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The Effect of Drug Resistance on Plasmodium falciparum Transmission and Gametocyte Development

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The Effect of Drug Resistance on *Plasmodium falciparum* Transmission and Gametocyte Development

by

Samantha Aylor

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science Department of Global Health College of Public Health University of South Florida

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Abstract

In order to reduce malaria prevalence worldwide, a better understanding of parasite transmission and the effect of drug resistance is needed. The effect of drug resistance on malaria transmission has been examined for some drugs, but not for mitochondrial inhibitors such as atovaquone and the current basis of malaria therapy, artemisinin. Therefore, the goal of this study was to produce gametocytes, the life cycle stage that transmits from mosquito to human, in several different drug resistant patient isolates as well as to determine the effect of drug resistance on gametocyte development and transmission. Previous studies have shown that the mutation that confers resistance to atovaquone, a common antimalarial, occurs de novo after treatment and transmission of this resistance is not seen in the field. Therefore, to determine whether or not the resistance mutation can be transmitted, mosquito-feeding experiments were conducted using atovaquone resistant parasites and resulting oocyst DNA was analyzed. In addition to these atovaquone studies, artemisinin resistant gametocytes were also grown in vitro and drug pressure was added to determine if resistance mechanisms affect gametocyte development. This study is the first examine gametocyte development in these resistant strains and the first to report that transmission of the atovaquone resistant mutation may be possible. However, data is currently inconclusive on the effect of artemisinin resistance on gametocyte development.
Introduction

Malaria is a parasitic disease that has proven to be a major global health problem for centuries. This disease, which first appeared over 4,000 years ago, poses a threat to nearly half of the world’s population, particularly in developing countries where there is poor access to health care (1). Although the World Health Organization (WHO) characterizes malaria as both preventable and treatable, public health officials estimate that there are approximately 219 million cases and 660,000 deaths per year due to this disease, with 90% of these deaths occurring in children living in sub-Saharan Africa (2).

Malaria is caused by protozoan parasites belonging to the genus Plasmodium. There are five species of Plasmodium that are known to cause disease in humans, including *P. falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi*. Most malaria infections are due to *P. vivax*, a species that can be found in tropical regions throughout the world. However, the most severe form of malaria is caused by *P. falciparum* parasites, which are mostly found in Africa (3). Malaria parasites are transmitted from person to person via female *Anopheles* mosquitoes. There are more than 30 species of *Anopheles* mosquitoes that are capable of transmitting malaria. These mosquitoes thrive in tropical regions throughout the world, making malaria control efforts challenging, especially in developing countries (4).

The malaria life cycle is extremely complex and begins when an infected mosquito takes a blood meal from a human host. During the blood meal, the mosquito injects sporozoites into the bloodstream. These sporozoites then travel to the liver and
invade parenchymal cells where they undergo asexual reproduction. In *P. vivax* and *P. ovale*, sporozoites may enter a resting stage, in which they become dormant and do not undergo reproduction. These parasites, which are characterized as hypnozoites, may stay dormant for weeks or months before they are activated and able to undergo asexual reproduction. As multiplication occurs in the liver, thousands of merozoites are produced. Merozoites cause hepatic cells to rupture and are released into the blood stream. Once in the bloodstream, merozoites invade red blood cells and undergo asexual reproduction. During this erythrocytic cycle, the parasite first enters a ring stage, then develops into a trophozoite stage, and finally forms a schizont, which ruptures the red blood cell, releasing more merozoites into the bloodstream (3). After several rounds of asexual reproduction, sexual stages of the parasite known as gametocytes begin to form. When a mosquito takes a blood meal from an infected human, it ingests male and female gametocytes that, once inside the vector, undergo sexual reproduction. Male gametocytes invade female gametocytes forming diploid zygotes, which elongate and form ookinetes. These ookinetes then penetrate the mosquito midgut and form oocysts. Inside of oocysts, sporozoites form. Eventually sporozoites are released and make their way to the salivary glands where a mosquito can then take a blood meal and infect another human host (5).

Once infected with malaria, it takes several days for symptoms to develop. The incubation period varies between species and strains; however, for *P. falciparum* infections symptoms usually appear within 9-14 days and in *P. vivax* infections symptoms can appear 12-18 days and up to 6-12 months after infection (6). Malaria pathogenesis occurs during the asexual erythrocytic cycle. Prodromal symptoms are usually nonspecific and include fever, chills, headache, fatigue, muscle pain, and
anorexia. These symptoms often occur in cycles that correspond to the synchrony of the malarial parasite. When the life stages are synchronous, red blood cells rupture at the same time causing waves of symptoms that can occur every 36-48 hours. This hallmark sign of malaria is known as malarial paroxysm (3). All species of malaria can cause nonspecific, flu-like illness; however, *P. falciparum* infections can cause severe disease manifestations including cerebral malaria, anemia, renal disease, pulmonary edema, and dysenteric malaria. Cerebral malaria is the most severe form of the disease that usually results in severe headache, confusion, coma, and if not treated early enough, death. Infections due to *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* usually cause mild symptoms and complications normally arise due to pre-existing conditions (1). People with suspected malaria should obtain a definitive diagnosis to determine which species of malaria they are infected with. The WHO recommends that all suspected malaria cases receive a definitive diagnosis through microscopy and rapid diagnostic tests (RDTs) (2). This allows infected cases to receive prompt and proper treatment and helps to avoid problems such as drug resistance.

