia was calculated per 200 white blood cells according to the following formula.

$$\text{Parasitemia (per } \mu\text{l)} = \frac{\text{number of parasites x 8,000 / number of leukocytes}}{200\text{WBC}}.$$

Counting of parasites was adjusted for high and low. Accordingly if >500 parasites were counted without having reached a count of 200 WBC, the count was stopped after completing the reading of the last field and the parasitemia was calculated according to the formula above. Or if the parasite count was less than 10 parasites/200 WBC, counting was continued until 500 WBC were covered. A total of 300 fields were examined before a blood smear was considered negative (WHO, 2002).

4.4.7 Extraction of *Plasmodium vivax* DNA for PCR Analysis

Parasite DNA was extracted for PCR using a protocol for filter paper bound blood. The blood spot was soaked in a 1.5ml Eppendorf tube containing 1ml of phosphate buffer solution (8g NaCl, 2g KCl, 1.15g of NaH_2PO_4, 0.24g of K_2HPO_4 per liter, pH 7.4) containing 1% saponin, which increases DNA yield by aiding the release of hemoglobin from the WFP and left overnight at 4°C. The supernatant was discarded and 1ml of 1x phosphate buffer solution was added to the tube, centrifuged for 2 min at 13,000 rpm, and the supernatant was discarded. Fifteen µl of Molecular Biology Grade water and 50µl of a 20% suspension of chelex 100 resin (Bio-rad) were added. Then the tubes were placed in water bath at 98°C for 8 min and then centrifuged for 1 min at 13,000 rpm. The supernatant, which contains the DNA, was carefully removed to avoid transfer of the chelex resin and stored at -20°C (Kain and Lanar, 1991).

4.4.8 Parasite Genotyping of *Plasmodium vivax* by PCR-RFLP

P. vivax DNA was amplified with the following primers for MSP-3α gene (Bruce *et al*, 1999).

Primary reaction P1—5' CAGCAGACACCATTTAAGG 3'

P2—5' CCGTTTGTTGATTAGTTGC 3'

Nested reaction N1—5' GACCAGTGTGATACCATTAACC 3'

N2—5' ATACTGGTTCTTCGTCTTCAGG 3'

Nested PCR amplification of MSP-3α gene was performed for primary and relapse samples in a reaction volume of 20μl [1μl of each primer, 10μl of Hot start master mix (QIAGEN GmbH Germany) and 6μl the PCR buffer]. Two micro-liters of DNA extract from samples were used for primary reaction and 1μl of the primary amplicon was used for the nested round. A negative control, with no parasite DNA, was included in each set of amplification reactions. The thermocycling conditions for the amplification reaction consisted of denaturation of DNA at 94ºC for 45 seconds, 1 cycle, then 94ºC for 30 seconds, 55 ºC for 30 sec, 68 ºC for 2.5 min 30 cycles. The PCR product for both samples was loaded on to 0.8% agarose gel (Sigma) containing 0.25μg/ml of ethidium bromide. A 1Kbp DNA ladder (0.1μg/lane) was loaded with each run as molecular size marker. Gels were visualized on an ultraviolet transluminator and were photographed using a gel documentation system.

For RFLP analysis of PCR products, both primary and relapse samples were digested individually with the restriction enzymes *Hha* I in 20μl reaction volumes (5 units of enzyme per reaction) in the buffer supplied with enzymes at 37ºC for 4 to 5 hours. The restriction fragments of DNA were separated on 1.8% polyacrylamide gel containing 0.25μg/ml of ethidium bromide. For the determination of fragment lengths, 4μl of 1kb DNA ladder was run adjacently (0.1μg/lane). Determination of the samples, whether they are of the same parasite or not, was done by comparing bands. Then gels were visualized on an ultraviolet transilluminator and were photographed using a gel documentation system (UVP-imager, Epi-chemII, darkroom, Upland, with Camera and computer attached to it).

4.4.9 High Performance Liquid Chromatography (HPLC)

Blood levels of CQ and its metabolite, desethylchloroquine (DCQ), was measured using HPLC in sample blood on WFP according to protocol by Bell *et al.*, 2007.

Chromatographic System and Conditions

The chromatographic system consisted of a gradient delivery pump (Spectra System P2000, Spectra Physics Analytical Inc. CA USA) connected to an injector (Syringe loading injector, Size 50µl loop, Rheo Dyne Model 7125, CA USA). Chromatographic separation was performed using a 4µm Phenomenex® SynergI Polar-RP 18 150mm×4.6mm UK) column. Guard column (5µm Lichrosher® RP-18e Darmsedt, Germany) was inserted between the injector and the column. UV–vis detector (Spectra Series UV100 (UV/VIS), Spectra Physics Analytical Inc. CA USA) set at 340nm was used. Chromatograms were obtained on a ChromoJet CHI (Spectra Physics Thermo separation Products CA USA) data module integrator with attenuation set at 4.

The mobile phase consisted of water and acetonitril in a ratio of 90%:10% and 1% of triethylamine (organic modifier) was added to the mobile phase to prevent the interaction of the analytes with the packing material of the column. The mobile phase was attached with automatic degasser.

Drug Extraction Procedure from Dried Blood on Whatman Filter Paper

Twenty microliters of quinidine internal standard solution (10ηg/ml methanol) was added to the sample blood spots of 50µl on WFP and allowed to dry. Then it was chopped in to pieces and put into labeled silianized pyrex tube (screwcaps contained PTFE inserts). One ml of 2M HCl was added and the tubes vortexed briefly. One ml of 5M NaOH was added and vortexed briefly and then 5ml of Tert-Butyl-methyether: N-Hexane (v/v) was added. The tubes were inverted at a speed of 4 cycles per sec for 30 min using Rotor (Rotator Drive STR4, Stuart Scientific, UK). The tubes were centrifuged at 3000 rpm for 10 min at 20°C to separate the aqueous and organic solvents. The organic solvent was then carefully transferred to a new silanized tube using pasture pipette. Then it was evaporated at 37°C in water bath under a stream of nitrogen to allow CQ to settle at the bottom of the tubes. Then, the drug was reconstituted using 100-µl mobile phase to be injected to the HPLC. The peak area ratio was taken to quantify the amount using the standard curve.

HPLC Method Validation

The sensitivity of the HPLC method used (Bell *et al.,* 2007) was validated using the following validation methods.

Preparation of Standards, Calibration and Quality Control Samples

Standards were prepared by dissolving Chloroquine-diphosphate (Sigma chemical Co. St. Louis Mo USA), quinidine (Sigma chemical Co. St. Louis Mo USA) and desethylchloroquine internal standard (donation from Dr Michael Green CDC, Atlanta U.S.A.) to give final concentration of 1mg/ml of stalk solution. Then working solution was prepared by diluting serially to give 100µg/ml, 10µg/ml and 1µg/ml. Then standards were kept at -20°C until use.

Samples of dried blood spot that mimic clinical samples were prepared by spotting 50µl of human blood on Whatman® chromatography paper (17CHR, 46X57cm). Then known amount of chloroquine, desethylechoroquine and quinidine was added to the spots and were allowed to dry at 30°C in an incubator for 30 minutes. Dried blood spots cover the entire range of concentrations (10-200ηg CQ and DCQ). Then calibration curves were generated four times using CQ or DCQ to QD peak area ratios by least-squares regression.

Quality control samples, using three concentrations low (LQC = 25ηg of DCQ and CQ), medium (MQC = 90ηg of each compound) and high (HQC = 180ηg of each compound) were prepared and stored at room temperature in dark place to be used simultaneously as the patient samples are processed.

Validation procedure

Recoveries of the drug (absolute, paper elution and column extraction) from filter paper were evaluated at three different concentration levels for each compound (C1: 20ηg, C2: 100ηg and C3: 200ηg per 50µl of spotted blood). Absolute recovery was calculated by comparing peak area ratios obtained from dried blood spot controls with those obtained by direct injection of methanol standard solutions at the same concentrations. Paper elution recoveries were determined by comparing peak area ratios from dried blood spots with those from 50µl of whole blood extract at the same concentrations. Column extraction recovery was calculated by

comparing peak area obtained from whole blood sample against direct injection of the standards. Each concentration level was done six times and average was taken to calculate percentage of recovery.

Method precision and accuracy was evaluated using three different concentrations (LQC=25ηg, MQC=90ηg and HQC=180ηg) six times for each concentration level that were extracted on the same day and in duplicate for each concentration level simultaneously with the patient samples between consecutive five days.

Lower limit of detection and limit of quantification was determined by spiking 10, 20, 40, 50 and 100ηg of CQ in 1ml of whole blood, then 50µl of blood was spotted on the filter paper. CQ were extracted and quantified. LOD and LOQ were not determined for QD.

4.5 Quality Assurance

Slide Reading Quality

Ten percent of the total slides were read by a second laboratory technician and was found to be concordant with the first laboratory technician. However, two samples were discrepant, one Day 0 sample and one Day 28 sample. Discrepant samples were checked for the third time by the principal investigator. The mean parasite density was taken for Day 0 sample and the Day 28 sample was found to be negative and was reported as negative.

