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Assessing competition between drug resistant and drug sensitive parasites in natural populations of *Plasmodium falciparum*

By

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B.Tech, SRM University, India, 2009

Advisor: Dr. Jacobus C. de Roode

An abstract of

Assessing competition between drug resistant and drug sensitive parasites in natural populations of *Plasmodium falciparum* submitted to the Faculty of James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Master of Science in Biology

2012

ABSTRACT

Understanding the intra host dynamics of infections by multiple clones has been of great interest to studies of the dynamics of anti-malarial drug resistance in *Plasmodium falciparum*. The evolution and spread of drug resistance in high and low endemic regions of malaria is considered a public health calamity that requires immediate attention and resolution. In an attempt to broaden our understanding of the reasons for the patterns of drug resistance and efficient drug use, we characterized natural populations of *Plasmodium falciparum* in Tanzania and Kenya, both highly endemic regions, using quantitative Real Time PCR methods. In our attempt to assess within host dynamics in mixed infections, we also tested the hypothesis that drug resistant parasites suffer a lowered fitness in the absence of drug pressure. We optimized pre-existent techniques to suit our study conditions and analyzed our samples. Each of our study sites presented a different situation. While there is a trend towards competition and lowered fitness of resistant parasites in Tanzania, the analyzed Kenyan population lacks evidence for competition and lowered fitness of the resistant genotype. We describe our reasoning and methods; and report our results and speculate on reasons for these trends in our study.

Understanding the within-host dynamics of drug resistant parasites and the threshold for lowered resistance of resistant parasites can prove immensely valuable for the continued usage of antimalarial drugs, as well as effective drug policy making. This study is an exploratory attempt to understand these dynamics in natural populations.

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ACRONYMS

ACT	Artemisinin-based combination therapy
AL	Artemether-Lumefantrine
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
CQ	Chloroquine
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
EIR	Entomologic inoculation rate
HIV	Human immunodeficiency virus
MS	Microsatellite
PCR	Polymerase chain reaction
PFCRT	Plasmodium falciparum chloroquine-related transporter
PFMDR1	Plasmodium falciparum multidrug resistance gene
qPCR	Quantitative PCR
RT-PCR	Real Time PCR
SNP	Single nucleotide polymorphism
SP	Sulfadoxine-pyrimethamine
UNICEF	United Nations Children's Fund
WHO	World Health Organization

Introduction

Competition can be described as a relationship between organisms in which the presence of each one reduces the growth rate of the others. Classical ecological theory suggests that when there is a finite amount of resources shared between multiple species, one species acquires more than others, and eventually displaces the others that require the resource (Grant 1972; Tilman 1976).

Malaria is a disease with a global burden, with concentration in tropical and sub-tropical regions. A major obstacle in the treatment and control of malaria is the widespread prevalence of anti-malarial drug resistance. Often, populations contain both drug sensitive and resistant genotypes circulating, with infections that constitute both sensitive and resistant parasites. Studies lend support to the notion that regions with a high transmission intensity of malaria have a higher frequency of polyclonal infections (Daubersies, Sallenave-Sales *et al*. 1996; Arnot 1998).

Empirical studies in rodent models have provided evidence for the presence of competition between conspecific parasite clones (Read and Taylor 2001). There has been evidence for the distinct suppression of one clone by another (Snounou, Jarra *et al*. 1989; Snounou, Bourne *et al*. 1992; Snounou, Bourne *et al*. 1992; Taylor, Walliker *et al*. 1997) in mouse models, that have demonstrated a trend for competition (De Roode, Read *et al*. 2003; de Roode, Pansini *et al*. 2005).

In the wake of these findings, we test for the presence of competition in natural populations with the following motivation: If there is indeed competitive suppression

of resistant parasites in the absence of drug pressure, how do we manipulate its presence to our advantage- in order to control the spread of drug resistance? Answering this question in natural populations can be the key to revising current drug policies that clear out drug sensitive parasites in host populations, thereby also eliminating the future competitive suppression of resistant parasites when drug pressure is removed.

Background

Malaria

Malaria is a febrile disease caused by the blood parasites of the genus *Plasmodium*. While the disease has been eliminated in the United States and Western Europe, it is an extant health concern in tropical and sub-tropical regions of the world. Currently, 41% of the world's population is at risk of infection (Breman and Brandling-Bennett 2011). There were 216 million cases of malaria in 2010; 81% of these were in the WHO African Region. An estimated 3.3 billion people were at risk of malaria in 2010 (WHO World Malaria Report, 2011)

There are several reasons for the continued prevalence of malaria. The most important reasons are poorly funded control programs, the origin and spread of drug resistant parasites, insecticide resistant vectors, global climate change, failure of intervention measures, and the lack of resources to appropriately implement and maintain control measures. There is no effective vaccine for malaria. Current control methods are based on vector control and drug treatment. Emergence of resistance to

antimalarial drugs and insecticides has led to major challenges for malaria control efforts (Plowe 2003).

Malaria is caused by protozoan parasites of the genus *Plasmodium* (phylum Apicomplexa). Female mosquitoes of the genus *Anopheles* transmit the parasites. Five species of the genus *Plasmodium* account for most of the human infections: *P. falciparum, P. vivax, P. ovale, and P. malariae*. Recently, it has been determined that there is zoonotic transmission of *P. knowlesi* to humans especially in Southeast Asia (Cox-Singh and Singh 2008). Among human malarial parasites, *P. falciparum* is more virulent than other species and accounts for the largest burden of morbidity and mortality associated with malaria. *P. vivax* has the largest geographical distribution(Potkar, Kshirsagar et al. 1995), being the most common species in the world(1947). Relapses are common in *P. vivax* infections, due to the emergence of new blood forms from multinucleated schizonts in the liver(Imwong, Snounou et al. 2007). In tropical areas, this relapse can occur within three or four months. *P. vivax* is less fatal than *P. falciparum,* but it does contribute substantially to malaria morbidity(Karyana, Burdarm et al. 2008; Poespoprodjo, Fobia et al. 2009). *P.ovale* is found in Africa in the western Pacific(Cornu, Combe et al. 1986; Faye, Konate et al. 1998). *P.malairae* is found across the world(Westling, Yowell et al. 1997).

The life cycle of the malaria parasite (Figure 1) is complex and involves a vertebrate host and insect vector. When an infected mosquito bites a human it can introduce sporozoites into the human blood stream. Sporozoites infect liver cells, where they mature into schizonts and undergo asexual reproduction. This process takes about 10

to 15 days. *P.vivax* and *P.ovale* can also remain dormant in the liver and release merozoites later, causing new blood stage infection. This causes relapses of infections, making treatment and elimination much harder. It is important to point out here that liver stage infection remains generally without any symptoms. When the schizont matures, it ruptures and releases merozoites that can invade erythrocytes. The development of the merozoite within erythrocytes typically takes about 48 hours, but it varies for different species. In the red blood cell the merozoite develops into ring stage and trophozoite stage. The trophozoite undergoes multiple rounds of nuclear division and matures into a schizont, which ruptures, releasing merozoites. This cycle can continue for days or weeks until the infection is treated or cleared by the immune system. A distinct feature of *P. falciparum* infection is that trophozoite- and schizont-infected red blood cells adhere to endothelial cells in the microvasculature causing sequestration of parasites (David, Hommel *et al*. 1983). It is believed that this cytoadherence may be a protective mechanism to prevent the parasite from getting cleared in the liver or spleen (David, Hommel et al. 1983). Blood stage parasites also differentiate into male and female gametocytes. When a mosquito bites an infected host, gametocytes can be taken up during the blood meal. The male and female gametocytes then develop into gametes. The union of male and female gametes forms a zygote, which further matures into a motile ookinete. Ookinetes embed themselves within the midgut wall of the mosquito and develop into oocysts that undergo asexual reproduction and release infective sporozoites that migrate to the salivary gland. The mature sporozoites remain stored in the mosquito salivary gland and can be transmitted to the human host during mosquito blood

feeding. Of note, malaria parasites exist in the haploid form in the human host and undergo sexual recombination only in the mosquito.