In malaria endemic regions, young children and pregnant women are at the highest risk of infection. Children ages 1-5 account for nearly 85% of all malaria deaths worldwide (6). Researchers believe that the maternal antibodies that children receive through breast-feeding during infancy may offer some protection from infection in their first year of life. However, children in endemic regions do not develop natural acquired immunity until the age of 5, which greatly increases their risk for developing severe malaria infections (7). Pregnant women are also at higher risk of infection due to hormonal changes that affect the immune response. Furthermore, red blood cells infected
with *P. falciparum* often sequester along the placenta, greatly increasing the risk for severe disease manifestations in pregnant women (8).

The current recommended treatment for uncomplicated malaria is artemisinin combination therapy (ACT) (2). Artemisinin is derived from the Chinese herb *Artemisia annua* and has been shown to be effective against all stages of the parasite life cycle. Because of the short half-life of this drug, it is often given in combination with a longer lasting drug to reduce parasite burden (9). For patients with severe malaria, intravenous artesunate, which is a semi-synthetic derivative of artemisinin, is the recommended first-line treatment in countries with low to moderate disease transmission. However, in areas with high transmission rates, intravenous quinine may also be used as a treatment option (2). In addition to artemisinin derivatives, there are several other classes of drugs that are available as treatment options, including cinchona alkaloids (quinine and quinidine), 4-aminoquinolines (chloroquine and amodiaquine), 8-aminoquinolines (primaquine), diaminopyrimidines (pyrimethamine), sulfonamides (sulfadoxine), quinoline methanols (mefloquine), tetracyclines (tetracycline and doxycycline), biguanides (proguanil), phenanthrene methanols (halofantrine), and hydroxynaphthoquinones (atovaquone) (3). Although these drugs can still be used as treatment options in certain areas of the world, they are often limited to use as chemoprophylaxis options for high-risk individuals such as travelers and pregnant women or in combination with other drugs. Problems with drug resistance have been documented for all available malaria chemotherapeutic agents, limiting control efforts throughout the world.

There are many different malaria control strategies that are used throughout the world; however, problems such as disease transmission, drug resistance, and limited
access to healthcare have halted eradication efforts for over 50 years. In 1955, the WHO developed a massive plan to eradicate malaria worldwide, in which the drug chloroquine was widely distributed throughout endemic countries. At the time, chloroquine was the first-line treatment for malaria and had an extremely high cure rate. However, after several years of administration, widespread resistance rapidly developed. Drug resistance, as well as insecticide resistance in mosquitoes and loss of political support led to the deterioration of eradication efforts in the 1970s, causing the number of malaria cases to drastically increase (4). Since then, many drugs have been developed to treat malaria; however, *Plasmodium* has developed resistance to all of them, including first-line ACTs. ACT resistance has recently been documented in Southeast Asia and researchers are concerned that this will soon spread to Africa (10). Since the deterioration of the WHO program, many organizations, including the Bill and Melinda Gates Foundation and the Roll Back Malaria Program have actively been continuing eradication efforts in many countries throughout the world. These efforts include increasing access to healthcare, mass drug administration, and mosquito vector control. The WHO is hoping that these efforts will reduce malaria incidence by 75% by 2015 and that eradication can be achieved within the near future (2).
Background

Gametocytogenesis and Disease Transmission

In recent years, many advances have been made in the field of malaria research, including a better understanding of parasite biology and the discovery of more effective anti-malarial drugs. However, in the past 50 years research has mainly focused on asexual stages of the parasite, leaving a major gap in our understanding of Plasmodium sexual reproduction and how the parasite is transmitted through the mosquito vector. During Plasmodium development in the erythrocytic stage, only a small proportion of asexual parasites will form gametocytes, the sexual form of the parasite that transmits the disease from human host to mosquito vector. It is unclear exactly what triggers gametocytogenesis, but stress factors, such as high parasite density, changes in hematocrits levels, and addition of drugs or toxins have shown to induce gametocyte production in vitro. Some researchers hypothesize that when malaria parasites are under stressful conditions, gametocytogenesis is triggered as a way to escape those conditions and move onto the next host. However, others believe that these stress factors may be associated with gametocyte production, but not causal, due to the ability of many malaria strains to produce gametocytes in vivo prior to the onset of symptoms (5). When an asexual schizont has committed to sexual reproduction, all merozoites released form gametocytes in the next generation in either male or female form. Asexual stage parasites reach maturation in only 48 hours; however, sexual stages take 8-12 days to mature, during which time they progress through five different stages. Once stage V gametocytes
form, they can then infect a mosquito and malaria can be transmitted to another human host (11).

Because researchers have not determined what triggers gametocytogenesis, inducing gametocyte production in vitro is often a challenging process. Most gametocyte studies use 3D7 and its parental isolate, NF54, because they have consistently shown to produce viable gametocytes (12). However, very few studies have examined gametocyte production in drug resistant patient isolates. Blocking disease transmission by targeting gametocytes and reducing drug resistance are important strategies that should be utilized in all malaria control programs and it is crucial that researchers gain a better understanding of these mechanisms. Therefore, this study aims to gain better insight into how atovaquone-resistant and artemisinin-resistant *P. falciparum* is transmitted through the mosquito vector, as well as to better understand how resistance affects gametocyte development.