4.6 Data Entry, Management and Analysis

Laboratory results and other data were double entered into Excel by two different data entry clerks and verified using the validation program of the Epi-info software, version 6.04. Statistical analysis was done using STATA software version 7.0. All data and forms were kept at the data management unit of the Armauer Hansen Research Institute (AHRI), Addis Ababa, to which only the principal investigator and the data management staff have access.

4.6.1 Statistical Analysis

Data was analyzed using a traditional method of analysis of *in vivo* test. In this method analysis patients who were excluded from the study due to *P. falciparum* infection or lost-to-follow-up

during the course of the project are not included. The number and proportion of participants, who have therapeutic failure and adequate clinical response, was calculated. Potential risk factors (e.g., age, initial parasite density) were assessed for an association with the therapeutic failure and Wilcoxn rank-sum test was used to determine the level significance difference between initial parasitemia among the patients that have clear their parasitemia versus patients that failed to clear parasitemia during the follow up period.

4.7 Ethical Consideration

Before the study was commenced, ethical approval was obtained from the Addis Ababa University Ethical Review Committee, the AHRI/ALERT Ethical Review Committee and the National Ethical Review Committee (Ethiopian Science and Technology Agency) and the study also received a letter of support from the Oromia Regional Health Bureau.

Participants were selected from patients with clinical malaria due to *P. vivax* cases seeking treatment at the malaria sector in Debre Zeit. After the patients were informed clearly about the objectives of the study and the procedures, written informed consent/assent was obtained from the study participants prior to sample collection. They were well informed about the need for additional blood samples, for genotyping of *P. vivax* and determination of CQ-DCQ.

The sample collection procedures included finger pricking, which is the usual procedure for malaria diagnosis. To prevent transmission of infectious diseases such as HIV during sample collection, a new lancet was used for each individual. To minimize pain, finger pricking was done by an experienced laboratory technician. Patients were treated according to the guidelines of the Ethiopian Ministry of Health. Four patients who showed recurrent parasitemia during the follow-up were treated using Quinine as recommended by WHO (WHO 2003).

The study participants were followed for 28 days and were requested to come back for their scheduled follow-ups during which their transportation expense was covered by the project.

5. Results

Prior to the use of the chloroquine phosphate (Batch Number 0073, Adigrat Pharmaceutical Factory) for the *in vivo* drug efficacy study, its quality was checked. The quality indicators measured were rate of disintegration, rate of dissolution, identification and content of the tablets.

The drug was disintegrated within 5 minutes in a medium of water. Dissolution test showed the release of active ingredient above 75% in all the six tablets tested to the HCl medium, which mimics the gastric environment (Table 2). These parameters are in the acceptable values recommended by British Pharmacopoeia, 2004.

Table 2. Percentage of chloroquine released to the solvent medium (HCl)

Tablet No	Absorbance	Percentage (%)
1	0.466	90.48%
2	0.472	91.65%
3	0.586	113.86%
4	0.473	91.70%
5	0.452	87.76%
6	0.477	92.62%

The identification test also yielded Similar infrared spectra of the chloroquine phosphate (Fig 6) with the reference spectrum of chloroquine phosphate (Fig 7).

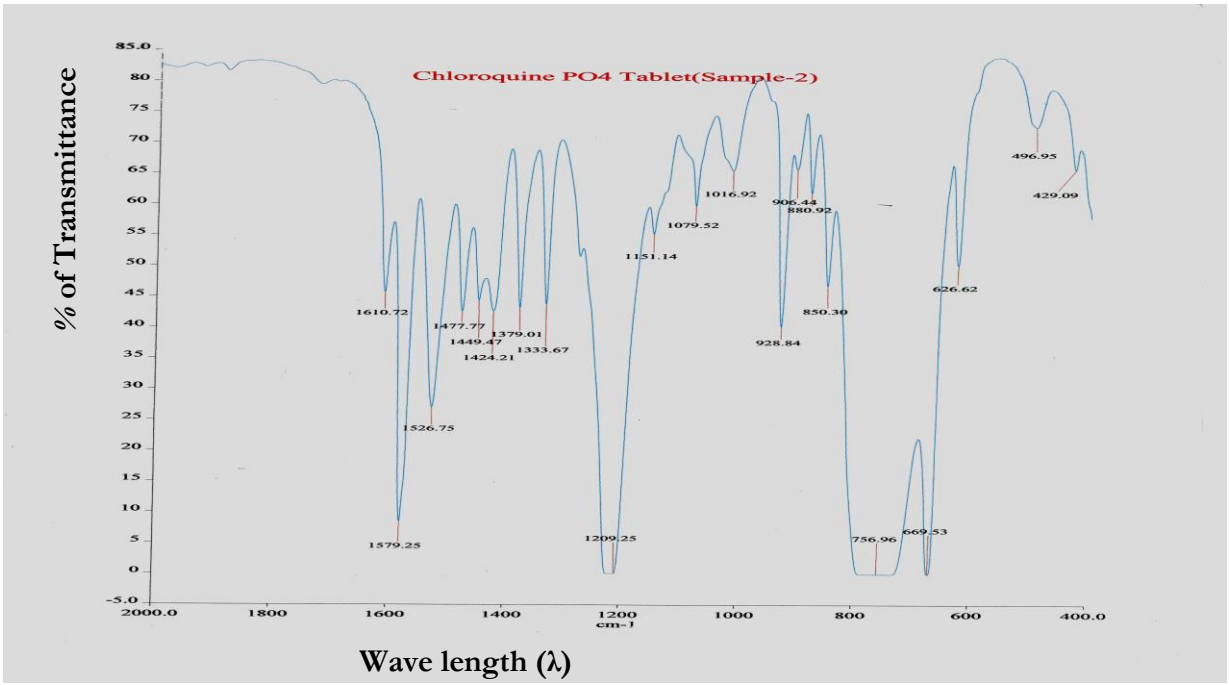


Figure 6 Spectrum of chloroquine phosphate 250mg containing 150mg of chloroquine base (Batch Number 0073, Adigrat Pharmaceutical Factory)

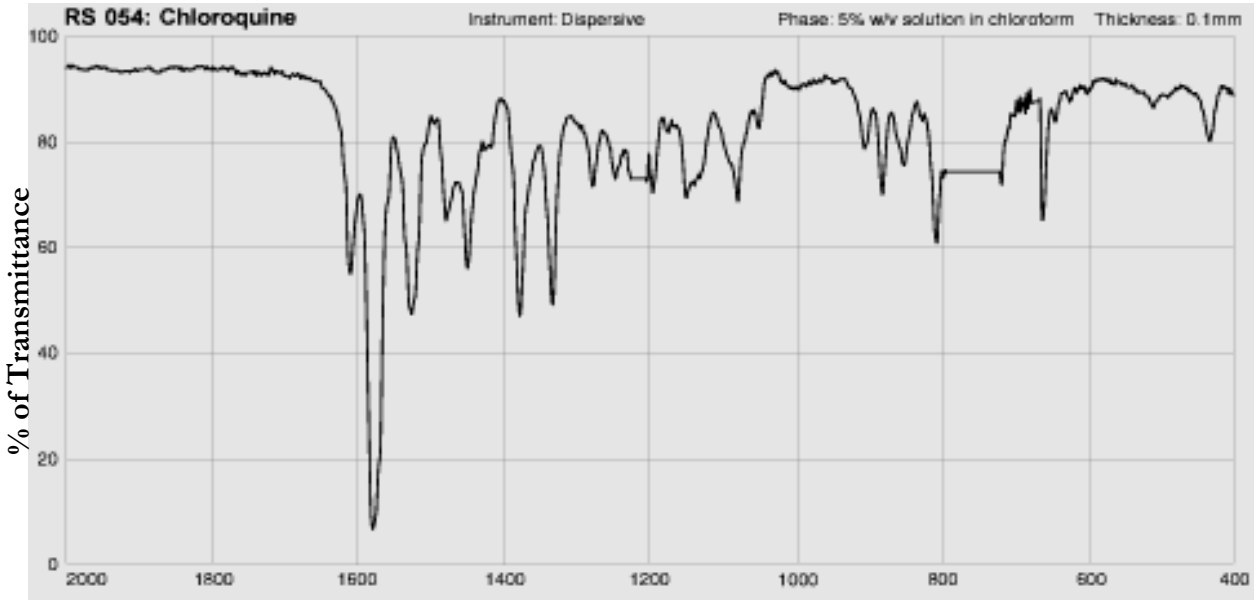


Figure 7. Reference spectrum of chloroquine phosphate (Source: British Pharmacopoeia, 2004)

The concentration in tablets was determined in triplicate samples and the results were 93.24%, 93.46% and 93.81%, which corresponds to the active ingredient of 300mg of chloroquine base. The tested batch of chloroquine tablets comply all the criteria for chloroquine phosphate tablet as per British Pharmacopeia, 2004. The same batch of chloroquine phosphate was used for *in vivo* efficacy test in Debre Zeit.

