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STUDY OF ANTI-MALARIAL DRUG RESISTANCE USING THE MUSE® RBC INVASION ASSAY

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The increasing resistance of malaria parasites to common anti-malarial drugs is a major concern for controlling the disease. The evolution of rapid, simple methods that can be used in diverse environments can help facilitate the discovery of new anti-malarial drugs. Current approaches include DNA-binding dyes and fluorometric approaches that result in loss of intact and parasitized cells. Flow cytometry can provide information on the percentage of parasitized cells; however, the cost and complexity of current instrumentation has been prohibitive for use in many environments. We have developed the research use only Muse® RBC Invasion Assay on the easy to use, low-cost Muse Cell Analyzer. The assay allows for the determination of the percentage of the RBCs invaded by *Plasmodium* parasites. The assay has been used for routine monitoring of RBCs infected by different *Plasmodium falciparum* strains, such as 3D7, D10, and Dd2 strains, as well as *in vitro* drug susceptibility studies. In this study, the *in vitro* impact of anti-malarial drugs on different *Plasmodium falciparum* strains was studied using the Muse RBC Invasion Assay. In particular, we looked at the dose response of chloroquinine and mefloquinine on 3D7 and Dd2 strains, and the impact on invaded RBC percentages. The IC50 for chloroquinine was 18.8 nM for 3D7 and 104 nM for Dd2 strains in this study, while mefloquine had IC50's in similar ranges for both strains. Our results demonstrate that the 3D7 strain showed susceptibility to chloroquine at low doses, while the Dd2 strain was resistant and only showed response at higher drug concentrations. Additional studies should help characterize the impact of more recent artemisinin drugs such as dihydroarteminisin on both strains. The studies performed demonstrate the importance of performing *in vitro* screening of antimalarial drugs on multiple strains, in order to truly characterize their action and utility. The Muse RBC Invasion Assay can be a useful and accessible method to characterize the *in vitro* impact of anti-malarial drugs, and can be a powerful drug discovery and development tool for malarial researchers.

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ARTEMISININ RESISTANCE MARKER OF *PLASMODIUM FALCIPARUM* IN OSOGBO METROPOLIS, SOUTH WEST, NIGERIA

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Artemisinin derivatives constitute a key component of the present-day treatment for *plasmodiumfalciparum* malaria and rsesistance with artemisinins is generally associated with S769N point mutation in the sarco-endoplasmic reticulum-dependant ATPase6 (SERCA ATPase6) gene of *Plasmodium falciparum*. However, few studies have been carried on the current level baseline and level of mutation of this drug in Nigeria most especially in Osun State. The present study determined the distribution of *plasmodiumfalciparum* and resistance marker for artmisinins drugs from the blood smears of 60 randomly sampled patients attending LAUTECH Teaching Hospital , Osogbo ,Osun state after obtaining ethical clearance from the relevant authority.The entire study period was divided into Pre-Treatment ,drug administration and post -Treatment phases .Blood smears of 70 consented participants were assessed microscopically using Giemsa ststaining technique for parasite identification and parasitaemia.. Samples found to have parasitaemia after drug administration were amplified and assessed for distribution of the *Pf*ATPase6 S769N mutation using Polymerase Chain Reaction (PCR) . 28 out of 30 samples subjected to PCR had successful amplification. However, none of the amplified samples harboured the *Pf*ATPase6 S769N mutation, suggesting 100% sensitivity of P. Falciparum population examined at the study area. There is therefore need for continuous surveillance for earlier detection of resistance as the use of ACT isbeing scaled up in the country.

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SELECTION OF *PLASMODIUM FALCIPARUM* DRUG RESISTANCE MARKERS POST-ACT INTRODUCTION AND A NEARLY COMPLETE REVERSION TO CHLOROQUINE SENSITIVITY