**Transmission of Atovaquone Resistance**

Although resistance is a major problem, many older drugs, like atovaquone, are still used to treat acute malaria cases in non-endemic regions and are used as chemoprophylaxis options to prevent infection in travelers. Atovaquone is a hydroxynaphthoquinone that was discovered in the 1980s for treatment of acute malaria. It inhibits electron transport in parasite mitochondria by targeting the cytochrome bc1 complex (13). When this drug went to clinical trials, researchers showed that it quickly eliminated *P. falciparum* from the blood; however, in one third of patients, recrudescence occurred. Because resistance rapidly developed in 30% of these patients, researchers proposed that the drug mechanism was directly responsible for inducing advantageous
mutations, conferring parasite survival. Because atovaquone blocks electron transport and causes the collapse of mitochondrial membrane potential, this most likely results in the build up of reactive oxidative species (ROS). ROS can then damage mitochondrial DNA (mtDNA) and cause mutations. Because mtDNA is highly conserved, especially within the same species, advantageous mutations that lead to resistance can quickly proliferate within that species under drug selective pressure (14). Due to the rapid emergence of atovaquone resistance, researchers combined the drug with proguanil, which allows atovaquone to be effective at a lower concentration, decreasing the rate of mutations within the mtDNA. They found that this combination, known as Malarone, had nearly a 100% cure rate with no recrudescence; however, like other antimalarials, widespread resistance has been reported throughout the last several years (15).

The mutation that confers parasite resistance to atovaquone is a single nucleotide polymorphism (SNP) in the cytochrome b gene of *P. falciparum* (*pfcytb*) located at amino acid 268 in the mitochondrial genome (16). Musset et al. analyzed *pfcytb* sequences of atovaquone failure patients before and after treatment to better understand how resistance came about. Before treatment with atovaquone, they found that the *pfcytb* gene did not contain atovaquone-proguanil resistant (APR) alleles; however, after treatment APR alleles were detected, suggesting that resistance arises de novo during treatment. Normally, drug resistance develops when a mutation leads to the presence of an advantageous allele in a population that confers resistance to a drug. This allele then increases in frequency due to directional selection. However, atovaquone resistance occurs due to parallel evolution of these advantageous mutations. In parallel evolution,
independent lineages repeatedly gain the same mutation when the same selective pressure is added (17).

Because resistance arises after treatment and apparently no transmission of new parasites is observed in the field, it is unknown whether or not *Plasmodium* is actually able to transmit atovaquone resistance. During *Plasmodium* development, the parasite undergoes asexual reproduction within the human host. Because the mutation propagates within humans after treatment, this suggests that the mutated mtDNA is inherited and highly conserved among these asexual stages, thus allowing for resistance (14). However, little is known about mitochondrial inheritance during sexual stages of the parasite within the mosquito. Because the mutation is present during asexual stages and transmission is not seen in the field, it is possible that *Plasmodium* is incapable of transmitting the mutation to next generation parasites during sexual stages inside the mosquito. Therefore, the first aim of this study is to test whether or not atovaquone resistance in *P. falciparum* can be inherited during these sexual stages and thus be transmitted.

**Artemisinin Resistance and Gametocyte Development**

Since its discovery in the 1970s, artemisinin has proven to be one of the most effective drugs against malaria. Studies have shown that this drug has the ability to kill malaria parasites in very short periods of time with parasite reduction ratios of nearly 10,000 per erythrocytic cycle, leading to impressive clinical outcomes. Artemisinin is classified as a sesquiterpene trioxane lactone and its antimalarial properties result from the presence of an endoperoxide ring. The parent drug of artemisinin has poor solubility in both water and oil. Therefore, many derivatives, including dihydroartemisinin (DHA), artemether, and arteether have been
developed. These derivatives are not only soluble in oil or water, but they are also more potent and have shown to have greater antimalarial activity. Although artemisinin is a very fast acting drug, it also has a short half-life. When absorbed into the body, artemisinin derivatives are quickly broken down into their bioactive metabolite, ultimately resulting in elimination through glucuronidation. This short half-life often leads to recrudescence following artemisinin treatment. This has led public health officials to recommend artemisinin in combination with another long-lasting drug as the first-line treatment option for *P. falciparum* malaria cases (9).

Although the WHO has recommended artemisinin be used in combination with a longer lasting drug, many pharmaceutical companies are still producing it as a monotherapy and signs of drug resistance are rapidly developing on the Thai-Cambodia border. In this region, lenient drug policies, lack of regulation, and the distribution of fake malaria drugs have drastically contributed to the emergence of artemisinin resistance (18).