From the total of 3,655 patients who self reported to have symptoms of malaria and gave blood samples to Debre Zeit malaria center from June to August 2006, 87 patients who satisfied the inclusion/exclusion criteria were recruited. The study participants originated from 23 Kebeles of the Adea Woreda. Among these, 20% of the participants came from Kaliti followed by 14% from Keta Worogenu (Kebele 12). The median age of participants was 16 years (ranged from 8 months to 52 years). Twenty three percent (n=20) of the participants were under the age of 5 years, and 39.8% were female (60.2% male). Their mean ±standard deviation (SD) duration of illness was 3.15 (±1.85) days. Among the participants, 60.2% had a history of fever and 39.8% had documented fever at the time of diagnosis. Their geometric mean parasite density at Day 0 was 7,045 parasites/µl (Table 3). Sixteen point one percent of the patients presented with gametocytes at a time of diagnosis that ranged from 80 to 600 gametocytes/µl. Two patients who did not have gametocyte at the time of recruitment showed gametocytemia on Day 1 and one patient on Day3.

Symptoms such as fever, headache, chills/rigor, back pain, vomiting, myalgia, poor appetite, abdominal pain, cough, diarrhea and joint pain were reported by the patients at the time of recruitment. Among these, the major ones were fever (96.5%), headache (86.2%) and chills/rigor (78%).

Table 3. Characteristics of patients enrolled in the *in vivo* efficacy test of CQ in *P. vivax* malaria in Debre Zeit, June-August 2006.

Characteristics		
Total number of patients completed the study		83
Age	Median age	16 years
	Range	8 Months- 52 Years
Sex	Female	39.8% (n=33)
	Male	60.2% (n=50)
History of fever		60.2% (n=50)
Axillary Temperature > 37.5°c (Day 0)		39.8% (n=33)
Duration of illness, Mean (±SD)		3.15 (±1.85) days
Geometric mean parasite density/µl (Day 0)		7,045 parasites

Seven percent of the study participants vomited their first dose of chloroquine and they were treated with equal dose and none vomited twice. Other symptoms reported after treatment with CQ were diarrhea with 10% followed by 5% itching. Only one patient had skin rash.

Among the 87 study participants, three patients were excluded from the study due to *P. falciparum* infection during follow up. One was found positive for asexual stages of *P. falciparum* on the 21st day and the other two were positive for *P. falciparum* gametocytes on the 2nd and 3rd day. Only one participant was lost to follow-up while the remaining 83 participants completed the 28 day follow-up successfully.

Parasitemia cleared rapidly, 42% of the patients having cleared their parasitemia by day 1 and 98% by day 3 and hundred percent of parasite clearance was observed by day 7. Five percent showed recurrent parasitemia on Day 28 (Fig 8). These patients were all children aged 2.4, 3.4, 5, and 7 years. Among these, only one patient complained of having diarrhea on Day14. All the others had no complaints or symptoms of disease and had not taken any drug during the follow-up period, except on the day of recurrent parasitemia.

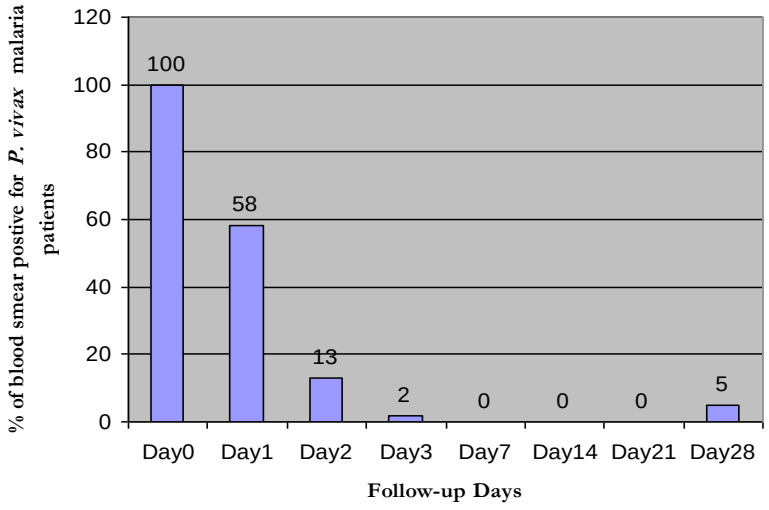


Figure 8. Percent of positive blood smears for *P. vivax* malaria parasites during the 28 Day follow-up of patients treated with chloroquine in Debre Zeit, June-August 2006.

Even though the correlation is not strong, significant negative correlation was observed between age and parasitemia at Day 0 (r=- 0.3136 p=0.003). The parasite density at Day 0 of patients with recurrent parasitemia during the follow up were 30,000, 14,000, 28,400 and 6, 520 parasites/µl. However there was no significant difference (Willcoxn rank sum test p=0.0613) observed in the parasitic density at day 0 between the patients who were eventually cured versus the patients who failed to clear parasites.

According to the WHO (2002) classification of treatment response, 95% of the 83 participants have been treated successfully. However, five percent treatment failure was observed.

To confirm the cause of treatment failure observed in the present study blood drug level was determined using HPLC. Prior to the determination of drug level in the patient samples, the HPLC method to be used was validated and standard calibration curve was generated. The results are shown below.

The assay performances were assessed by specificity, accuracy, precision and linearity studies. The specificity of the peaks of the analytes (CQ and DCQ) and internal standard (quinidine QD) was determined by injecting standards dissolved in methanol directly to the HPLC. The retention time determined for DCQ, CQ and QD was 4.91, 6.56 and 12.45 min respectively

(Table 5). An example of a chromatogram standard is shown below (Fig 9).

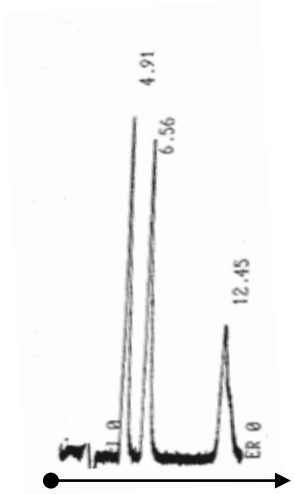


Table 4. Retention time of CQ, DCQ and QD and their respective peak area of the chromatogram shown on Fig 9.

Peak	Retention Time	Area
CQ	4.91	64644
DCQ	6.56	68931
QD	12.45	29681

Figure 9. Chromatogram showing peaks after extraction of the drug from filter paper and retention time in minutes are shown adjacent to the peaks of standard sample.

To determine the accuracy and precision of the method used, quality control samples (QCs) using three concentrations, low (25ηg, LQC), medium (90ηg, MQC) and high (180ηg, HQC), were run. Then accuracy and precision within QCs processed on the same day (6 times for each concentration) and between QCs processed in different days that were run simultaneously with patient samples (five days) (Table 6) was determined. The reproducibility for intra-assay was in the acceptable range of RSD of less than 10% for MQC and HQC. A slight increase was observed for LQC. The accuracy was also in the acceptable range ±20% of the expected value for LQC and ±15% for MQC. A slight increment was observed for HQC. The method accuracy for inter-assay was all in the acceptable range. However, the precision was not as expected except for LQC in determination of DCQ.

Table 5. Precision and accuracy as measured by %bias and relative standard deviation of blood spot samples spiked with known amounts of CQ and DCQ (25, 90 and 180ηg) extracted from filter paper.

Intra-assay				
Compound	Concentration added	Concentration found	Accuracy (% bias)	Precision RSD
CQ	25 LQC	29.51±3.46	18.04	11.73
	90 MQC	102±10.59	14.04	10.32
	180 HQC	251±12.04	19.68	5.59
Inter-assay				
Compound	Concentration added	Concentration found	Accuracy (% bias)	Precision RSD
CQ	25 LQC	23.72± 6.61	15.85	22.82
	90 MQC	99.35±21.42	10.39	21.56
	180 HQC	162.77± 24.08	-9.57	14.80
DCQ	25 LQC	22.75±1.29	-9.00026	5.65
	90 MQC	101.12±16.58	12.35289	16.40
	180 HQC	165.92±32.06	-7.82479	19.32

Recovery test was done at three different concentrations (20, 100 and 200ηg per 50μlof blood) each six times and percentage of absolute recovery, paper elution and column extraction was determined. The absolute recovery ranged from 87.82 to 94.7 for CQ and 84.2 to 91.2 for QD from filter paper against direct injection. When extraction recovery from filter paper was compared against whole blood (Paper elution), it ranged 83.13 to 95.66% for QD and 78.1% to 85.99% for CQ and was found as good as extraction from whole blood (Table 7). The column extraction as measured by whole blood against direct injection gave a recovery percentage that ranged 96.4 to 98.3 for QD and 102 to 125 for CQ.