The blood stage of the malaria infection is the pathological stage of malaria. In this stage, there is a synchronous lysis of red blood cells that leads to periodic spikes in body temperature. Owing to the extensive lysis of red blood cells, malaria can often result in anemia (Jakeman, Saul *et al*. 1999; Price, Simpson *et al*. 2001; Gwamaka, Fried *et al*. 2011). Anemia can also be caused by other mechanisms associated with malaria such as immune disregulation (Othoro, Lal *et al*. 1999). The major complications of severe malaria include cerebral malaria, pulmonary edema, acute renal failure, severe anemia, and/or bleeding. Acidosis and hypoglycemia are the most common metabolic complications. Any of these complications can develop rapidly and progress to death within hours or days (Trampuz, Jereb *et al*. 2003).

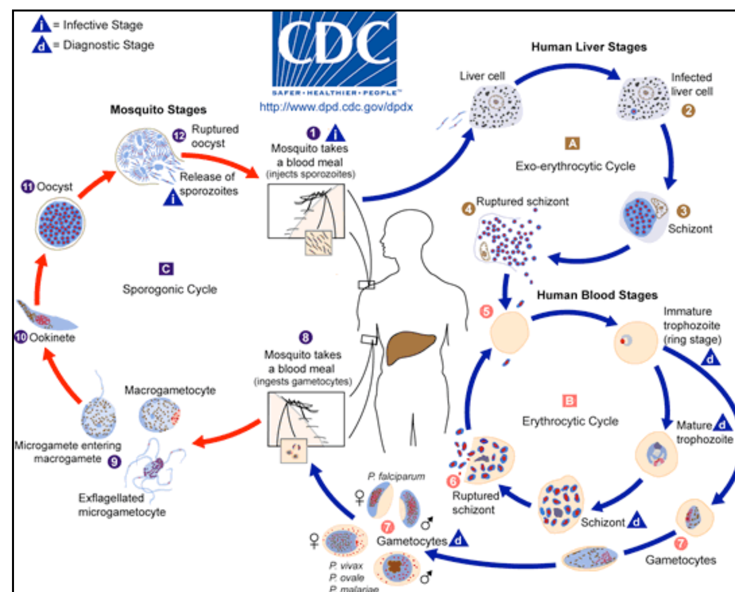


Figure 1 Life Cycle of the malarial parasite (Image from CDC Malaria website)

Antimalarial Drugs and Drug Resistance

Chinese wormwood was the earliest known treatment for malaria, used as early as the 4th century in China. Although this treatment was not widely known outside of China, artmesinin, the active ingredient, was isolated and characterized in 1972 and is used currently as a key drug in combination therapy (Miller and Su 2011). Another early treatment was quinine, extracted from cinchona tree bark in 1632; it was widely used for primary treatment until chloroquine (CQ) was discovered (Krafts, Hempelmann *et al*. 2012). After World War II, CQ was widely used throughout the world as the primary treatment of malaria.

Chloroquine was inexpensive, safe and successful until drug resistance became widespread. *P. falciparum* resistance to chloroquine emerged in Thailand and South America in late 1950's and eventually spread to rest of the world including Africa (Plowe 2003; Awasthi, Satya *et al*. 2012). After the wide spread occurrence of CQ resistance, several countries began to adopt the usage of sulfadoxine-pyramethamine (SP) to treat *P. falciparum* infections. SP is an antifolate drug combination that became an alternative to CQ for several reasons, which include effectiveness of treatment, affordability, ease of administration, and safety of usage. Resistance to antifolate drugs originated rapidly after they were introduced. Resistance to SP emerged early in South America and Southeast Asia (Lumb, Das *et al*. 2011). Subsequently resistance to SP spread to Africa. Although other drugs for the primary treatment of malaria replaced SP, its use for prophylaxis in pregnant women, especially in Africa, continues, owing to the limited number of alternatives (Thera,

Sehdev *et al*. 2005). There are recent reports indicating lowered efficacy of SP for prophylaxis in pregnant women in some parts of Africa, and alternative drugs for this purpose are being investigated, with the growing concern that SP may ultimately lose its prophylactic efficacy (Peters, Thigpen *et al*. 2007).

As resistance to SP became wide spread, the World Health Organization (WHO) adopted Artemisinin based combination therapy (ACT) for the primary treatment of *P. falciparum* malaria (Global Plan for Artemisinin Containment, 2011). Artemisinin is a short acting drug and it is combined with a long acting drug to effectively treat malaria and possibly delay the onset of resistance to combination therapy. Unfortunately, recent findings from Cambodia and Thailand show evidence for development of resistance to Artemisinin, and it is feared this will be a major challenge for malaria control (Noedl, Se *et al*. 2008; Phyo, Nkhoma *et al*. 2012).

Genetic mutations that lead to drug resistance

CQ resistance: The molecular basis for CQ resistance was initially thought to involve mutations in *P. falciparum* multi drug resistance gene (*pfmdr1*) (Slater 1993; Awasthi, Satya *et al*. 2012). Later it was proven that pfmdr1 only plays a secondary role in modulating CQ resistance. It has been established that *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) plays a key role in resistance to CQ (Fidock, Nomura *et al*. 2000; Cooper, Ferdig *et al*. 2002; Sidhu, Verdier-Pinard *et al*. 2002). The key mutation is a K to T transition in codon 76, and other secondary mutations in codons 72-76 also are associated with CQ resistance (Figure 2). The ancestral CQ sensitive codons CVMNK are mostly found in Central American

countries, the only region where CQ still works. In Africa and Southeast Asia the most commonly found CQ resistant genotype is CVIET. In South America and South Asia SVMNT is the most commonly observed CQ resistant genotype (Bacon, McCollum *et al*. 2009; Escalante, Smith *et al*. 2009; Griffing, Mixson-Hayden *et al*. 2011). Mutations in multiple genes play a role in CQ resistance, although the exact mechanisms leading to resistance remain unclear. It is speculated that mutations interfere with drug transport and reduced concentration in the parasite digestive vacuole(Fidock, Nomura *et al*. 2000; Cooper, Ferdig *et al*. 2002; Sidhu, Verdier-Pinard *et al*. 2002).

A study using microsatellite markers around *pfcrt* in samples from different areas of the world showed that CQ resistance appeared independently at least four times: once in Papua New Guinea, twice in South America, and once in the Thailand/Cambodian region (Wootton, Feng *et al*. 2002; Griffing, Mixson-Hayden *et al*. 2011). The resistant CVIET genotype that evolved in Southeast Asia eventually spread to Africa. The SVMNT genotype that arose in Papua New Guinea was found to spread to India and Pakistan (Bacon, McCollum et al. 2009; Griffing, Mixson-Hayden et al. 2011).) The CVMNT allele that arose in the Colombia/Venezuela region has spread to the western coast and then to south as far as Peru. On the other hand, the SVMNT allele that originated in the Amazon region has spread to Brazil, Venezuela, Guyanas and eventually to the Peruvian Amazon (Bacon, McCollum *et al*. 2009; Griffing, Mixson-Hayden *et al*. 2011).