Characteristics such as decreased drug susceptibility and prolonged parasite clearance times have been shown to be associated with artemisinin resistance; however, researchers are still unclear about the exact mechanisms and phenotypes involved. Several studies have shown that ring stages still persist after 48 hours of artemisinin treatment; therefore, many researchers hypothesize that prolonged clearance times are due to reduced susceptibility of ring stage parasites (19). Teuscher et al (20) have also shown that artemisinin resistant malaria can enter a dormancy state in which ring-stage parasites are temporarily arrested and can recrudesce several days after treatment. Research in this field has mainly focused on
asexual stage phenotypes involved in artemisinin resistance and it is unclear how resistance affects sexual stages. Therefore, the final aim of this study is to add to the current understanding of artemisinin resistance by examining how artemisinin resistance affects gametocyte development.
Materials and Methods

Parasites

*P. falciparum* isolates or clones used in this study included NF54-F7, TM90C2B, TM92C1088, TM92C1086, and Thai50B5. Of these, NF54-F7 was used as a control due to its ability to produce viable gametocytes and the others are atovaquone resistant isolates collected from Phase II studies in Thailand. Cultures were maintained, as previously described by Trager & Jensen (21), in O+ red blood cells at 4% hematocrit in RPMI 1640 media containing L-Glutamine, 25 mM Hapes, 50 µg/ml Hypoxanthine, and 10% AB+ serum at 37°C in a 90% N2, 5% CO2, and 5% O2 environment.

Production of atovaquone resistant *P. falciparum* gametocytes in vitro

To induce gametocytogenesis, two cultures of each strain (G1A and G1B) were started at 0.5% parasitemia and 7.0% hematocrit in 15 ml (Day 0). Media was changed daily and culture volume was increased to 25 ml on Day 3 to reduce the hematocrit. Two additional cultures of each strain (G2A and G2B) were started on Day 3 at 0.5% parasitemia and 7.0% hematocrit. On Day 6, the volume of G2A and G2B was increased to 25 ml to reduce the hematocrit. G1A and G1B cultures were combined on Day 7 and maintained in 25 ml. G2A and G2B were combined on Day 10 and maintained in 25 ml. Media was changed daily until Day 17 when gametocytes were fully mature (See Figure 1). Gametocyte maturation was assessed using thin smears and by checking for exflagellation.
Figure 1. Schematic representation of the production of atovaquone resistant *P. falciparum* gametocytes in vitro.

**Mosquito Feedings**

On the day of mosquito feed experiments, a 3:1 mixture of AB+ serum and O+ red blood cells was prepared and pre-warmed to 37° C. Mature gametocyte cultures were centrifuged at 4,000 g for 3 minutes and the culture pellet was diluted in the serum/blood mixture at a 1:1.6 ratio. Diluted gametocyte cultures were fed to 3-4 day old *Anopheles stephensi* mosquitoes for approximately 20 minutes at room temperature using a membrane feeding system (Hemotek) that was pre-warmed to 37° C. After receiving the
blood meal, mosquitoes were stored in a 28°F C and 70% humidity environment and fed
10% sucrose solutions daily. Midgut dissections were performed 8-10 days after infection
to look for the presence of oocysts. Dissected midguts were stored in PBS solution and
frozen at -20°F C until used for DNA analysis.

**P. falciparum oocyst DNA analysis**

For oocyst genomic DNA (gDNA) extraction, midgut samples were digested in
1 mL of digestion buffer (0.5% SDS, 0.1 M EDTA pH 8.0, 10 mM Tris-HCL) and 50 μL
proteinase K at 50°C overnight. Digests were centrifuged at 13,000 g for 1 minute
and the aqueous phase was removed. DNA was extracted using the Qiagen DNeasy
Blood and Tissue kit according to the manufacturer’s instructions, with the
exception of an additional wash step with buffer PE (Qiagen) and decreasing elution
volume to 35 μL. Concentration and purity of gDNA for each sample was assessed by
a NanoDrop spectrophotometer.

To confirm the presence of the Y268S cytochrome b mutation, DNA
amplification was performed using PCR reactions containing primers cytbFOR 5’—
TGCCTAGACGTATTCCTG—3’ and cytbREV 5’—GAAGCATCCATCTACAGC—3’ with
Taq DNA Polymerase HF (Invitrogen). The final reaction contained 10X AccuPrime
PCR Buffer II (5 μL), PCR primers (0.2 μM final concentration), template DNA (500
ng), and AccuPrime Taq HF (1 U). Nuclease-free water was added to each reaction to
give 50 μL total volume. The *P. falciparum* cytochrome *b* thermocycling conditions
were as follows: initial denaturation of 94°C for 1 min, 32 cycles of 94°C for 30s,
54°C for 20s, and 68°C for 1:20s. All PCR products were run out on 1% Tris-acetate-
EDTA (TAE) agarose gels stained with SYBR Safe DNA Gel Stain (Invitrogen) and
visualized on a BioRad GelDoc imaging system. PCR products were purified with the QIAquick PCR purification kit (Qiagen), and the concentration was assessed using a NanoDrop spectrophotometer. Sequencing of PCR products was performed by Genewiz. Each parasite sample was PCR amplified and sequenced in duplicate. Sample sequences were analyzed and aligned using ApE (A Plasmid Editor) software, and mapped to the Pf-3D7 cytochrome b gene for mutation detection.