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The emergence and spread of artemisinin resistance in South East Asia are of great concern as drug resistance renders drugs ineffective in the fight against malaria. Additionally, resistance to previous first-line treatments, including chloroquine (CQ) and sulfadoxine-pyrimethamine (SP), was imported to Africa from South East Asia. Genetic markers of resistance are important molecular epidemiological tools used for early detection and monitoring of the spread of drug-resistant parasites. We carried out a temporal analysis of changes in allele frequencies of 8 drug resistance markers and 4 putative artemisinin resistance background markers over two decades of changing anti-malarial drug policy in Kenya. Using the *crt*-76 and *mdr1*-86 and *mdr1*-1246 molecular markers, we found that the withdrawal of CQ and SP as first-line treatment in Kenya shifted the parasite population from CQ resistant to a nearly fixed (99%) CQ sensitive population. On the other hand, *dhps* mutations associated with SP resistance (A437G and K540E) were maintained at a high frequency (greater than 75%). Something that may be attributable to the fact that SP is still readily available in Kenya, with a recent survey reporting a market share of 57% in the private sector. We did not detect any of the artemisinin resistance markers nor the background mutations that precede artemisinin resistance except mdr2 I492V mutation. However, we show a gradual decline in the novel *nfs* (K65 allele) marker, which potentially confers resistance to lumefantrine, from 38% to 20% with a significant allele frequency difference pre- and post-ACT introduction. In summary, the high frequency of CQ sensitive parasites circulating in the population supports the on-going debate on re-introduction of chloroquine for the treatment of malaria and the impact of the decreasing frequency of *nfs* (K65 allele) on the efficacy of Artemether-Lumefantrine combination warrants close monitoring.

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INFLUENCE OF OXYGEN AND CARBON DIOXIDE ON *PLASMODIUM FALCIPARUM IN VITRO* RESISTANCE TO ARTEMISININ

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With the goal of malaria eradication firmly on the table, several aspects are undermining this, including the resurgence of malaria cases in some malaria endemic countries and artemisinin combination therapy (ACT) failure within the greater Mekong subregion of Asia. *Plasmodium falciparum* (*Pf*) ACT resistance presents as a slow parasite clearance time (> 5 hours) *in vivo* and *in vitro* greater than 1% survival of early ring stage parasites, 0-3 hours post red blood cell invasion, after a 6-hour dose of 700nM of artemisinin derivative (ART). Both these phenotypes are correlated to mutations within the *Pf*K13 propeller domain, but not exclusively as some genetic predisposition is proposed to pre-empt the K13 mutation acquisition. The *in vitro* acquisition of K13 mutations through escalating ART challenge has only resulted from one study where O_2 levels

throughout incubation were 21% in comparison to 1-5% in other studies. Routinely the *in vitro* culture of *Pf* utilizes a single gaseous environment that is constant and maintained at, for example, 5% O_2 and 5% CO_2. However, *in vivo* the gaseous microenvironment has a diverse range of concentrations and ratios of O_2 and CO_2 depending on the location within the circulatory system and the presence of certain pathologies. To study the impact of alterations in the gaseous environment on parasite susceptibility to ART, the ring stage survival assay (RSA) was performed on *Pf* parasites, designated resistant or sensitive (K13 mutant or WT), cultured under different O_2 and CO_2 concentrations. To advance knowledge on the effect changing gaseous environments has on ART sensitivity and resistance at different stages of *Pf* asexual lifecycle, the RSA was also performed at a range of O_2 and CO_2 concentrations at selected times post red blood cell invasion. The effects of gaseous environments on parasite *in vitro* resistance and susceptibility profiles, relationship to K13 mutations, and biological evaluation of the underlaying mechanisms behind the altered responses will be discussed.

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PREVALENCE OF ANTIMALARIAL DRUG RESISTANT MARKERS IN KWARA, NORTH-CENTRAL, NIGERIA: A DECADE AFTER REPLACEMENT OF CHLOROQUINE AND ANTIFOLATES AS FIRST-LINE REGIMEN