SP resistance: Nonsynonymous point mutations in *dhfr* codons 50, 51, 59, 108 and

164 alter the parasite's susceptibility to pyrimethamine (Plowe, Kublin *et al*. 1998; Plowe 2003). A mutation in codon 108 that changes the amino acid from serine to asparagine is sufficient to confer a low level of resistance to pyrimethamine both *in vitro* and *in vivo*. When paired with the mutation 108, mutations at codon 50 (cysteine to arginine), codon 51 (asparagine to isoleucine), codon 59 (cysteine to arginine), or codon 164 yield higher levels of SP resistance both *in vitro* and *in vivo*. In Southeast Asia, the highly resistant 51I/59R/108N/164I genotype was found to be widely distributed while in Africa triple mutant allele 51I/59R/108N is commonly found (Brooks, Wang *et al*. 1994; Triglia, Menting *et al*. 1997; Wang, Read *et al*. 1997; Triglia, Wang *et al*. 1998). In South America, two different triple mutant genotypes have evolved. In Peru, parts of Bolivia and Brazil a triple mutant genotype 51I, 108N, 164I has been found while in other parts of South America a triple mutant genotype 50, 51, 108 has been reported (Vinayak, Alam et al. 2010).

Microsatellite marker analysis around the *dhfr* gene led to the finding that the triple mutant 51I/59R/108N allele originated in Thailand/Cambodian region and eventually spread to Africa as in the case of CQ resistance (Roper, Pearce *et al*. 2003; Roper, Pearce *et al*. 2004). On the other hand, triple mutant *dhfr* alleles observed in South America evolved locally and they are distinct from their counterparts in Africa and Asia (Alam, Vinayak et al. 2011).

Point mutations in *dhps* confer resistance to sulphadoxine. Mutations in codons 436 or 437 seem to be the most common mutations that have been observed early during the development of sulphadoxine resistance. Additional mutations in codons 540 and

581 lead to a high level of resistance. The most common highly resistant genotype is a triple mutant genotype with mutations in codons 437, 540 and 581. Mutations in other codons such as 631 have also been reported and in Southeast Asia quadruple mutants have been reported. The triple resistant *dhps* allele has been found in high proportions in Southeast Asia and South America but rarely in Africa. The triple mutant dhps genotypes found in Southeast Asia, South America, and double mutant genotypes from Africa have evolved independently based on their microsatellite haplotype (Vinayak et al., 2010). SP belongs to a class of folate antagonist drugs that inhibit enzymes in the *P. falciparum* folate pathway, ultimately resulting in reduced parasite DNA synthesis. Drug resistance to SP develops as a result of the accumulation of step-wise mutations in genes encoding enzymes involved in the parasite folic acid pathway. It is hypothesized that these mutations alter the structure of the enzyme's active site such that the drug can no longer bind and inhibit enzyme action (Olliaro 2001).

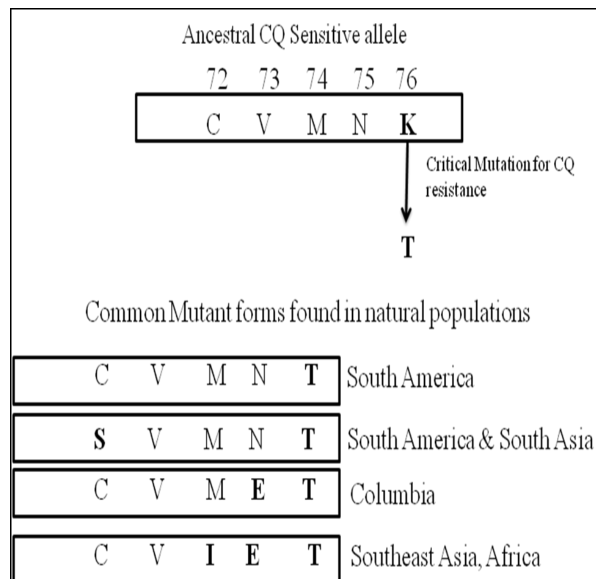


Figure 2 Illustration of K to T mutation and mutations in codons 72-76 that confer CQ resistance in *Plasmodium falciparum*. The mutant amino acids are in bold.

Selection, fitness and fixation of drug resistant alleles in natural populations

Understanding the selection process and biological fitness costs associated with drug resistant alleles is pertinent to devise appropriate drug policies to manage resistance owing to the limited number of drugs available. Origins of resistance to two major drugs such as CQ and SP primarily occurred in South America and Southeast Asia (low endemic countries) but not in Africa where intensity of malaria transmission and burden of disease is much higher than other parts of the world. There is recent evidence that resistance to Artemisinin has also evolved in Southeast Asia (Noedl, Se et al. 2008). Understanding the reasons for these patterns of the emergence of drug resistance in low endemic countries may provide important information that will help mitigate the spread of drug resistance.

Studies propose multiple causative factors that contribute to this pattern. These include, but are not limited to, host immunity, complexity of infection, extent of drug pressure, and potentially the intra-host dynamics of parasites with sensitive and resistant alleles. Mathematical modeling has been instrumental in providing a deeper insight into these factors (Koella 1998; Hastings and D'Alessandro 2000; Babiker, Hastings *et al*. 2009; Huijben, Sim *et al*. 2011). A few of these factors are discussed here.

Influence of immunity on the evolution of resistance

The immune response to malarial infections is pro-inflammatory, and the clearance of the parasite is thought to occur both by antibodies (specific immunity) and by phagocytosis by macrophages (non specific immunity). Different RBC variants are involved in the innate immune response to malaria that aims at reducing parasite growth and phagocytosis(da Silva Santos, Clark et al. 2012). Non-specific mechanisms involving neutrophils, macrophages and natural killer cells control parasite growth before the development of acquired immunity(Lanier, Buck et al. 1988; Druilhe and Perignon 1994). Specific immunity is mediated by antibodies, which block the invasion of the sporozoites to the liver, as well as merozoites to RBCs. They also mediate the lysis of gametes(Stevenson and Riley 2004).

Individuals who reside in areas of low transmission have lowered acquired immunity, which contributes to symptomatic as opposed to asymptomatic infections. Asymptomatic individuals do not receive treatment, and hence lower the amount of drug pressure in the population. However, owing to the lack of clinical immunity, individuals in low endemic area present with more symptomatic infections that require treatment, increasing the drug pressure in the population. In areas with intense malaria transmission, children (who have not yet acquired clinical immunity) develop symptomatic infections and older children and adults develop very few symptomatic infections due to development of clinical immunity. Owing to this, treatment is concentrated in the younger age group and asymptomatic infections remain a reservoir of infection, while not being subjected to high drug pressure. As a

result, it takes a longer time to get drug resistant alleles established in areas with high levels of malaria transmission(Gupta and Day 1994; Schofield and Mueller 2006). Several modeling studies also support these assumptions (Hastings, Watkins *et al*. 2002). Models have explored several factors that contribute to the transmission intensity as well as drug usage. These include climate change (Artzy-Randrup, Alonso *et al*. 2010), vector control interventions (Talisuna, Okello *et al*. 2007), and the right types of combination therapy among other things (Pongtavornpinyo, Yeung *et al*. 2008). Mathematical models hence emphasize the high level of drug use and low transmission intensity as strong predictive factors in understanding the dynamics of drug resistance and spread.

Multiplicity of infection and its relevance

Several studies indicate the impact of transmission intensity on the development and spread of drug resistance because of its relationship with multiclonal or multi-genotypic infections (Babiker and Walliker 1997; Hastings 1997; Hastings and Watkins 2005). Regions with high transmission have a higher frequency of polyclonal infections (Daubersies, Sallenave-Sales *et al*. 1996; Arnot 1998), which facilitate transmission of gametocytes with different genetic make-up to mosquitoes and increase the chance for recombination between drug sensitive and resistant alleles. Therefore, parasite populations in these regions exhibit a lower amount of linkage disequilibrium, because of a greater amount of recombination and this can break up drug resistant alleles. On the contrary, in low transmission settings there are fewer polyclonal infections that can lead to the transmission of gametocytes from

monoclonal population of parasites and as a consequence low chances for recombination. This can also contribute to the rapid selection of resistant parasites in the presence of high level of drug pressure. McCollum and colleagues have shown evidence for differences in the linkage disequilibrium in resistant alleles comparing parasite populations from different transmission areas (Escalante, Grebert *et al*. 2002; Escalante, Cornejo *et al*. 2004; McCollum, Basco *et al*. 2008; Mixson-Hayden, Jain *et al*. 2010; McCollum, Schneider *et al*. 2012).