Table 6. Recovery of extraction of chloroquine from filter paper and whole blood samples spiked with known amount of the drug.

Concentration added	Concentration found	Absolute recovery		Paper elution		Column Extraction	
		QD	CQ	QD	CQ	QD	CQ
Low concentration (20ηg)	Mean	91.2	94.7	95.66	85.99	96.4	125.5
	CV%	19.7	7.2	19.70	19.65	9.5	6.8
Medium concentration (90ηg)	Mean	82.49	83.62	83.13	78.09	95.49	105.0
	CV%	12.34	17.67	14.95	19.69	6.25	4.5
High concentration (180ηg)	Mean	84.20	87.82	85.65	85.30	98.31	102.9
	CV%	10.95	9.01	10.95	9.01	7.79	6.3

QD= quinidine CQ= chloroquine

The lower limit of detection was 50ηg/ml of blood and the lower limit of quantification was found to be 100ηg/ml of blood for CQ. Lower limits of DCQ were not determined due to the short period of stay in Kenya.

The HPLC method was found to be in the acceptable range in its precision and validity to measure CQ and its metabolite DCQ. Therefore the amount of CQ and DCQ was determined using a calibration curve that was generated based on the analysis of the data from extraction of samples spiked with known amount of CQ and DCQ standards in the range of 10-200ηg. The peak area was linear over the concentration range. Linear equation was obtained by least square linear regression. The linear equations are $y = 0.014x - 0.167$ and $y = 0.015x - 0.17$ for CQ and DCQ respectively, where x is amount of CQ and DCQ, y is a peak area ratio of CQ to DCQ. Four calibration curves were generated and found to be consistent; among these two were selected to calculate CQ and DCQ amounts for the patient samples (Fig. 11 and 12)

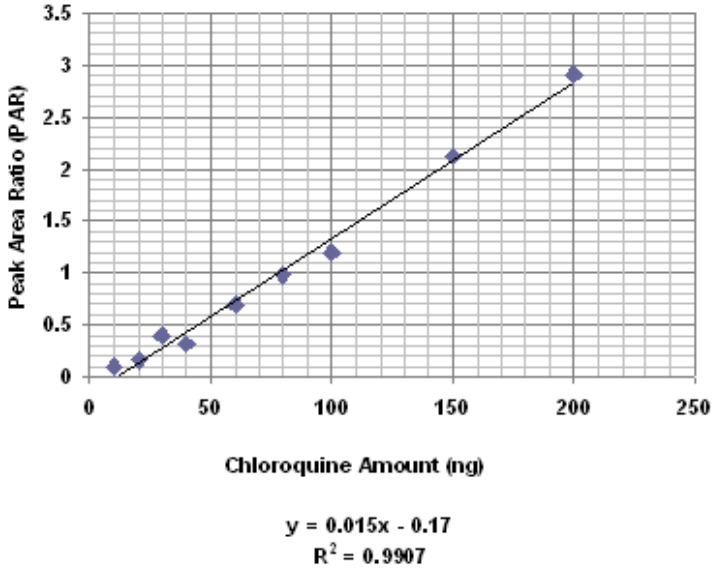


Figure 10. Calibration curve for Chloroquine

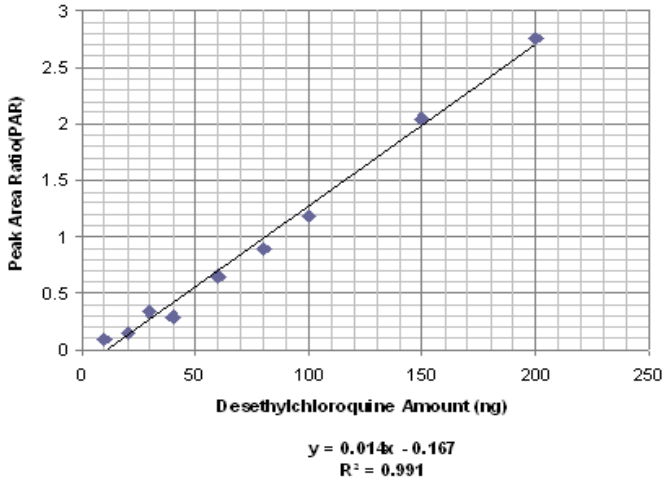


Figure 11. Calibration curve for Desetylchloroquine

Determination of CQ-DCQ level was performed for eighty-three blood samples that were taken on Day 2 before intake of the third dose. The median CQ plus DCQ level was 882.3ηg/ml that ranged from 166.8 to 6,714.6ηg/ml. There was no significant difference observed in their CQ-DCQ level at Day 2 between the patients that showed recurrent parasitemia versus patients that have cleared their parasitemia throughout the 28 days follow-up (Wilcoxn rank-sum test, p=0.52).

The levels of CQ -DCQ in the blood at day 28 for patients who cleared their parasitemia were 218.4, 297.3, and 521.8 ηg per ml (Table 8). For patients that showed recurrent parasitemia were 524.6, 672.1, 521.8, 868.6 and 1,164.0ηg per ml (Table 7), all above the MEC.

Table 7. Chloroquine plus desethylchloroquine level in the blood of *P. vivax* malaria patients with adequate clinical and parasitological response, Debre Zeit, 2006.

Case No	Age (years)	Blood CQ+DCQ level (ηg/ml)	
		Day 2	Day 28
61	2.7	759.8	218.4
79	5	816.6	297.3
46	8	1,447.1	521.8

Table 8. Chloroquine plus desethylchloroquine level in the blood of *P. vivax* malaria patients with late clinical failure, Debre Zeit, 2006.

Case No	Age (years)	Blood CQ+DCQ level (ηg/ml)	
		Day 2	Day 28
02	2.4	829.9	524.6
37	7	486.1	672.1
57	3.4	1,557.8	868.6
84	5	1,611.9	1,164.0

In addition to the determination of drug level PCR was done to determine whether the parasite strains at a time of recruitment and recurrent parasitemia are similar or different.

PCR was run on five paired samples that were collected from the patients who showed recurrent parasitemia. It was only possible to amplify five isolates: four Day 0 samples and one Day 28 sample. Comparison of Day 0 and 28 was possible for only one patient (code 57). Day 0 samples showed a PCR product of 1.9kb. However Day 28 samples showed 1.9kb and 1.5kb showing the existence of two parasite populations in which one strain was not detected from samples that were collected at the time of recruitment (Fig.12).

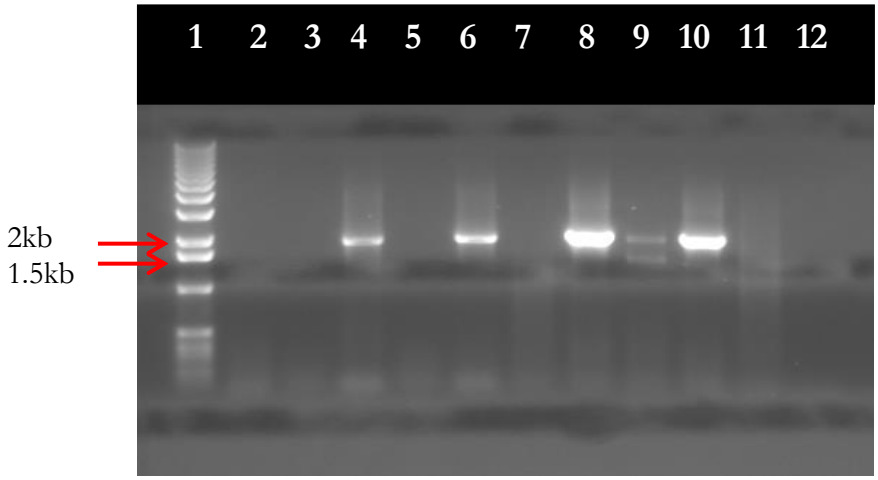


Figure 12. Uncut polymerase chain reaction amplification product of *Plasmodium vivax* merozoite surface protein-3α gene from patients that showed recurrent parasitemia during the 28 days follow-up (L1 DNA ladder, L12 negative control lane 2-11 clinical isolates)(Lanes 2, 4, 6, 8 and 10 are Day 0 samples whereas Lanes 3, 5, 7, 9 and 11 are Day 28 samples).

RFLP was intended to determine whether Day 0 and Day 28 samples have same or different parasite populations. However, the PCR products of Day 28 sample from one patient gave different bands suggesting the presence of different strain of a parasite population that was not observed in Day 0 sample.

To determine the stains of circulating parasites in the patients, RFLP was done on the five PCR products obtained from the patient samples. Three strains were observed from five positive PCR samples using restriction enzyme *Hba I* (Fig 13).