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The emergence of “super malaria” parasites, resistant to artemisinins based combinations (ACT), portend danger to malaria control efforts. While measures to curtail the spread must be addressed, preparedness, also becomes necessary. One premonition is to evaluate background status of parasites resistant to previously used drugs, especially as artemisinin resistance selects for sensitive alleles of chloroquine resistant markers. This study was designed to establish the current profile of *Plasmodium falciparum* resistant markers of chloroquine (*Pfcrt*), pyrimethamine (*Pfdhfr*) and *Pfmdr1* in field isolates. In a cross sectional survey, 305 participants were enrolled from four communities in Kwara state, Nigeria. Participants were randomly tested for malaria infections, using rapid diagnostic detection. Parasite densities were determined from thick blood smears by microscopy. Dried blood spots samples were collected on Whatman FTA cards, from which parasite genomic DNA was extracted. Polymerase chain reaction (PCR) and restriction fragment length polymorphism were conducted on genomic samples. The prevalence of *P. falciparum* infections by microscopy was 22.30% (68/305), with a PCR corrected value of 23.28% (71/305). *P. falciparum* chloroquine resistant transporter (*Pfcrt*) mutation (K76T) was predominantly significant ($p<0.05$) in all positive samples (100%), compared to 56% and 38%, for dihydrofolate reductase (*Pfdhfr*) primary point mutation (S108N) and the *P. falciparum* multidrug resistant gene (*Pfmdr1*) mutation (86Y), respectively. Background detection of *Pfcrt* wild-type K76 was recorded in 41% (29/71) of the positive samples. More than ten years after withdrawal of chloroquine (CQ) and PYR from first-line treatment of malaria in the region, there is still a strong presence of resistant markers to these drugs in *P. falciparum* isolates. However, the presence of CQ sensitive strains in mixed population with resistant cousins may suggest development of reversal processes by parasites probably due to fitness cost in the absence of chloroquine and/ or evolving mechanisms towards artemisinin resistance due to ACT pressure.

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IDENTIFYING MOLECULAR MARKERS OF *PLASMODIUM FALCIPARUM* ARTEMISININ RESISTANCE USING THE CRISPR-CAS9 GENOME EDITING SYSTEM

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The emergence and spread of *P. falciparum* to artemisinin and the artemisinin combination therapy partner drugs threatens to upend the gains made by the control program in recent times especially in Africa. With hindsight from the devastation chloroquine and sulphadoxine-pyrimethamine resistance wreaked in Africa due to an unprepared public health system, real time monitoring of resistance has therefore been recommended in other to forestall any similar occurrence. Monitoring of the molecular markers of resistance is less logistically and financially constraining compared with *in vivo* and *in vitro* monitoring especially in resource limited setting. However, the kelch 13 molecular markers for monitoring artemisinin resistance have arisen independently, with multiple mutations either conferring resistance or otherwise. Gene-editing system has previously been utilised to validate some of these mutations, we therefore undertook to use the CRISPR-Cas9 genome editing system validate kelch 13 mutations detected from recrudescing parasites sampled from the monitoring sentinel sites in Ghana. We have successfully edited kelch 13 V568G and C580R previously detected in Ghana in addition to C580Y and R539T commonly found in Southeast Asia and C580C (silent mutation) into Dd2 *P. falciparum* strain. We utilised a single plasmid system carrying a chimeric short-guide RNA, a codon-optimised Cas9 sequence and a donor DNA.Cloning by limiting dilution method has been used to clone out isogenic parasites, which would be utilised to perform ring stage survival assay to validate these mutations. In addition, conventional SYBR green drug assays of commonly used antimalarial, fitness and growth assays.

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THERAPEUTIC RESPONSE OF SINGLE *PLASMODIUM FALCIPARUM* SPECIES VERSUS MIXED SPECIES INFECTIONS TO ARTEMETHER-LUMEFANTRINE AS COMPARED TO OTHER ARTEMISININ COMBINATION THERAPIES IN KISUMU COUNTY, WESTERN KENYA

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Information on treatment outcomes of infections comprising non-*falciparum* species is scanty despite it being reported to be responsible for around 25% of imported malaria from Africa. The recommended first line treatment in Africa is artemether-lumefantrine (AL) with dihyroartemisinin-piperaquine (DHAPPQ) as second line whereas in Asia, artesunate-mefloquine (ASMQ) is the preferred choice of treatment. The aim of this study was to compare the treatment outcomes of mixed *Plasmodium* species versus pure *falciparum* species infections during dosing with AL, DHAPPQ & ASMQ. Samples collected at hours 0, 4, 8, 24 and 30 from individuals enrolled in an ACT efficacy study in Kisumu County, Western Kenya, between 2013 and 2015 were analyzed for species composition using real time polymerase chain reaction (rt-PCR). The assay targeted detection of *Plasmodium falciparum* (*Pf*), *Plasmodium malariae* (*Pm*), *Plasmodium ovale curtisi* (*Poc*) and *Plasmodium ovale wallikeri* (*Pow*). Therapeutic response was evaluated using parasite clearance parameters as well using the World Health Organization criteria and compared with species composition of each infection. Recurrent parasitemia for the subsequent time points specifically hours 24 and 30 was also characterized to rule out re-infection. There was a steady decline in parasites for