Rationale for current study: Competition between parasite clones and Evidence for loss of fitness in drug resistant alleles in natural populations

Studies in rodent malaria models have indicated evidence for the presence of competition between conspecific parasite clones, as discussed earlier. The virulence level of a clone has also been shown influence its competitive success(de Roode, Pansini et al. 2005). There is also evidence for immune mediated differential competitive exclusion or coexistence in mixed infections (Raberg, de Roode et al. 2006), as well as its effect on the outcome of the competition(De Roode, Read *et al*. 2003; de Roode, Culleton *et al*. 2004; de Roode, Helinski *et al*. 2005; de Roode, Pansini *et al*. 2005; Bell, de Roode *et al*. 2006; Raberg, de Roode *et al*. 2006; Wargo, de Roode *et al*. 2007). The dynamics of the competing clones change with the order of infection, with infections that are sequential being subjected to higher competition. With these studies demonstrating competitive exclusion among conspecific clones, a natural question is whether there is an expansion of each of the competitors in the absence of others. This phenomenon, referred to as competitive release, has been

demonstrated by Culleton *et al* in drug resistant rodent malarial parasites after chemotherapeutic treatment. Further, Nelson et al explored the dynamics of competitive release in a study, where sub curative dosages of anti malarial drugs have led to a lowered competitive release of the resistant parasite (de Roode, Culleton et al. 2004)

A recent investigation of competition between conspecific clones has also been conducted in *in-vitro* models. In a study by Wacker, Turnbull *et al*, in-vitro cultures of *P.falciparum* have shown patterns of competitive suppression and release in the absence of drug pressure, dependent on the parasite being sensitive or resistant in a mixed culture. The density of the resistant strain remained the same in single as well as mixed cultures, but the sensitive strain was competitively suppressed by the resistant strain in mixed cultures. Upon drug treatment, there was competitive release of the drug resistant strain, that varied with the dosage and time point of drug introduction (Wacker, Turnbull *et al*. 2012).

Infections are typically treated only when symptoms surface. This could be after simultaneous/succeeding infections of different clones, the dynamics of which are already at play by the time treatment is administered. If this is the case, there is the possibility that owing to the competitive release of the resistant genotype, it expands to the ecological niche of its competitor, thereby doubling it's relative fitness (suppressing the drug sensitive clones) (Gryer 1974; Gupta and Maiden 2001; Read and Taylor 2001; Frank 2002; Raberg, de Roode et al. 2006; Read, Day et al. 2011). This is an important consideration for disease dynamics in natural populations in

order to optimally exploit competitive suppression to our advantage. When drug resistant mutants suffer a worse disadvantage in untreated patients, resistance will spread slower (Hastings and D'Alessandro 2000; de Roode, Culleton et al. 2004). The extent of using this to affect the spread of resistance, and accordingly revise drug policy can only be investigated with empirical evidence in natural populations.

In the wake of these findings, we test for the presence of competition in natural populations with the following motivations: If there is indeed competitive suppression of resistant parasites in the absence of drug pressure, how do we manipulate its presence to our advantage- in order to control the spread of drug resistance? Answering this question in natural populations can be the key to revising current drug policies that clear out drug sensitive parasites in host populations, thereby also eliminating the future competitive suppression of resistant parasites when drug pressure is removed. If the competitive ability of a clone is what is being selected for, are we worsening the situation with vigorous chemotherapy that aids competitive release (Read, Day et al. 2011)?

It has been observed in various studies that the development of mutant drug resistant alleles help parasites get selected positively in the presence of drug pressure. It has been hypothesized that when the drug pressure is removed, the mutant alleles will be at a disadvantage in competing against wild type alleles as the mutation may have compromised the fitness of the parasite population (Taylor, Walliker *et al.* 1997; Read and Taylor 2001).

There has been evidence for a decrease in the frequency of drug resistant alleles in a

population following the removal of drug pressure. For instance, a decrease in the frequency of the CQ resistant genotype has been observed in Malawi after the removal of CQ for the treatment of malaria. In Malawi, this decline has been observed in the K76T allele from 85% to 13% in the eight years after removal of CQ for the treatment of malaria (Kublin, Cortese *et al*. 2003; Mita, Kaneko *et al*. 2003). This decline was shown to be due to the expansion of the existing wild type allele in the population rather than a back mutation to the wild type. A recent clinical trial confirmed the return of CQ efficacy for the treatment of *P. falciparum* malaria in Malawi. The observed decline in the frequency of K76T and supporting evidence of the wild type population expansion suggests that this mutation carries a significant fitness cost in the absence of drug pressure (Laufer, Thesing *et al*. 2006). Similar results were seen after the withdrawal of SP for treatment of malaria in Peru that led to a decline of SP resistant genotypes in this country (Zhou, Griffing et al. 2008; Bacon, McCollum et al. 2009). Although a decline in the resistant alleles in some populations has been noticed following a change in the drug pressure, it has not been observed in all populations. There may be several reasons for such observations, including transmission intensity, drug policy and subsequent fixation of resistant alleles before the drug pressure is changed.

Studies from certain countries with seasonal transmission of malaria also indicate varying levels of sensitive and resistant alleles depending upon the seasonality of transmission. It has been reported that resistant alleles were found more commonly during the peak transmission season and sensitive alleles were more frequently seen during the dry season. This reduction of resistant alleles during dry seasons has been

used to argue a fitness cost to being resistant (Ord, Alexander *et al*. 2007).

These observations from the above mentioned studies provide indirect evidence for the presence of fitness costs for drug resistance in natural populations. However, currently, there is no direct evidence to demonstrate fitness costs in natural human infections due to logistical challenges. Analyzing competition between drug sensitive and resistant alleles can be an interesting approach for looking at the fitness costs of resistance in natural infections and it can help to answer at least two questions: Is there competition between drug sensitive and resistant parasites in mixed infections in the host? When there is a cost to being drug resistant, parasites may perform worse on their own, but do they suffer disproportionately worse in competition than sensitive strains do? There is a distinct paucity of evidence in this direction for natural populations of *P. falciparum*. Further, there is also a lack of evidence to show differences in the growth rates of sensitive and resistant parasites. This study is an attempt to investigate the potential competition between drug sensitive and resistant parasites by determining the relative levels of CQ sensitive and CQ resistant alleles in humans with natural *P. falciparum* mixed infections using real time PCR methods. Competition is analyzed as a reduction in parasite density in a mixed versus single infection, similar to approaches in rodent models (De Roode, Read et al. 2003; de Roode, Culleton et al. 2004; de Roode, Helinski et al. 2005; de Roode, Pansini et al. 2005).

In order to effectively detect and analyze competition, there is a need to ascertain that there is an influence of the presence of one clone on the others'. Hence, an important

consideration is the measurement of parasite densities in single infections of each genotype being analyzed, as well as the measurement of the parasite densities of the genotypes in mixed infections, in order to. With this in mind, the study aims at using quantitative real time PCR to differentially quantify parasite densities of the two genotypes, followed by comparative analyses of the densities.

In order to manipulate intra-host dynamics to our advantage, an accurate assessment of potential competition between clones in a mixed infection is required. A preliminary approach to this is presented below.

Hypothesis: Our study aimed to test the hypothesis that there is competition between drug-sensitive and drug-resistant parasites in mixed infections of *Plasmodium falciparum* in natural populations. In the absence of drug pressure, the resistant parasite suffers a fitness cost, which can be characterized by its competitive suppression within the host.