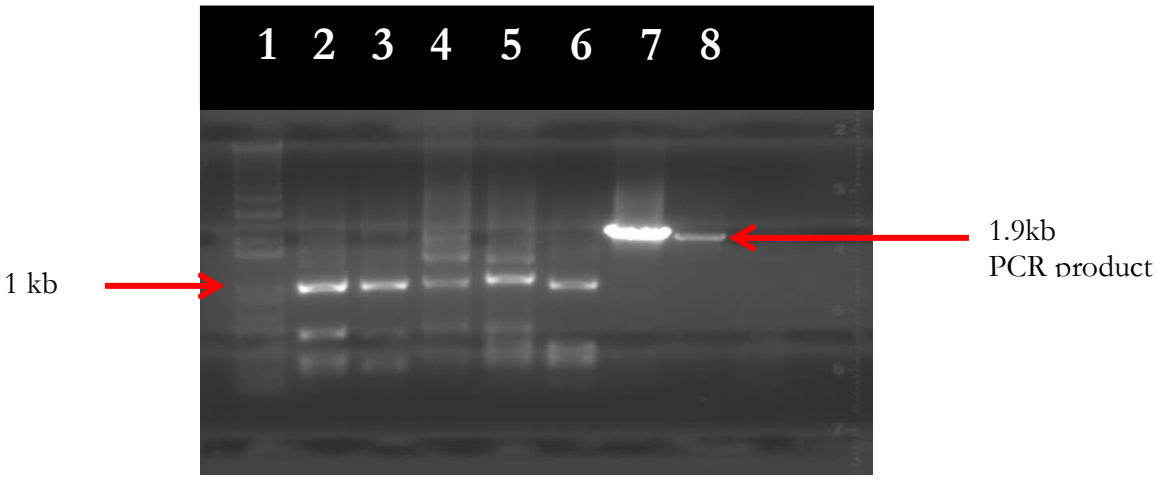


Figure 13. Restriction fragment length polymorphism (RFLP) patterns after digestion with the restriction enzymes *HhaI.* (Lane 2-6) (Lane 1 DNA ladder) (Lane 7&8 uncut PCR products)

6. Discussion

Chloroquine has been in use for over forty years in Ethiopia and it is still the first line drug for the treatment of *P. vivax* malaria. Thus the emergence of drug resistance in *P. vivax* in Debre Zeit, which has prevalence of 70%, would be a major public health concern.

Poor quality drugs have been one of the factors that contribute to the treatment failures and emergence of drug resistant parasites (Basco, 2004). In the present study to rule out the use of poor quality of drug as being one of the reasons for the treatment failure, drug quality was determined. When a batch CQ- phosphate tablets comply dissolution test with the percent of active ingredient release above 75% to the HCl medium, disintegrated within 5 minute in water, having a spectrum identical to Fig. 7 (using infrared spectroscopy) and a concentration of chloroquine phosphate that fall in the range of 92.5 to 107.5% of the stated amount is of a standard quality of CQ-phosphate tablets as per British Pharmacopoeia (2004). Based on these criteria the tested drug in the present study is of a good quality. Therefore, same batch of tablets was used to treat study participants at Debre Zeit.

Among the 87 *P. vivax* positive study participants recruited, majority of them came from two Kebeles, Kaliti (20%) and Ketaworogenu (K-12) (16%) among the 23 Kebeles of Adea District. This might be due to the irrigated horticulture introduced in Kaliti and the presence of Wodech River that crosses the Ketaworeogenu Kebele, which may provide a suitable environment for the breeding of vector mosquitoes.

Although history of sudden onset of fever was recorded, 62% of the patients had no detectable fever at the time of recruitment. This can be explained by considering the common manifestation of malaria illness - high fever followed by chills - in which the patients might be in the afebrile state at the time of physical examination. Since temperature measurement included use of a digital thermometer, which allowed for sufficient time before reading, inadequate duration of temperature measurement as a cause for the high proportion of afebrile patients can be ruled out.

The treatment failure observed in patients who were under the age of seven years was similar with reports from Turkey and Peru in which treatment failure was observed in children than adults (Ruebush *et al.,* 2003; Kurcer *et al*., 2004). In addition, the negative correlation between age and initial parasite density, observed in this study, could be interpreted as an indication of age related acquired immunity. It is known that long-term exposure to both *P. vivax* and *P. falciparum* confers partial immunity (Cole-Tobian, 2002; Baird, 1998). Therefore, observed treatment success in the adults might be due to acquired immunity, which has an effect on the outcome of treatment response (White, 1997).

Even though parasite density at Day 0 was higher in the patients with recurrent parasitemia, compared with patients who cleared parasitemia, the difference was not statistically significant. A similar finding was reported from Colombia; whereby 44% treatment failure of CQ in treating *P. falciparum* was not related to initial parasitemia (Osorio *et al.,* 1999). In contrast, in a study from North Ghana, among 225 children under the age of five, initial high parasite density was an independent predictor of early treatment failure (Ehrhardt *et al*., 2002).

The rationale of *in vivo* efficacy study is based on the recurrent parasitemia within 28 days of supervised CQ therapy. In this period blood level of CQ is believed to be above the MEC. Confirmation of this requires proof of adequate compliance to and absorption of the drug by reliable supervision or, ideally, by determination of the levels of drug in blood. In places where drug determination is not available, knowledge of pharmacokinetics of CQ would give indicative information on the presence of drug resistance using the *in vivo* efficacy only. In the present study although measurement of CQ-DCQ level was done only for three patients who were treated successfully, it was above the MEC at Day 28. This finding was similar with the reports from Indonesia where the level of CQ-DCQ in patients treated with a standard dose of Chloroquine was above the MEC (Baird, 1997a).

In the present study therapeutic response was interpreted using the criteria developed for the diagnosis of resistance using an *in-vivo* test (Baird *et al*., 1997b). Accordingly, a recurrent parasitemia within 28-day follow-up after full compliance with standard CQ therapy was taken as the defining diagnosis for drug resistance. In addition, evidence of CQ-DCQ level above the minimal effective concentration (MEC) in the blood demonstrates the presence of

resistance regardless of the origin of parasite; whether it is relapsed from liver or is a recrudescence from blood stage (Baird *et al.*, 1997b; Baird, 2004). Therefore, based on the above criteria the recurrent parasitemia observed was due to resistance, as these parasites are present in the face of chloroquine level above the MEC.

The appropriateness of this method as a conformation of resistance has been shown by other studies from different geographical areas. Eighty four percent of resistance was reported from Indonesia, 14% from Myanmar, 10.1% from Brazil and 1 % from Peru, using 28 days follow-up on 29, 50, 109 and 177 patients, respectively. In all these studies resistance was confirmed using determination of CQ-DCQ level at a time of recruitment except Brazil where only CQ level was measured (Marala *et al.*, 1995; Sumawinata *et al.*, 2003; Ruebush *et al.*, 2003 Filho *et al.,* 2007).

Although the published report from Debre Zeit showed treatment failure of CQ, their findings were not confirmed by either measurement of CQ-DCQ or genotyping (Tulu *et al.*, 1996).

In the present study prior to the determination of CQ-DCQ level from the patient samples; the method to be used was validated according to standard method of validation. An HPLC method is valid when the accuracy of QCs for inter- and intra-assay are within the acceptable range of ±20% for LQC and ±15% for MQC and HQC, and a precision below 10% for LQC, MQC and HQC. In addition, recovery test must yield above 75% of extraction when compared to the direct injection of the analyts. Even though, the precision was not below 10% for some tests based on the standard criteria the method used has accuracy within the acceptable range. The recovery test has also shown that the extraction method yields the recovery of the drug from filter paper above 82% (http://www.fda.gov/Cder/guidance/cmc3.pdf). In general, the method was valid to determine the blood level of CQ and DCQ from the patient samples. The lower precision might due to the slight modification in the method used.

In addition to the determination of drug level in patient blood, molecular genotyping has been recommended by the WHO on studies of anti-malarial drug efficacy (WHO, 2003). This method would enable to distinguish between relapse/recrudescence and re-infection in

P. vivax. The method assumes that primary parasite population is the same as in recurrent parasitemia due to relapse (Craig and Kain*,* 1996). However, a recent study from three different countries has shown the presence of heterologous hypnozoites in the liver (Imwong *et al.,* 2007) in which a single allelic type of hypnozoites is being activated at a time (Chen *et al.,* 2007). In addition, this assumption does not take into account the possibility of some strains circulating at low parasite density that cannot be detected in primary samples but could emerge to cause treatment failure, thus leading to misclassification of a recrudescence as a new infection (Baird, 2004). Moreover, lack of a standardized classification of the outcome in categorizing mixed genotypes as recrudescence or re-infection is a limitation of this method.

In some studies, mixed genotypes have been classified as new infection (Cattamanchi *et al.,* 2003). However, a study from Uganda (Slater *et al.,* 2005) has shown that the classification of mixed genotypes as recrudescence or new infection without taking the transmission dynamics of the study area into consideration will lead to wrong reporting. In the study, data from 3,000 patients enrolled in clinical trials at seven sites in Uganda from 2002-2004 was analyzed. Using this they suggested that a classification of mixed infections as recrudescence will increase the treatment failure from 38% to 84% for a high transmission area. Simply classifying mixed infections as new infection will also affect the decision as different stains of parasite emerge from the liver at different times. Based on these studies, evidence from genotyping using PCR-RFLP in *P. vivax* can only help us in confirming whether the genetic identity of the recurrent parasites is identical to those from the primary infecting parasite at Day 0 or not. In the context of the present study, although only one paired sample was amplified, the finding was an evidence of the presence of mixed strains.