**Production of artemisinin resistant gametocytes in vitro**

To assess the effect of artemisinin resistance on gametocyte development, gametocytogenesis was induced in both artemisinin resistant and artemisinin sensitive strains. NHP-2065 (4A) and NF54 parasites were grown as artemisinin sensitive controls. 4A is an artemisinin sensitive patient isolate from Mae Sot, Thailand. ARC08-22 (4G) is an artemisinin resistant strain from Tasan, Cambodia that was used to assess the effect of resistance on gametocyte development. All strains were started on Day 0 in two 15 ml cultures (G1 and G2) at 0.3% parasitemia and 6% hematocrit. Media was changed daily and temperature was maintained at 37°C. On Day 3, media volume was increased to 25 ml to drop the hematocrit level. On Day 7, when early gametocytes began to form, all G1 cultures were separated into three 5 ml flasks to undergo drug treatment. One flask was used as a control, another was used for treatment with DHA, and the final flask was used for treatment with artelanic acid (AL). 100nM of DHA and 100nM of AL were given to their respective flasks and parasites were kept under drug pressure for 48 hours. After 48 hours, cultures were grown continuously until Day 16. This process was repeated for G2 cultures on Day 10 to examine the effect of artemisinin resistance on late
stage gametocytes. This experiment was also repeated in its entirety using 10nM concentrations of DHA and AL (See Figure 2). Thin smears were taken throughout the experiment to assess gametocyte development and on Day 16, exflagellation events were documented to evaluate the effect of drug pressure on transmission potential.

**Figure 2.** Schematic representation of the production of artemisinin resistant *P. falciparum* gametocytes *in vitro*
Results

Gametocyte Composition

Gametocytogenesis was successfully induced in most of the atovaquone resistant parasites. Only the lines that produced gametocytes were used for these studies. For each parasite, the percentage of gametocytes, gametocyte stages, male to female ratio, and exflagellation events were documented. All of the numbers reported are for Day 17 mature gametocyte cultures that were fed to *A. stephensi* mosquitoes.

Two separate TM90C2B cultures (C2B-A and C2B-B) were grown and fed to mosquitoes on separate occasions. C2B-A had a gametocytemia of 2.1% with 33% stage II, 11% stage IV, and 56% stage V gametocytes and a 3:1 male to female ratio. C2B-B had a gametocytemia of 2.3% with 22% stage IV, 78% stage V gametocytes, 18% male gametocytes (microgametes), and 82% female gametocytes (macrogametes). No exflagellation was observed for either TM90C2B culture.

Two separate TM92C1086 cultures (1086-A and 1086-B) were grown and fed to mosquitoes on separate occasions. 1086-A had a gametocytemia of 2.8% with 23% stage III, 25% stage IV, and 52% stage V gametocytes and a male to female ratio of 1:2. 1086-B had a gametocytemia of 3.2% with 46% stage IV and 54% stage V gametocytes and 45% microgametes and 55% macrogametes. No exflagellation events were observed for either TM92C1086 culture.
Two NF54 cultures (NF54-A and NF54-B) were grown on separate occasions and used as positive controls. NF54-A had a gametocytemia of 2.2% with 46% stage IV and 54% stage V gametocytes, 43% microgametes and 57% macrogametes, and 7 exflagellation events per 40x microscopic field. NF54-B had a gametocytemia of 2.5% with 33% stage IV and 67% stage V gametocytes, 38% microgametes and 62% macrogametes, and 15 exflagellation events/field.

Several mixed experiments were performed in which NF54 was crossed with either TM90C2B or TM92C1086 before mosquito feeding. Two cross experiments were performed with NF54 and TM92C1086 (NF54+1086-A and NF54+1086-B). On Day 17, NF54+1086-A had a gametocytemia of 2.1% with 2.1% stage II, 30% stage IV, and 50% stage V gametocytes, 43% microgametes and 57% macrogametes, and 3 exflagellation events per 40x microscopic field. NF54+1086-B had a gametocytemia of 3.1% with 12% stage II, 25% stage IV, and 63% stage V gametocytes, 36% microgametes and 64% macrogametes, and 10 exflagellation events per 40x microscopic field. One mixed experiment with NF54 and TM90C2B was performed in which the resulting gametocytemia was 2.5% with 11% stage II, 32% stage IV, and 57% stage V gametocytes, 23% microgametes and 77% macrogametes, and 5 exflagellation events per 40x microscopic field.

Gametocytogenesis was attempted in Thai50B5 and TM92C1088; however, both were unable to produce mature gametocytes. TM92C1088 produced early stage gametocytes, but did not survive past 10 consecutive days in culture. Although Thai50B5 had viable parasites by Day 17, the gametocytemia was less than 1% and there were no stage V gametocytes present in culture.
Figure 3. Gametocyte composition of atovaquone-resistant strains on day 17 prior to mosquito feeding experiments.

Oocyst counts in *A. stephensi* feeding experiments

All cultures that produced mature gametocytes were fed to *A. stephensi* mosquitoes to determine the transmission potential of atovaquone resistance. To check for *P. falciparum* infection, dissected midguts were stained to look for the presence of oocysts. Oocyst numbers reported are number of oocysts out of twenty midguts. There were no visible oocysts in C2B-B or NF54+1086-A infections. C2B-A had one oocyst, NF54+1086-B had 6 oocysts (one oocyst in one midgut and five oocysts in another midgut), NF54-A had two oocysts in one midgut and three oocysts in another, NF54-B had five oocysts in one midgut and two midguts with one oocyst each, and NF54+C2B had two oocysts in one midgut (See Figure 4).
**Figure 4.** Developing oocysts in *A. stephensi* midguts positive for *P. falciparum* infection. NF54 (Top left), TM90C2B (Top right), NF54+TM90C2B (Bottom Left), NF54+TM92C1086 (Bottom Right).