Study design: This study was designed to compare the relative levels of parasite density between parasites harboring CQ sensitive and CQ resistant alleles in areas where both alleles are present. In most parts of Asia and South America CQ resistant alleles have reached fixation and this study cannot be performed in such areas. In Africa, CQ was replaced about a decade ago and there is evidence from the literature(Mulligan, Mandike et al. 2006; Kabanywanyi, Mwita et al. 2007; Porter-Kelley, Cofie et al. 2010) that both CQ sensitive and resistant alleles are present. Therefore, it was decided to use parasite samples available from other studies

conducted in Tanzania and Kenya for the purpose of this investigation.

Study Sites

Samples were analyzed from study sites in two regions of Sub-Saharan Africa. The first set of samples was available from an on-going *in vivo* drug efficacy study designed to investigate the efficacies of two different artemisinin based combination therapies in Tanzania (Figure 3). Samples were collected in the period of 2010-2011 from Miono Health Center and Msata Health Center, both in the Bagamoyo District. The drug policy in Tanzania was changed from CQ to SP in 2000 and from SP to ACT in 2006 (Schonfeld, Barreto Miranda *et al*. 2007). There were 75 samples available that were collected at the time of enrollment (day 0 of the trial).

Figure 3 Study Site 1: Tanzania. Samples were obtained from Bagamoyo region from an in vivo drug efficacy trial. (Image from CDC website- CDC in Tanzania)

The second region that was analyzed for the dynamics of multiple infections within the human host was Kenya. Samples were collected from Kenya as a part of an on-going study aimed at monitoring antimalarial drug resistance using an *in vitro* drug

sensitivity assay. Samples were collected in the period of 2010-2011 from Siaya District Hospital, Kenya. There were 66 samples available, obtained from participants at the time of enrollment in the study (before any treatment was given). The participants' ages were from 6 months to 5 years old. The study site is located in a holoendemic area in Western Kenya near Kisumu (Figure 4). The epidemiology of this area has been well characterized by several investigators (McElroy, Lal et al. 1999; McElroy, ter Kuile et al. 2000; Aidoo, Terlouw et al. 2002; UNICEF and WHO 2003; Hay, Okiro et al. 2010). In Kenya, SP replaced CQ in 2000 and in 2006 ACT was introduced (Mwai, Ochong *et al*. 2009).

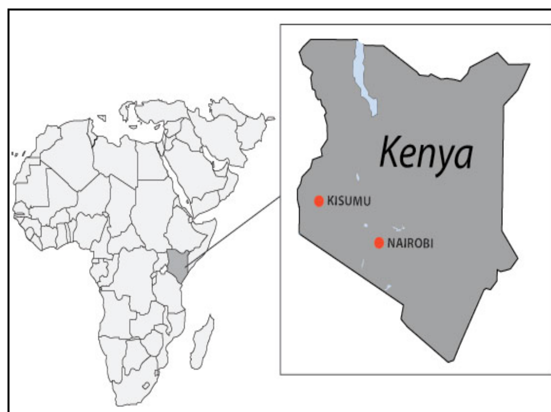


Figure 4 Study Site 2: Kenya. Samples were obtained from a pediatric in-vitro drug response study for Artemisinin based combination therapy (Image from CDC website- CDC in Kenya)

Quantitative Polymerase Chain Reaction and Quantification

DNA from all blood samples was extracted using the QIAamp DNA minikit (Qiagen, Valencia CA-(Qiagen method)) according to the manufacturer's recommendations. All extracted samples were stored at -20°C. Double-labeled probes for the detection and quantification *pfcrt* were adopted from Gadalla *et al* (Gadalla, Elzaki *et al*. 2010);

each probe was labeled with a reporter dye at the 5' end and a quencher at the 3' end. Probes were obtained from the Core Facility, CDC, Atlanta GA.

Probe 5'	Fluorophore	Sequence 3'	Quencher
crt76-CVMNK	FAM	TGT GTA ATG AAT AAA ATT TTT GCT AA	BHQ1
crt76-CVIET*	HEX	TGT GTA ATT GAA ACA ATT TTT GCT AA	BHQ 1

Table1 : Double-labeled probes for pfcrt genotyping. (Adapted from (Gadalla, Elzaki et al. 2010))

A 25µl reaction volume contained 12.5µl of Invitrogen Platinum qPCR mix, 5.5uM of $MgCl_2$, 0.3uM of forward and reverse primer, 0.1uM of each probe, and 0.3uM of ROX dye for normalization. DNA from 3D7 and Dd2, representing the two *pfcrt* alleles CVMNK and CVIET at codons 72-76 was obtained from lab cultures. Reactions were set up with 5 concentrations of each genotype and optimized for concentration of reagents and reaction conditions. PCR conditions were as follows:

Temperature	**Time**	
95 °C	6 min	
95 °C	15 s	50 Cycles
57 °C	1 min	50 Cycles

A standard curve was obtained by multiplex reactions that were run on five concentrations of DNA with known parasite density (Figure 6; Figure 7). The lowest concentration of parasite density on the standard curve was 100parasites/µl. The

technique was optimized by varying reagent concentrations, annealing temperatures and the amount of template DNA added, until reproducible standard curves were obtained. A principal example of standard curves for each probe is shown in Figure 6. Following this, the technique was tested to quantify mixed DNA of known concentrations. This was done for two reasons: to validate the technique for use on samples with mixed infections; and to negate any effects of interactions between probes when run in multiplex. 50:50 mixes of 3D7 (positive control for sensitive genotype) and Dd-2 (positive control for resistant genotype) were made, and a serial log dilution was set up from these, for four concentrations of the DNA, starting from a log density of 10^5 and going down to 10^2. Log parasite density was then computed from these Ct values as described below. These experiments validated our techniques by returning log parasite densities that were correspondingly added to each of these mixtures. The results of the mean log parasite densities from these experiments are included in Table 2. Once the technique was optimized to work for mixed DNA of known concentrations, it was employed in field samples to identify and quantify the corresponding alleles.

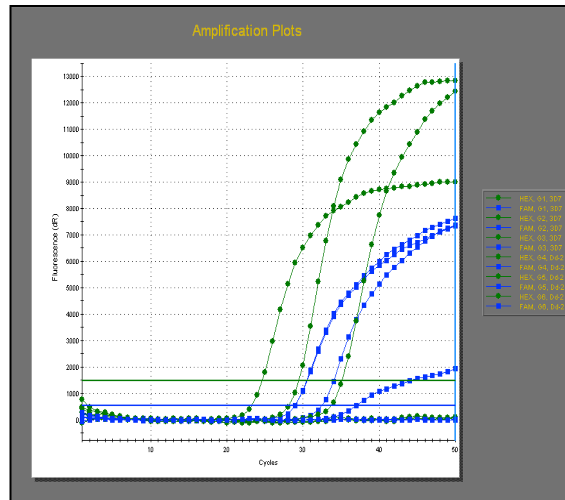


Figure 5 Amplification plots for qPCR. FAM labeled curves are blue, and HEX labeled curves are green. FAM dye used in probe for sensitive allele found in 3D7, used as positive control for the drug sensitive genotype. HEX dye used in probe for resistant allele found in Dd-2, used as positive control for the drug resistant genotype. C_T values are read from the x-axis, at the point where the fluorescence exceeds the threshold.