Until molecular markers, which correlate with resistance in *P. vivax*, are identified an *in vivo* test combined with determination of CQ-DCQ levels in the blood will provide plausible information on resistance.

In order to rule out the degradation of DNA as one of the possible reasons why we couldn't amplify the DNA we repeat the extraction procedure and load the DNA sample directly on a gel containing 0.25μg/ml of ethidium bromide and intact DNA samples have been observed (no smear). The inability to amplify 5 samples from patients with recurrent parasitemia in this

study may have been due to the mismatch at the 3' end of the primers, which could thus not be attached to the parasite DNA. In order to rule out technical problems, the samples were processed again starting from sample extraction. This problem of failure to get PCR signals had also been observed in the study by Bruce *et al.*, (1999). They could not amplify DNA in 17 of 39 samples using these primers. In addition, one sample could not be amplified in a study by Gizaw (2005) using the same primer and protocol even though the specimen was microscopically positive for parasites.

Although RFLP was performed only on five isolates, three patterns were observed which indicated the presence of diverse strains. This is in agreement with the presence of diverse stains of *P. vivax* reported from 20 isolates from different parts of Ethiopia (Ziway, Dera, Arbaminch and Bure) (Gizaw, 2005).

In summary, CQ is still highly efficacious in the treatment of *P. vivax* malaria in Debre Zeit. However, the presence of confirmed 5% resistance in this area rings the alarm in the need to monitor the pattern of resistance in parts of the country where *P. vivax* is prevalent. The trend in the pattern of resistance in Indonesia showed a high resistance of this parasite within 14 years. If transmission of the drug resistance continues unabated in our country, similar situation will happen in the near future. Therefore, further investigation in drug resistance pattern will help in the proper management of resistance cases and in detecting the possible rise of treatment failures.

7. Conclusions

1. Chloroquine is effective with 95% efficacy and well tolerated in patients with *P. vivax* malaria in Debre Zeit.

2. HPLC determination of CQ-DCQ level in the blood of patients with recurrent parasitemia provided evidence of the existence of clinically resistant *P. vivax* malaria in Debre Zeit.

3. Even though it is difficult to conclude from data of only three patients with adequate treatment response, CQ-DCQ level above MEC on Day 28 was noted in a supervised treatment of CQ.

4. PCR showed the existence of mixed parasite population at the time of recurrent parasitemia of which could be an early relapse of a resistant parasite population or a recrudescence from primary parasitemia.

8. Recommendations

1. Further multi-centered investigation should be done to substantiate the present result from other parts of Ethiopia.

2. The pattern of *P. vivax* resistance should be monitored using the existing regular surveillance system for *P. falciparum*.

3. Sequencing *P. vivax* isolates from Ethiopia must be considered to design specific primer for a more specific and sensitive PCR amplification.

4. Future studies on assessment of CQ resistance to *P. vivax* should consider drug quality test and determination of serum CQ and DCQ for confirmation of the presence of drug resistance.

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Annex 1: Case Record Form

Section A

Date: _____ Participants Study Number:_____

Health Center: _____

Patient's Name: _____

(For children) Mother's Name: _____

Father's Name: _____

(Address) Direction to home: _____

_____

_____Transport cost (round trip): _____

Age: ☐☐ Sex: 1.Male ☐ 2.Female ☐ Weight: ☐☐.☐ (Kg)

History of Patient's Illness (if a child, ask parent/guardian)

Have you had fever during the last 48 hours? Yes ___ No___ Duration:_____days

What symptoms do you or your child (if the patient) have?

1. _____ Duration: _____days

2. _____ Duration: _____days

3. _____ Duration: _____days

Have you taken any medications for the illness or has any medication already been given to the child for the illness: 1.Yes ☐ 2.No ☐

(If the answer is "YES") What medications? What doses? When? (Ask specifically chloroquine, Fansidar, other antimalarial drug traditional drugs anti-acid or cemetidin):

No	Drug	Dose (mg or tabs)	Date
1			
2			
3			

Evaluation of the Patient

Axillary temperature (°C): _____ Respiratory rate (30 sec.) (Children < 5 years): _____

Is there evidence of any other medical problem? 1. Yes ☐ 2. No ☐

Which ones? _____

Is additional medication needed? 1. Yes ☐ 2. No ☐

What medication? _____

Laboratory Evaluation (Day 0)

Parasite species: _____

Asexual parasites/ WBC: _____/_____

Gametocytes: _____

Treatment (Day 0)

CQ: _____ Tablets = _____mg

Time of the first dose: _____ vomiting (within30 min.)? 1. Yes ☐ 2. No ☐

Time of the second dose: _____ vomiting (within30 min.)? 1. Yes ☐ 2. No ☐

Did the patient receive paracetamol? 1. Yes ☐ 2. No ☐ Time: _____

Axillary temp after paracetamol: _____ Time: _____

Section B Case Record Form

Participant Study Number: _____ Weight (kg): _____

Follow-up day	Day 0	Day 1	Day 2	Day 3	Day ____	Day ____	Day 7	Day ____	Day 14	Day ____	Day 21	Day ____	Day 28
Date (Day/Month)													
Chloroquine dose (mg)				-	-	-	-	-	-	-	-	-	
Axillary temperature (o C)													
Since your last visit, have you had: Fever? (Y/N)													
Vomiting? (Y/N)													
Diarrhea? (Y/N)													
Itching? (Y/N)													
Skin Rash? (Y/N)													
Any other drug given? (Y/N)													
(If other drug given) Type and amount.													

Follow-up day	Day 0	Day 1	Day 2	Day 3	Day ____	Day ____	Day 7	Day ____	Day 14	Day ____	Day 21	Day ____	Day 28
Any other treatment? (ex., blood transfusion) (Y/N)													
Better, Same or Worse (B/S/W)													
Parasite species (V, F, NPF*)													
Parasite Density													
Gametocyte Density													
Patient condition (Continue, Withdrawn, Abandoned) (C/W/A)													

**-Ve= Negative

Definition of diarrhea: The abnormal frequency and liquidity of fecal discharge.

If patient is excluded from the study or is lost to follow up, please list reasons:

_____

_____

_____

Annex 2: Schedule of Study Procedures

The following chart outlines the procedures and treatment for study subjects:

	Day 0 (Enrollment)	Day 1	Day 2	Day 3	Day 7	Day 14	Day 21	Day 28	Any other day (after Day 3, if fever)	Day of any recurrent parasitemia
Procedures										
Blood smear	X		X	X	X	X	X	X	X	X
Axillary temperature	X	X	X	X	X	X	X	X	X	X
History and physical exam	X	X	X	X	X	X	X	X	X	X
Blood for PCR	X									X
Blood for CQ levels (EDTA-coated tube)			X							X
Treatment										
CQ	10 mg/kg	10 mg/kg	5 mg/kg							

Annex 3: Classification of Treatment Response

Definition of Treatment Failure (TF):-

- Clinical deterioration due to *P. vivax* illness requiring hospitalization in the presence of parasitemia.
- Presence of parasitemia and axillary temperature ≥37.5 °C any time between Day 3 and Day 28.
- Presence of parasitemia on any day between Day 7 and Day 28, irrespective of clinical condition.

Annex 4: Consent/Assent Forms/Information sheet

Information Sheet Form

Assessing the efficacy of chloroquine in the treatment of *Plasmodium vivax* malaria in Debre Zeit, Ethiopia.

Principal investigator:	W/t Hiwot Teka
Supervisors:	Prof. Beyene Petros (AAU)
	Dr. Howard Engers (AHRI)
	Dr. Abraham Aseffa (AHRI)
	Dr. Lawrence Yamuah (AHRI)
Name of the health center:	_____
Name of sponsor:	Armauer Hansen Research institute (AHRI)
	Addis Ababa University, School of graduate studies (AAU)

Purpose of the study

P. vivax represents 40% of all malaria infection in Ethiopia. It is a major cause of morbidity. High-risk groups are pregnant women and children under five years of age. The disease causes low birth weight and anemia in pregnant women; anemia in children. The main control strategy is to treat malaria cases with effective drugs. To attain this goal drug resistance surveillance must be done to use this approach as effective control strategy. Recently, few chloroquine failure cases have been reported. Therefore the main purpose of this research is to assess the efficacy of chloroquine, which is a first line drug in treatment of *P. vivax* malaria in Debre Zeit, Ethiopia.

Procedure

To do this volunteer participants age greater than 6 months who are with *P. vivax* mono infection will be recruited and treated under supervision with the first line drug for three days and followed up to 28 days. Blood samples will be taken from the participants.