**Oocyst DNA Analysis**

PCR was used to amplify the cytochrome b gene in *P. falciparum* oocyst DNA. When PCR reactions were first run, the only two products that appeared on a 1% agarose gel were the NF54 positive controls. Because oocysts counts were very low, it was suspected that any *P. falciparum* DNA was only present in very small quantities. Therefore, the PCR reaction was repeated using purified PCR products from the first reaction. When these products were run on a gel, 1.38 kb bands appeared for all samples except the uninfected midgut negative control and C2B-A (Figure 5). Genewiz sequence analysis revealed that the NF54 positive controls, as well as all cross experiments, produced PCR products that contained wildtype DNA sequences for the cytochrome b gene. DNA analysis of the C2B-B reaction revealed the presence of the Y268S atovaquone resistance mutation, along with wildtype sequences of the cytochrome b gene.
Figure 5. Gel electrophoresis analysis of atovaquone resistant *P. falciparum* oocyst DNA.

**Effect of artemisinin resistance on gametocyte development**

The effect of artemisinin resistance on gametocyte development was evaluated by determining the gametocytemia and proportion of different gametocyte stages before and after drug treatment, as well as by counting exflagellation events on Day 16. In the first experiment, all strains except 4G were treated with 100nM of DHA and 100nM of AL. During early stages of culturing, one of the 4G cultures crashed; therefore, there was only enough culture to create a control and DHA treatment group. This parasite did not get treated with 100nM AL. When 4G (Figure 6) and 4A (Figure 7) early stage gametocytes were treated with 100nM of DHA or AL, they had a 100% clearance rate after 48 hours of drug
pressure. Examination of thin smears on Day 16 revealed that the parasites were completely killed and did not recrudesce after drug pressure was removed. When early stage NF54 gametocytes were treated with 100nM DHA, the parasitemia was reduced by 87.5% and there was a 96.5% reduction in gametocytes. When early stage NF54 gametocytes were treated with 100nM AL, parasitemia was reduced by 71% and gametocytemia was reduced by only 9%. By Day 16, there were no viable parasites in NF54 cultures treated during early stages of gametocyte development (Figure 8).

When the artemisinin resistant 4G strain was treated with 100nM DHA during late stage gametocyte development, there was no significant difference in parasitemia or gametocytemia (Figure 9). 4A late stage gametocytes treated with 100nM DHA experienced a 70% decrease in parasitemia; however, there was no significant difference in gametocytemia. Similarly, 4A late stage gametocytes treated with 100nM AL experienced a 79% decrease in parasitemia and no significant difference in gametocytemia (Figure 10). When NF54 late stage gametocytes were treated with DHA and AL there was no significant difference in either parasitemia or gametocytemia before and after treatment (Figure 11).

Exflagellation events were counted for each culture on Day 16 of the experiment. The exflagellation numbers reported correspond to the average number of events per 40x microscopic field. 4G cultures that were treated with 100nm of DHA during early stage gametocyte development had zero exflagellation events on Day 16, compared with an average of 10 exflagellation events in the control group. However, cultures treated during late stage gametocyte development
had approximately 2 exflagellation events per field, which is an 80% less than exflagellation events in the control cultures.

4A cultures that did not receive drug treatment had approximately 4 exflagellation events per field; however, early stage gametocytes that were treated with either DHA or AL had no exflagellation. Late stage gametocytes that were treated with DHA or AL had approximately 2 exflagellation events per field, which is 50% less than what was seen in control flasks.

NF54 control cultures had an average of 35 exflagellation events per field. There was no exflagellation in cultures treated during early stage gametocyte development. In cultures treated with 100nM DHA or AL during late stages, exflagellation was reduced by 75%.

Because 100 nM drug treatments were effective at killing the 4G artemisinin resistant early stage gametocytes, it was assumed that the drug concentration was too high to evaluate the effect of drug resistance on gametocyte development. Therefore, this experiment was repeated using only 10nM of drug treatment. When thin smears for these experiments were examined, it was revealed that this concentration was too low to be effective at killing either stages of gametocytes in both the sensitive and resistant strains. There were no significant differences in gametocytemia before and after treatment; however, there were differences in exflagellation numbers. For early stage 4G cultures treated with 10nM of either DHA or AL, exflagellation numbers were decreased by 71%, but there were no significant differences between the control groups and parasites treated during late stage gametocyte development. There were no significant differences in exflagellation
numbers between NF54 cultures. 4A cultures that did not undergo drug treatment had an average exflagellation number of 8. However, early stage gametocytes treated with either DHA or AL had a 62.5% reduction in exflagellation. Similarly, late stage gametocytes treated with 10nM DHA or AL had a 50% reduction in exflagellation.