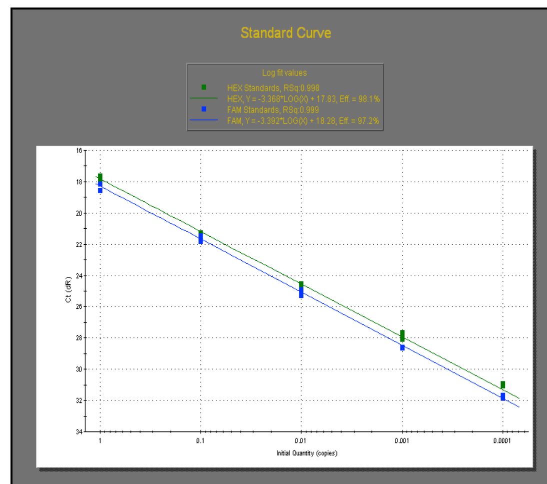


Figure 6 Standard Curve with 5 concentrations of DNA. Sensitivity and efficiency of qPCR was tested by detecting serial dilutions of purified DNA from each control clone. FAM dye used in probe for sensitive allele found in 3D7, used as positive control for the drug sensitive genotype. HEX dye used in probe for resistant allele found in Dd-2, used as positive control for the drug resistant genotype.

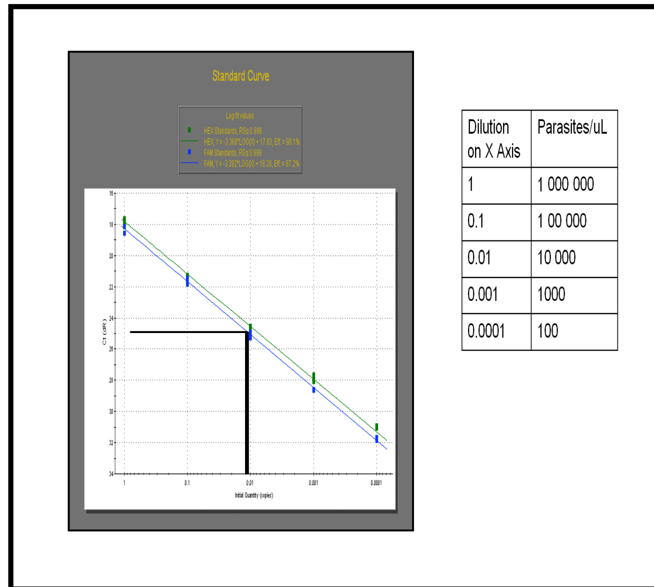
Log Concentration of mixture	Measured mean log sensitive parasite density	Measured mean log resistant parasite density
10^5	5.073448	5.085229
10^4	4.019074	4.067089
10^3	3.06535	3.088108
10^2	2.148307	2.116614

Table 2 Parasite densities for serial log dilutions of 50:50 mixes of 3D7 (positive control for sensitive genotype) and Dd-2 (positive control for resistant genotype)

Owing to the concentrations of DNA used in generating standard curves, all samples that had a parasite density below the detection limit of the technique (our lowest concentration, 100 parasites/µl) were excluded from the analysis. This reduced the sample sizes in both of the analyzed populations. In Tanzania, the total number of single infections of the sensitive type was reduced from 56 to 52 due to this exclusion. The number of single infections of the resistant type remained the same at 2. The number of mixed infections dropped from 17 to 12. Similarly, in Kenya, the total number of single infections of the sensitive type dropped from 35 to 34; and the total number of single infections of the resistant type declined from 13 to 11. The number of mixed infections dropped from 18 to 14.

5µl of DNA from each sample was added to the reaction components and analyzed as described above. Quantitative experiments contained multiplex qPCR reactions, run in triplicate for each standard dilution and in duplicate for each sample. All reactions were run using the Mx3000P™ Real-Time PCR System. Relative quantification was performed by using the standard curves and interpolation of the parasite densities

using the standard curve for each experiment (Figures 5-7).



Dilution on X Axis	Parasites/uL
1	1 000 000
0.1	1 00 000
0.01	10 000
0.001	1000
0.0001	100

Figure 7 Determination of unknown parasite density values by using a standard plot. In this plot, C_T values from the test results of individual samples can be used (on the Y axis) to deduce parasite density on the X-axis as shown in this example. Each point on the X-axis represents a known parasite density as indicated in the table on the side.

Statistical Tests and Analyses

Statistical analysis of results was done using GraphPad Prism (v5). The parasite densities were log transformed and normalized, and an unpaired Student's t-test was performed for the following comparisons: parasite density of each genotype in single infections; parasite density of each type in mixed infections; parasite density of each genotype in single and mixed infections. To further test the relationship between the sensitive and resistant parasites in mixed infections, a regression analysis was performed, testing the response of the parasite density of resistant genotype to the parasite density of the sensitive genotype. These analyses were performed using R.

Statistical significance for all analyses was considered at an alpha of 0.05.

Results

I. Tanzania:

Parasite density levels of CQ sensitive and resistant parasites were compared among samples with single detectable infections based on their *pfcrt* genotype (Figure 8a). The median parasite density of single infections of the sensitive genotype was 2365 parasites/µl and the median parasite density of single infections of the resistant genotype was 6067 parasites/µl. Statistical analyses were not possible due to the low sample size of the single infections with the resistant type (n=2). A similar comparison between sensitive and resistant alleles in the mixed infection group showed a different pattern of results (Figure 8b). In patients that carried both the genotypes, the resistant genotype reached lower parasite density then the genotype with the sensitive allele (p=0.0366). As shown in the figure, the median parasite density in the sensitive allele group was 1687 parasites/µl and in the resistant allele it was 426 parasites/µl.

In order to assess competition, the parasite density of each genotype needed to be compared between single infections and mixed infections. For the resistant genotype, the median parasite density in single infections was 6067 parasites/µl and in mixed infections it was 426 parasites/µl. Statistical analyses were not possible due to the low sample size of the single infections with the resistant type (n=2) (Figure 8c) Comparison of the parasite density of the sensitive genotype (Figure 8d) between single and mixed infections did not provide statistically significant results

(p=0.5947). The median parasite density of single infections of the sensitive type was 2365 parasites/µl, and in mixed infections it was 1687 parasites/µl.

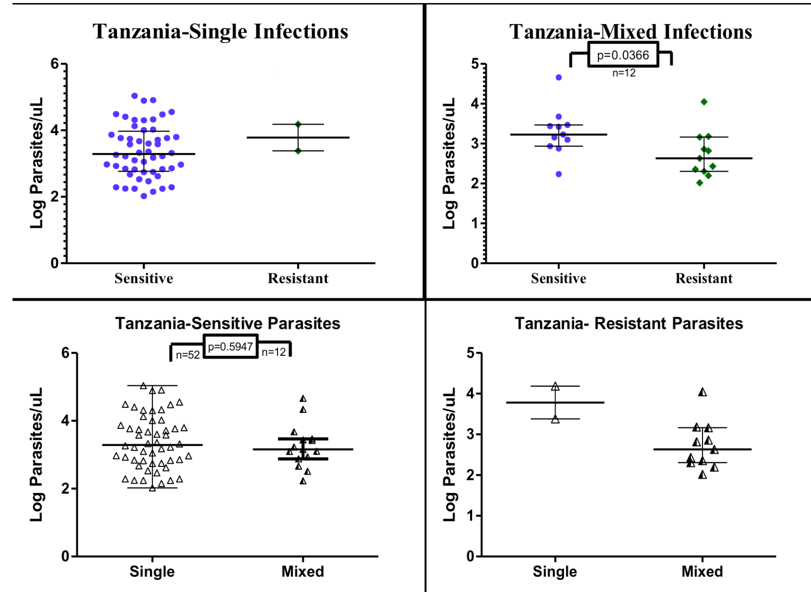


Figure 8a. Comparison of parasite densities of single infections of Sensitive (CVMNK) parasites and resistant (CVIET) parasites in Tanzania; Figure 8b. Comparison of parasite densities of Sensitive (CVMNK) parasites and resistant (CVIET) parasites in Mixed Infections in Tanzania. The lines indicate the medians with inter quartile ranges of each parasite density distribution**;** Figure 8c. Comparison of parasite density of the wild type (CVMNK allele) parasites in single and mixed infections in Tanzania; Figure 8d. Comparison of parasite density of the resistant (CVIET allele) parasites in single and mixed infections in Tanzania. The thicker lines indicate the medians with inter quartile ranges of each parasite density distribution

The regression analysis (Figure 10a) provided information on the relationship between the sensitive and resistant genotype within mixed infections (Table 3a). In mixed infections, there was a 74.79% increase in the parasite density of the resistant

genotype for each 100% increase in the parasite density of the sensitive genotype ($F_{1,9}$= 10.06 with a p-value of 0.01133).