If you agree/ agree for your child to participate in the study: You will be asked about you or your child's health status and other related questions. Additional blood samples (400µl) will be taken for laboratory tests such as drug level and to know the type of parasite you have by finger pricking.

You/your child should come seven days within 28 days for follow up.

Risks

There are some risks associated with this study. The patient may experience a brief moment of physical discomfort and/or fear during the finger prick procedure and the site of the finger prick rarely might become infected.

A minority of subjects might experience side effects from the drug. The most commonly reported side effects from CQ are nausea, vomiting, headache, slightly blurred vision, and pruritus. A severe hypersensitivity reaction is rare, seen in less than 1 in 10,000 people.

Commonly reported side effects of quinine include tinnitus, nausea, blurred vision, and headache. These symptoms clear with cessation of therapy.

Benefits

Participants enrolled in this study will benefit in several ways. All subjects will receive their treatment doses under supervision. In addition, the subjects will be examined and treated for other concurrent illnesses. At each visit, the patient or parent/guardian (in the case of children) will be informed as to the status of the patient's health, and the procedures and/or treatments that will occur during that visit.

Participants will immediately receive treatment for adverse drug reactions or alternative antimalarial therapy with a known effective drug, as soon as a problem is identified.

If the participants have a resistant parasite in their blood they will be treated with other effective drug.

Incentives

Participants will not be provided any incentives to take part in the research however 15 Birr per day will be reimbursed for transport and the lost time during participation in the study.

Confidentiality

If you can decide to take part/decide for your child to take part in this study any personal information about you/your child will be kept private, and neither your name nor your child's name will be used in any report that results from this study. Information about you/your child will be filed with code. Only the physician and the investigator will know the codes.

Rights to refuse or withdraw

You have full rights to refuse taking part in the study. Even if you do not want to take part/do not want your child to take part in this study, you/he/she will still be able to be treated at this clinic. You can also withdraw yourself/your child from the study at any time and for any reason you have. You will still be able to get the treatment according to the usual standard of care here and will not lose any benefits.

Whom to contact

This thesis project is approved by the AHRI/ALERT, Department of Biology, Addis Ababa University and National Ethical committees. If you want to get more information about the project or if you have any complaints you may contact the ethical committee through the following address

1 **Prof. Beyene Petros Addis Ababa University, Department of Biology info@bio.aau.edu.et or Tel. 011-1239471.**
2 **Dr. Howard Engers (Director of AHRI) AHRI/ALERT**
Tel. 011-3211334 or E-mail engersh@ahrialert.org
3. **Dr Yimtubezinash W/Amanuael Tikur Anbessa Hospital/Medical Faculty,**
Tel. 0115-528726

If you don't understand or if you have any questions about the information given above you may contact the principal investigator:

Ms Hiwot Teka Tel. 0911-614602 or hiwtek@yahoo.com

Consent Form for Adults (English Version)

Efficacy of Chloroquine for the Treatment of *Plasmodium vivax*

Identification No: _____ Date: _____

Health Institution: _____

A study will be conducted to assess the efficacy of chloroquine in the treatment of *Plasmodium vivax* malaria parasites in Ethiopia. To do this, a group of patients with malaria will be treated and then followed for 28 days to see if their malaria is cured or not.

If you agree/agree for your child to take part in this study, we will need to ask you about you/your child's health, do a medical examination, and take a small amount of blood from your finger and test it to find out if you/your child have/has malaria. All patients who join the study will receive chloroquine, the standard treatment for malaria *P. vivax* in Ethiopia.

We would like you to come back/bring your child back to the clinic seven times over the next four weeks so that we can follow the progress of the treatment. On seven of these visits we will again take a small amount of blood from you/your child's finger. In addition, on the second day we will take blood to check the level of the medicine, chloroquine in your blood. If you still have malaria on one of the follow up blood smears, we will want to take the same amount of blood for the same purpose. If you are a woman, additionally we will test your urine for pregnancy.

I understand that I/my child may feel a bit of pain or fear as my/my child finger is pricked. On rare occasions this can cause an infection in your finger. Sometimes the drugs used to treat malaria have side effects. I am informed that there are risks and if I/my child get worse after taking the study medicine, there will always be a health worker at the clinic who can give me/my child treatment for these problems. And if I /my child have bleeding problem care will be taken while blood sample collection.

If I decide/decide for my child to take part in this study, I will benefit because I /my child will have close follow-up over the next 28 days. If I/my child still have/has malaria after taking the treatment with chloroquine, I/my child will receive an alternative treatment.

If I decided to take part/my child to take part in this study, I understand that any personal information about me or my child will be kept private, and neither my name nor my child's name will be used in any report that results from this study.

Even if I do not want to take part/do not want my child to take part in this study, I/my child will still be able to be treated at this clinic if I want. I understand that taking part in this study will not cost me anything, but I will not get paid anything for joining the study however treatment cost will be covered by the project and I will be reimbursed 15 Birr per day for my lost time during participation in the study and for transportation.

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a subject in this study and understand that I have the right to withdraw from the study at any time without in any way affecting my further medical care.

_____ _____ _____

Name/Signature of Patient (Parent/Guardian) *Date* *Name of Child*

_____ _____

Witness's Signature *Date*

_____ _____

Witness's Signature (if verbal consent) *Date*

Child's Assent Form (English Version)

Efficacy of chloroquine for the treatment of *Plasmodium vivax*

Identification No: _____ Date: _____

Health Institution: _____

To know the medication for treatment of *P. vivax* malaria is still effective, we are doing a research study.

If I agree to take part in the study, I will be treated for malaria with chloroquine. And I understand that I have to come back to the clinic seven times over the next four weeks so that they can follow the progress of the treatment. On seven of these visits we will again take a small amount of blood from you/your child's finger. In addition, on the second day we will take blood to check the level of the medicine, chloroquine in your blood. If you still have malaria on one of the follow up blood smears, we will want to take the same amount of blood for the same purpose. If you are a woman, additionally we will test your urine to see if you are pregnant.

I understand that I may feel a bit of pain or fear as my finger is pricked. On rare occasions this can cause an infection in my finger. I was also told sometimes the drugs used to treat malaria have side effects. However, if I get worse after taking the study medicine, there will always be a health worker at the clinic who can give me treatment for these problems. And if I have bleeding problem care will be taken while blood sample collection.

The study was explained and I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a subject in this study.

_____ _____ _____

Child's name/Signature *Age* *Date*

_____ _____

Witness's Signature (for verbal consent) *Date*

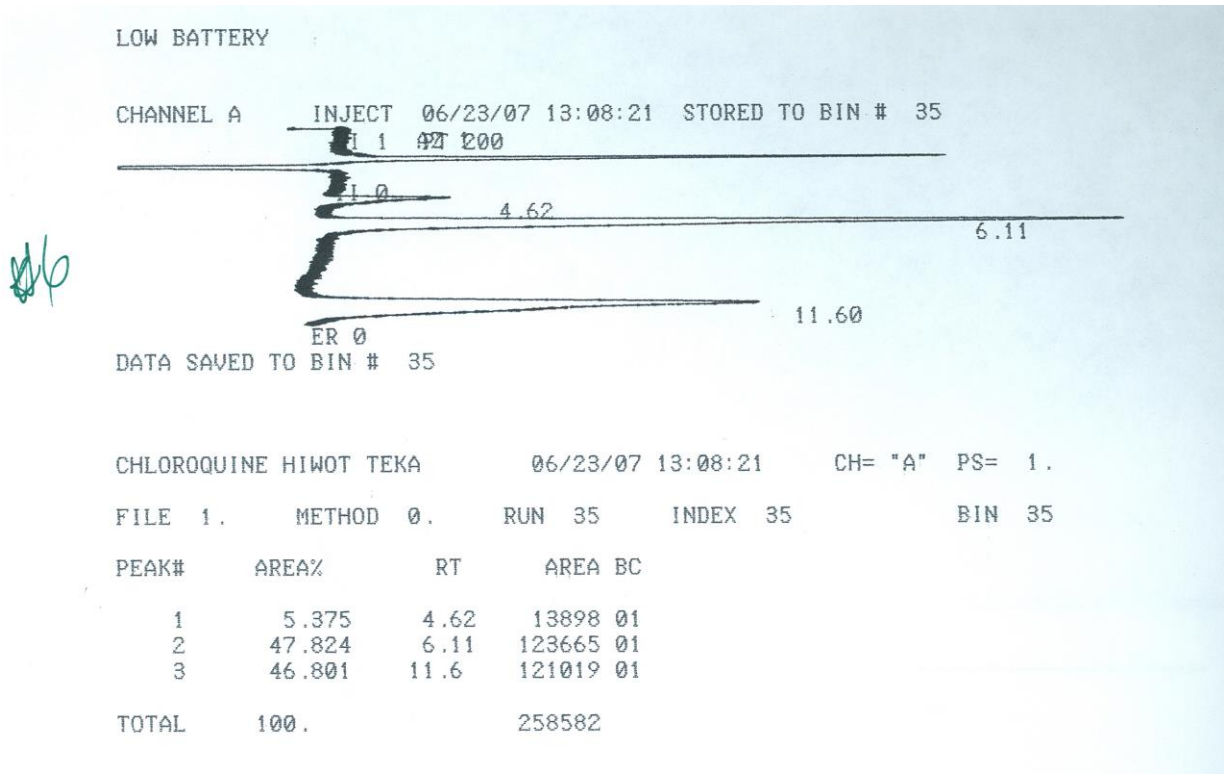
Annex 5: Parasite density at a time of recruitment