Because 10nM proved to be too low of a concentration to determine the effect of artemisinin resistance on gametocyte development, previous IC50 data was used to determine what a proper drug concentration would be. The latest hypoxanthine assay data for the 4G resistant strain revealed that this strain is actually sensitive to artemisinin. Therefore, these experiments did not provide any insight into how gametocyte development is effected by artemisinin resistance since all three strains ended up being sensitive to the drug. This experiment should be repeated with a strain that is confirmed to have resistant phenotypes.
Figure 6. Early stage gametocytes before and after artemisinin treatment in artemisinin resistant clone 4G.
Figure 7. Early stage gametocytes before and after artemisinin treatment in artemisinin sensitive clone 4A.
Figure 8. Early stage gametocytes before and after artemisinin treatment in NF54 control.
Figure 9. Late stage gametocytes before and after artemisinin treatment in artemisinin resistant clone 4G.
Figure 10. Late stage gametocytes before and after artemisinin treatment in artemisinin sensitive clone 4A
Figure 11. Late stage gametocytes before and after artemisinin treatment in NF54 control
Discussion

In order to better understand sexual reproduction and transmission potential of malaria parasites, it is important to assess gametocyte development in different drug resistant strains of malaria. This study demonstrated that gametocytes can be produced *in vitro* from many different patient isolates, including atovaquone and artemisinin resistant *P. falciparum*. In our experiments, gametocytogenesis was induced by starting parasite cultures at high hematocrit levels and mimicking symptoms of anemia by drastically decreasing hematocrit levels several days later. This induced stress triggered the production of early stage gametocytes and allowed them to mature into transmissible stage V forms. When compared to the gametocyte-producing NF54 control, all strains that produced mature gametocytes had similar proportions of gametocyte stages and percent gametocytes by Day 17. This study indicates that gametocytemia levels between 2% and 3% are sufficient to produce successful mosquito infections; however, it is hypothesized that levels lower may also be sufficient and higher levels may produce even better infection rates. Interestingly, TM92C1086 parasites had a higher gametocytemia than NF54 controls; however, when fed to *Anopheles* mosquitoes, there was no evidence of oocyst production. Because no visible oocysts were detected, mosquito midguts were not dissected for DNA analysis. However, this experiment should be repeated to include PCR analysis on these samples because there may have been infection in low levels.
Stage V gametocytes were produced in all strains except atovaquone-resistant Thai50B5 and TM92C1088. Thai50B5 was able to produce early stage gametocytes, but they did not fully mature to stage V by Day 17. This may be due to several factors. When hematocrit levels are decreased on Day 3, it is important that parasite cultures be growing logarithmically and that the parasitemia is approximately 5%. There should also be evidence of stressed out parasites in order for the decrease in hematocrit to induce gametocyte production. It is possible that the Thai50B5 strain was not started at a high enough parasitemia to induce gametocyte production. Patient isolates usually thrive in low hematocrit conditions; therefore, if the parasite was not stressed enough when the level was dropped, gametocytogenesis may be harder to induce. Furthermore, many cultures lack the ability to produce gametocytes because of the length of time they have been in culture. When cultures have high in vitro passage numbers, they lose telomere length on their chromosomes (22). Day et al (23) have shown that decreasing telomere length on chromosome 9 may inhibit gametocyte production in strains that have been in culture for long periods of time. It is unknown how long either of these strains was in culture before we received them, so this may be a possible explanation for why they did not produce gametocytes. There are also several other factors, including defects in adhesion surface proteins or other molecules involved in gametocyte development that may have limited the production of gametocytes (24). Although these two strains did not produce gametocytes in these experiments, it cannot be concluded that they are incapable. There are several ways to induce gametocytogenesis in vitro, including adding certain drug pressures, adding lysed
red blood cells, and trying different starting hematocrit and parasitemia levels (12). Perhaps, the methodology used in these experiments was simply not appropriate for these strains.

The male to female ratios observed in these experiments closely resemble results from previous studies. Studies have traditionally shown most malaria cultures to be female-biased (25). When male gametocytes are ready for fertilization, 8 microgametes are released for every one microgametocyte. Therefore, to achieve an approximately 1:1 sex ratio during mosquito infection, there are usually many more female gametocytes present in culture (5). This study shows that nearly all strains produced female-biased sex ratios. C2B-B was the only culture that had a higher male to female ratio. It was also one of the only cultures that did not produce successful mosquito infection as indicated by oocyst counts and PCR analysis. This indicates that a lower male to female gametocyte ratio may be essential for successful transmission of the parasite.