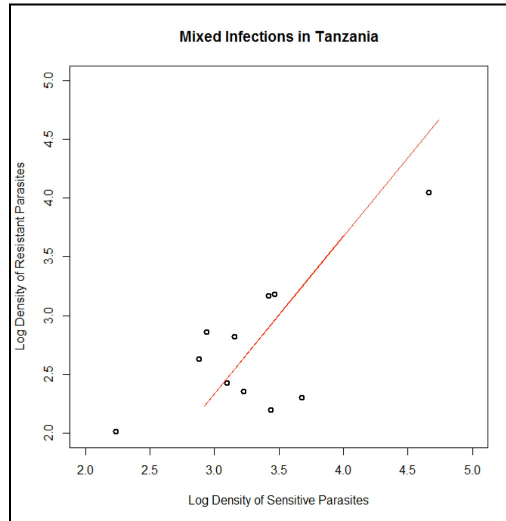


Figure 10a Relationship between log parasite density of drug sensitive and resistant parasites in mixed infections in Tanzania. The line shows the least-squares regression line.

II. Kenya:

Similar to analyses in Tanzania samples, parasite density levels of CQ sensitive and resistant parasites were compared among samples with single detectable infections based on their *pfcrt* genotype (Figure 9a). Among single infections, the median parasite density in the sensitive allele group was 4325 parasites/µl and in the resistant allele group it was 2805 parasites/µl. This difference was not statistically significant (p=0.4486). A similar comparison between sensitive and resistant alleles in the mixed infection group showed a similar pattern of results (Figure 9b) with no significant difference in the median parasite density between sensitive and resistant genotypes in mixed infections (p=0.6853): the median parasite density in the sensitive allele group was 3083 parasites/µl and in the resistant allele group it was 3580 parasites/µl.

In order to assess competition, the parasite density of each genotype needed to be compared between single infections and mixed infections. With the resistant genotype, the median parasite density in single infections was 2805 parasites/µl and in mixed infections it was 3580 parasites/µl (Figure 9c), a non-significant difference (p=0.3302). Comparison of the parasite density of the sensitive genotype between single and mixed infections (Figure 9d) did not provide statistically significant results either (p=0.9294): the median parasite density of single infections of the sensitive type was 4325 parasites/µl, and in mixed infections it was 3083 parasites/µl.

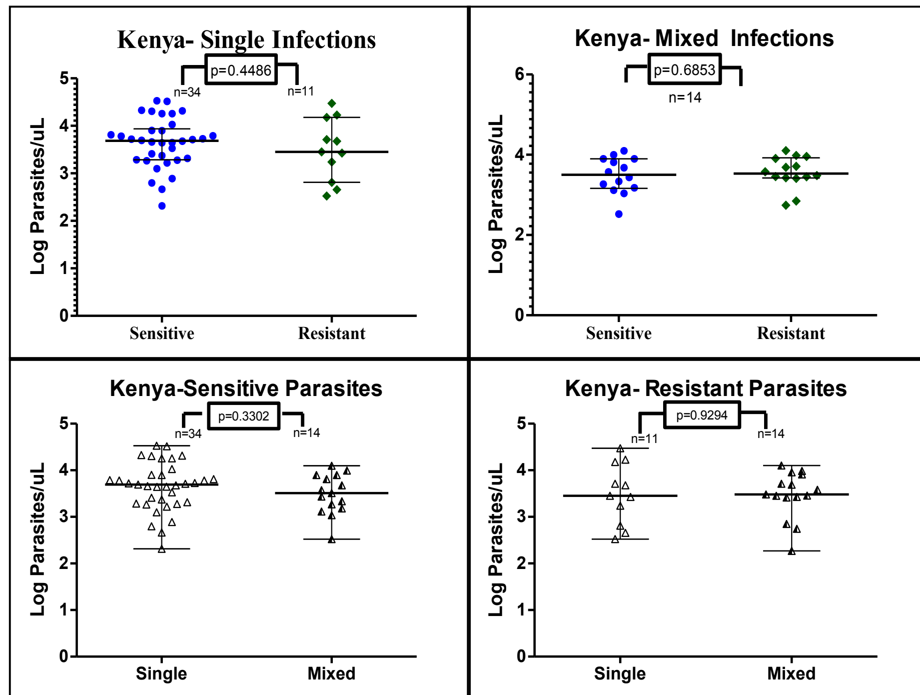


Figure 9a Comparison of parasite densities of single infections of Sensitive (CVMNK) parasites and resistant (CVIET) parasites in Kenya; Figure 9b Comparison of parasite densities Sensitive (CVMNK) parasites and resistant (CVIET) parasites in mixed infections in Kenya. The thicker lines indicate the medians with inter quartile ranges of each parasite density distribution; Figure 9c Comparison of parasite density of the sensitive (CVMNK allele) parasites in single and mixed infections in Kenya; Figure 9d Comparison of parasite density of the resistant (CVIET allele) parasites in single and mixed infections in Kenya. The thicker lines indicate the medians with inter quartile ranges of each parasite density distribution

The regression analysis (Figure 10b) showed that there was no significant relationship between the densities of the sensitive and resistant genotype within mixed infections.

($F_{1, 10}$=2.485, p=0.146). The results are provided in Table 3b.

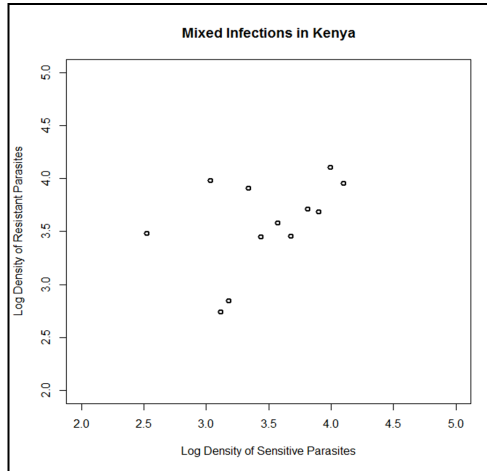


Figure 10b Relationship between log parasite density of drug sensitive and resistant parasites in mixed infections in Kenya.

3a. Tanzania				
Coefficients:	Estimate	Std.Error	t-statistic	p value
Intercept	1.2502	0.7431	0.546	0.5983
Resistant Parasite density	0.7479	0.2225	3.172	0.0113
Residual standard error: 0.4202 on 9 degrees of freedom Multiple R-squared: 0.5279, Adjusted R-squared: 0.4754 F-statistic: 10.06 on 1 and 9 DF, p-value: 0.01133				
3b. Kenya				
Coefficients:	Estimate	Std.Error	t-statistic	p value
Intercept	2.1464	0.9142	2.348	0.0408
Resistant Parasite density	0.4117	0.2611	1.576	0.146
Residual standard error: 0.3996 on 10 degrees of freedom Multiple R-squared: 0.1991, Adjusted R-squared: 0.119 F-statistic: 2.485 on 1 and 10 DF, p-value: 0.146				

Table 3: Regression Analyses of resistant parasite density as a response to sensitive parasite density in mixed infections done on R.