Patient code	Parasite Density at Day 0/µl	Parasite Density at Day 28/µl
1	31250	-ve
2	30000	24,105
3	35928	-ve
4	3480	-ve
5	1320	-ve
6	6240	-ve
7	4640	Excluded
8	4280	-ve
9	19440	-ve
10	19240	-ve
11	256	Excluded
12	17640	-ve
13	3560	-ve
14	256	-ve
15	6200	-ve
16	19480	-ve
17	26666	-ve
18	3040	-ve
19	1280	-ve
20	3000	-ve
21	1160	-ve
22	6000	-ve
23	8960	-ve
24	1760	-ve
25	9080	-ve
26	14600	-ve
27	14160	-ve
28	3920	-ve
29	30255	-ve
30	400	-ve
31	18320	Excluded
32	8120	-ve
33	52631	-ve
34	25000	-ve
35	600	-ve
36	3640	-ve
37	14840	11,760
38	1800	-ve
39	760	-ve
40	12200	-ve
41	16160	-ve

42	10800	-ve
43	9080	-ve
44	4560	-ve
45	16280	-ve
46	35714	-ve
47	1800	-ve
48	7880	-ve
49	6080	-ve
50	40816	-ve
51	10240	-ve
52	12080	-ve
53	920	-ve
54	2760	-ve
55	21621	-ve
56	43234	-ve
57	28400	27,727
58	2360	-ve
59	12800	-ve
60	6260	-ve
61	13280	-ve
62	25316	-ve
63	2000	-ve
64	3720	-ve
65	1280	-ve
66	41237	-ve
67	10240	-ve
68	6960	-ve
69	2360	-ve
70	11920	-ve
71	12800	-ve
72	15760	-ve
73	10480	-ve
74	7840	-ve
75	760	-ve
76	25974	-ve
77	14480	-ve
78	13800	-ve
79	4240	-ve
80	6240	-ve
81	6360	-ve
82	5600	-ve
83	4440	-ve
84	6520	3840
85	5440	-ve
86	7640	-ve
87	960	Excluded

Annex 6: Representative Patient Chromatograms

Day 02 Samples of two patients (Code 6 and 7)



LOW BATTERY

CHANNEL A INJECT 06/23/07 13:08:21 STORED TO BIN # 35

I 1 AT 200

I 0

4.62

6.11

11.60

ER 0

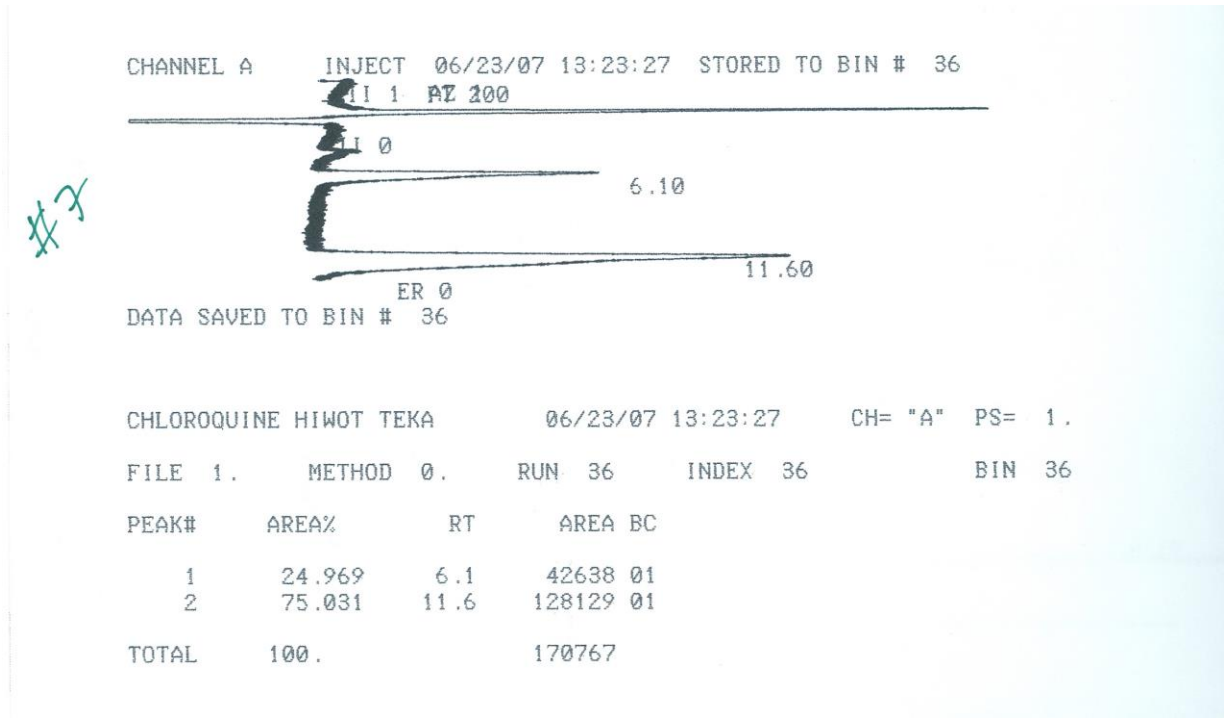
DATA SAVED TO BIN # 35

#6

CHLOROQUINE HIWOT TEKA 06/23/07 13:08:21 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 35 INDEX 35 BIN 35

PEAK#	AREA%	RT	AREA BC
1	5.375	4.62	13898 01
2	47.824	6.11	123665 01
3	46.801	11.6	121019 01
TOTAL	100.		258582



CHANNEL A INJECT 06/23/07 13:23:27 STORED TO BIN # 36

I 1 AT 200

I 0

6.10

11.60

ER 0

DATA SAVED TO BIN # 36

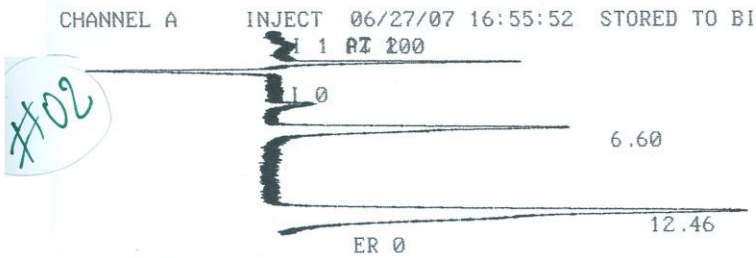
#7

CHLOROQUINE HIWOT TEKA 06/23/07 13:23:27 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 36 INDEX 36 BIN 36

PEAK#	AREA%	RT	AREA BC
1	24.969	6.1	42638 01
2	75.031	11.6	128129 01
TOTAL	100.		170767

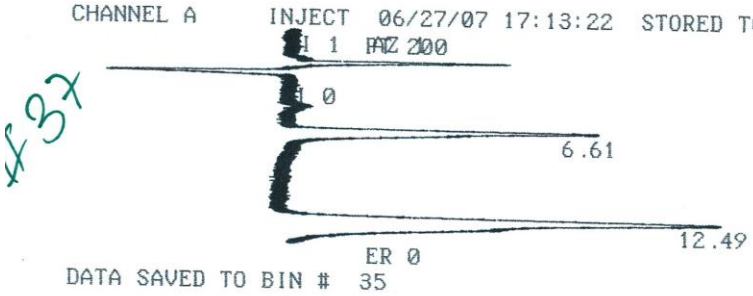
Day 28 Samples of two patients (Code 02 and 37)



CHLOROQUINE HIWOT TEKA 06/27/07 16:55:52 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 34 INDEX 34 BIN 34

PEAK#	AREA%	RT	AREA	BC
1	24.483	6.6	47001	01 ✓
2	75.517	12.46	144975	01 ✓
TOTAL	100.		191976	



CHLOROQUINE HIWOT TEKA 06/27/07 17:13:22 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 35 INDEX 35 BIN 35

PEAK#	AREA%	RT	AREA	BC
1	29.946	6.61	52044	01
2	70.054	12.49	121751	01
TOTAL	100.		173795	

Declaration

I the undersigned declare that this thesis is my original work. It has not been presented for degree in this or any university and all the source materials used for this thesis have been duly acknowledged.

Name of the candidate	Hiwot Teka
Signature	_____
Place	Addis Ababa
Date	____/_____/_____