Because these experiments allowed us to successfully produce mature gametocytes in atovaquone-resistant strains, we wanted to then test whether or not these strains would be able to be transmitted. Resistance to atovaquone is conferred with a single mutation in the cytochrome b gene. Although this mutation confers resistance and protects asexual stages from being killed by atovaquone, it is possible that this SNP may also be associated with a fitness cost. Vaidya et al (15) have suggested that mitochondrial function is limited during asexual stages and is only essential for pyrimidine biosynthesis, leading to the hypothesis that impaired mitochondrial function has little impact on asexual stage development and
reproduction. However, during gametocyte development, studies have shown that
the mitochondria greatly expand and undergo morphological changes. Okamoto et
al (26) demonstrated that gametocyte mitochondria develop tubular mitochondrial
cristae that are not observed in asexual stages and that the expansion of the
mitochondrion is associated with the increase in metabolic activity during these
sexual stages. Studies have also shown that enzymes of the tricarboxylic acid (TCA)
cycle are upregulated during sexual reproduction (27) and that complex II of the
electron transport chain is essential for oocyst development in the mosquito midgut
(28). These findings suggest that, unlike asexual stages, energy metabolism in sexual
stage parasites is driven by oxidative phosphorylation and that mitochondrial
activity is much more essential. Therefore, the presence of a mitochondrial DNA
mutation may impair mitochondria function, thus decreasing the parasite’s ability to
undergo sexual reproduction and transmit. Exflagellation is an essential part of
disease transmission that is triggered in response to changing environmental
conditions within the mosquito. Previous studies have indicated several different
molecular pathways involved in this process; however, it is unclear exactly which
pathways trigger this event (5). Our study reveals that exflagellation is visible in all
NF54 control groups, but not in atovaquone-resistant strains alone. This suggests
that the resistant strains may not be able to be transmitted to the mosquito.
Therefore, cross experiments were performed with NF54 to help identify a negative
result. Because the resistance mutation may affect male and female gametocytes
differently, cross experiments would allow the mutation to still be transmitted if
there was a defect in atovaquone-resistant male development.
Sequencing analysis of resulting oocyst DNA revealed that wildtype sequences were present in all NF54 control experiments and all crossed infections. This suggests that although resistant strains were crossed with NF54, the NF54 parasites most likely had a fitness advantage allowing them to be the only ones to develop oocysts. Because mitochondrial DNA is maternally inherited, these results may also suggest that resistant males were able to cross with NF54 females and produce successful mosquito infections; however, because there was no exflagellation, the former hypothesize is most likely correct. Sequencing analysis also revealed that the successful TM90C2B mosquito infection contained mostly wildtype cytochrome b DNA; however, there was also a small peak indicating the presence of the atovaquone resistance mutation. It is possible that the presence of wildtype DNA indicates a contamination issue during the PCR reaction; however, it is also possible that the parasite is undergoing reversion. This parasite may be reverting back to a wildtype sequence and losing its resistance genotype; however, this experiment needs to be repeated to determine the rationale behind these results.

All mosquito-feeding experiments resulted in extremely low infection rates. Most infections were so low that DNA samples had to be amplified twice in PCR reactions in order to detect a *P. falciparum* DNA on a gel. *P. falciparum* mosquito infections are often very difficult to do in a laboratory setting. Because infections were low with NF54 control groups, this may indicate that there were issues with the actual mosquitoes used in the experiments. Low infection rates also may suggest that gametocyte culturing methods used were not optimal for infection studies.
Perhaps factors such as freshness of blood and serum, temperature, and pH levels affected infection rates. Furthermore, *A. dirus*, which is the natural vector for these resistant patient isolates, was not available to use during the course of this study. Therefore, this study should be repeated using this vector to see if higher infection rates can be achieved.

Because infection rates were so low, it is unclear whether or not these parasites would have been able to be transmitted from mosquito vector to human host. The sexual stages of the parasite’s life cycle are considered to be the bottleneck stages because many parasites are lost during development inside the mosquito. This means that although oocysts were present, it is possible that the parasite may not have progressed to sporozoite form, which transmits the disease from mosquito to human. This experiment should be repeated and both oocyst and sporozoite DNA should be quantified and analyzed using pyrosequencing methods. This will determine the proportion of DNA that contains the resistance mutation and give better insight into the transmission potential of these mutations.

The results for artemisinin gametocyte development in the presence of drug did not produce significant differences between the susceptible and artemisinin resistant parasite lines. *In vitro* susceptibility testing of the 4G strain used to produce gametocytes suggested that the asexual stages had reverted to sensitivity of artemisinin. It is not clear if this reversion happened prior to the gametocyto genesis studies. Therefore, further studies need to be conducted to determine whether or not the parasite lost the resistance phenotype or if it was just a careless mistake that resulted in cultures being mixed up. Although this study did not yield desired
results, there is still much that can be learned from this data. First, this study confirmed previous results indicating that artemisinin is effective against early stage gametocytes, but not late stages. This has important implications in the field because it means that individuals treated with artemisinin still have the ability to transmit the parasite. This further supports the recommendation of combining artemisinin with another drug that has transmission blocking capability. This study also showed that drug concentrations between 10 nM and 100 nM should be used in future experiments to look at artemisinin sensitive and resistant phenotypes. The lower concentration had no significant effect on gametocyte development, whereas the higher concentration eventually cleared parasites treated during early stages of gametocytogenesis, suggesting that a concentration in this range will be appropriate for future studies. An interesting observation from this study is that although artemisinin was not effective at killing late stage gametocytes, it did greatly reduce exflagellation events by Day 16. This suggests that artemisinin may have some transmission blocking potential; however, the amount of exflagellation present is most likely enough to still transmit the disease.

Battling drug resistance and reducing transmission are major obstacles in controlling and preventing malaria infections. However, with recent global funding and emerging interest in transmission stages of the parasite life cycle, public health officials now have more insight into the mechanisms of transmission and how drug resistance affects this process. Although the results of this study did not provide total insight into these mechanisms, they have shown the ability of resistant strains to produce gametocytes in vitro, confirmed previous study results, and have given a
solid foundation for future research. This study is the first to report the possibility of atovaquone resistance transmission and the first to examine the role of artemisinin resistance on gametocyte development. Hopefully, future studies will provide deeper insight into these problems and contribute to the overall reduction of disease transmission.
List of References