Discussion

Within-host dynamics and the lowered fitness of drug resistant parasites have been explored extensively in rodent malaria (De Roode, Read et al. 2003; de Roode,

Helinski et al. 2005; de Roode, Pansini et al. 2005) and in vitro models (Wacker, Turnbull et al. 2012). The aim of our study was to analyze within host competition in natural populations of *Plasmodium falciparum*. In order to achieve this, our first step was to optimize and employ a technique that would enable effective relative quantification of multiple genotypes within the same infection. Parasite densities of resistant and sensitive genotypes in single as well as mixed infections were quantified using real time quantitative PCR and were compared in the samples collected from each of two highly endemic malaria regions. The results from each region provided different situations, discussed in detail below.

Tanzania:

Analysis of the results obtained indicated a trend for competition between drug sensitive and resistant parasites, which is consistent with previous evidence in rodent and *in vitro* models (De Roode, Read et al. 2003; Escalante, Cornejo et al. 2004; de Roode, Helinski et al. 2005; de Roode, Pansini et al. 2005; Wacker, Turnbull et al. 2012), with a statistically significant difference in the parasite densities of sensitive and resistant parasites in mixed infections. It has been agreed upon by numerous authors that multiplicity of infection and within host dynamics play an important role in the spread of drug resistance (de Roode, Culleton et al. 2004; Huijben, Nelson et al. 2010; Huijben, Sim et al. 2011). A major contributing factor in this consideration is the hypothesized (de Roode, Culleton et al. 2004; Huijben, Nelson et al. 2010; Huijben, Sim et al. 2011) lowering of fitness of the drug resistant parasite in the absence of the drug pressure. Several studies stress the importance of being able to experimentally quantify this lowered fitness (Hastings and D'Alessandro 2000;

Hastings, Watkins et al. 2002; Hastings and Watkins 2005; Bell, de Roode et al. 2006; Babiker, Hastings et al. 2009; Huijben, Sim et al. 2011). The significant lowered parasite density of the resistant genotype in mixed infections than in single infections can be considered direct evidence for the lowered fitness of the resistant parasite in the absence of drug pressure (De Roode, Read et al. 2003; de Roode, Helinski et al. 2005; Read and Huijben 2009; Huijben, Sim et al. 2011). In our study, there is preliminary evidence for competition within mixed infections between resistant and sensitive parasites in natural populations of *Plasmodium falciparum*.

The regression analyses on the parasite density of sensitive and resistant genotypes in mixed infections provided two key pointers. Within mixed infections, there is a positive relationship (slope=0.7479, p=0.0113) between the parasite density of the sensitive genotype and the parasite density of the resistant genotype. This is counter to our expectation that there would be a negative correlation between the two, given that we expect the resistant genotype to suffer in the presence of the sensitive genotype. However, the value of the slope (0.7479) obtained suggests that in mixed infections, for a 100% increase in parasite density of the sensitive genotype, there is only a 74.79% increase in the parasite density of the resistant genotype. This is an indication that the response of the resistant genotype is slower than the change in the sensitive genotype. The positive relationship between the two types of parasites across patients can then be explained by the fact that patients that allow for greater overall parasite populations allow higher populations of both genotypes.

As discussed earlier, the presence of within-host competition and the competitive

suppression of the resistant genotype in natural populations of *Plasmodium falciparum* can lead to a subsequent competitive release following the re-entry of drug pressure (de Roode, Culleton et al. 2004; Wargo, Huijben et al. 2007; Read and Huijben 2009; Read, Day et al. 2011). Tiago Antao discusses the implications of modifying chemotherapeutic policy by incorporating the effects of relative fitness of parasites with disparate resistance profiles that coexist in natural populations. (Antao 2011). In order to effectively avert and eliminate parasites with exceedingly fit drug resistant alleles, understanding the effect of intra host competition is imperative, (Wargo, Huijben et al. 2007; Read, Day et al. 2011) and our study suggests a viable technique as well as preliminary data in this direction.

Kenya:

The analyses of samples from Kenya presented a different picture. Analyzing the parasite density of sensitive and resistant genotypes in mixed infections, there seemed to be no significant difference in the median parasite density (p=0.6853). In order to analyze the presence of competition and its extent, comparative analyses were done of the median parasite density of the sensitive as well as resistant genotypes in single and mixed infections. The unpaired student's t-test on log transformed values of parasite densities of sensitive parasites in single and mixed infections produced statistically non-significant results (p=0.9294), as did the comparison of parasite densities of the resistant genotype in single and mixed infections (p=0.3302).

Regression analysis of the parasite density of the resistant genotype within mixed infections as the dependent variable to the parasite density of the sensitive genotype within mixed infections was performed similar to the approach to the data from

Tanzania. In Kenya, the analysis did not provide a statistically significant relationship between the parasite densities of the sensitive and resistant genotypes within mixed infections. This distinct lack of evidence for a significant linear relationship between the parasite density of the sensitive and resistant genotypes within mixed infections suggests that the growth of each genotype (sensitive and resistant) within a mixed infection was independent of the other.

Studies have shown that the decrease in resistant alleles after the change in drug policy has been slower in Kenya than in other countries (Mwai, Ochong *et al*. 2009). Field observations on the fitness of drug resistant *P.falciparum* consider the reduction in frequency of the resistant genotype and the revival of sensitive parasites following the withdrawal of drug pressure an important piece of evidence that the fitness of drug resistant genotypes in the absence of drug pressure is lowered (Hastings and Donnelly 2005; Babiker, Hastings et al. 2009). This is relevant, because it may suggest that resistant strains in Kenya have a lower cost of resistance (and consequently a lowered reduction of fitness) than in Tanzania. This apparent slower reduction in the frequency of CQ resistant alleles after a policy change away from the CQ may be a reason for the lack of any competitive suppression of resistant strains in our study samples from that region.

Another possible explanation for our results could be the presence of compensatory mutations that reduce the costs of resistance may have occurred in Kenya. The occurrence of such mutations could result in a resistant parasite that has a fitness comparable to the fitness of a sensitive parasite, as predicted by Regoes *et al* and

other studies (Maisnier-Patin and Andersson 2004; Wijngaarden, van den Bosch et al. 2005; Handel, Regoes et al. 2006) and in *in-vitro* experiments (Levin, Perrot et al. 2000).

Summary

In Tanzania, there was a significant difference in the parasite densities of sensitive and resistant parasites within mixed infections, indicating that the resistant parasite performed worse than sensitive parasites in mixed infections. The regression analysis provided evidence to indicate a lag in the growth of the resistant parasites in mixed infections as well. The distinctly lowered density of the resistant parasite in mixed infections than in single infections is indicative of competition. Statistical significance could not be measured owing to the low number of single infections of the resistant type

The data from Kenya presented a different image, with significant differences lacking in both cases: the densities of sensitive and resistant parasites in mixed infections, as well as the density of each type in single and mixed infections. Consistently, the regression analysis did not yield a significant relationship between the growth rates of each genotype in mixed infections.

Future Directions

Further studies are necessary in two directions- first, there is a necessity to confirm these results with larger sample sizes from populations with both resistant and sensitive genotypes circulating; secondly, these dynamics have to be tested in

populations that harbor parasites of the different alleles that confer resistance to a single drug, discussed previously in the background.

Conclusion

Within host dynamics of drug sensitive and resistant parasites of natural populations of *Plasmodium falciparum* have not been characterized previously. Our study provides a useful technique that can be used for the simultaneous relative quantification of multiple infections in human hosts for the CQ sensitive genotype, as well as the genotype carrying the CVIET mutant allele for CQ resistance. While the population in Tanzania seems to show a trend for the CQ resistant genotype suffering in mixed infections, these patterns are not observed in the samples from Kenya. There may be multiple reasons for these differences, which include the slower reduction of resistant allele frequencies in the Kenyan population, as well as compensatory mutations reducing the fitness costs of resistance. We conclude that further studies are important in this area, with larger sample sizes.

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