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Characterization of PfCRT F145I in piperaquine-resistant Plasmodium falciparum isolates from Cambodia through zinc-finger nuclease-mediated gene editing

Item Type	dissertation
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- Huang F, Takala-Harrison S, Liu H, Xu JW, Yang HL, Adams M, Shrestha B, Mbambo G, Rybock D, Zhou SS, Xia ZG, Zhou XN, Plowe CV, Nyunt MM. Prevalence of Clinical and Subclinical *Plasmodium falciparum* and *Plasmodium vivax* Malaria in Two Remote Rural Communities on the Myanmar-China Border. Am J Trop Med Hyg. 2017 Nov;97(5):1524-1531.
- Adams M, Joshi SN, Mbambo G, Mu AZ, Roemmich SM, Shrestha B, Strauss KA, Johnson NE, Oo KZ, Hlaing TM, Han ZY, Han KT, Thura S, Richards AK, Huang F, Nyunt MM, Plowe CV. An ultrasensitive reverse transcription polymerase chain reaction assay to detect asymptomatic low-density Plasmodium falciparum and Plasmodium vivax infections in small volume blood samples. Malar J. 2015 Dec 23;14:520.
- Travassos MA, Coulibaly D, Laurens MB, Dembélé A, Tolo Y, Koné AK, Traoré K, Niangaly A, Guindo A, Wu Y, Berry AA, Jacob CG, Takala-Harrison S, Adams M, Shrestha B, Mu AZ, Kouriba B, Lyke KE, Diallo DA, Doumbo OK, Plowe CV, Thera MA. Hemoglobin C Trait Provides Protection From Clinical Falciparum Malaria in Malian Children. J Infect Dis.2015 Dec 1;212(11):1778-86.

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Abstract

Title of Thesis: Characterization of PfCRT F145I in piperaquine-resistant *Plasmodium falciparum* isolates from Cambodia through zinc-finger nuclease-mediated gene editing

Biraj Shrestha, Master of Sciences, 2019

Thesis Directed by: Dr. Shannon Takala-Harrison, Associate Professor, Medicine and Epidemiology & Public Health

Artemisinin-based combination therapies (ACTs) are the first-line treatment for clinical malaria in most of the malaria-endemic world and have played an indispensable role in reducing global malaria-associated mortality and morbidity. However, the recent emergence of *Plasmodium falciparum* that is resistant to both the artemisinins and key partner drugs, such as piperaquine, in Cambodia and other nearby countries in the Greater Mekong Subregion poses a threat to the control and elimination of malaria. Identification and validation of molecular markers of antimalarial drug resistance provides surveillance tools to monitor resistance and inform drug policy decisions, as well as insights into the molecular mechanisms underlying resistance.

Previous studies have found that the F145I mutation within the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT), and *plasmepsin 2/3* gene copy number are associated with resistance to the artemisinin partner drug piperaquine. When PfCRT F145I is introduced into the Dd2 strain of *P. falciparum,* a piperaquine-sensitive strain with a single copy of *plasmepsin 2/3,* it confers piperaquine resistance. In this study, we will use gene-editing approaches to remove PfCRT 145I from Cambodian field isolates that contain both this mutation, as well as amplified *plasmepsin 2/3,* in order to quantify the effect on malaria parasite susceptibility to piperaquine.

Characterization of PfCRT F145I in piperaquine-resistant *Plasmodium falciparum* isolates from Cambodia through zinc-finger nuclease-mediated gene editing

by
Biraj Shrestha

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
2019

Acknowledgements

This research was supported by NIH. We thank our colleagues from AFRIMS and Walter Reed Army Institute of Research for helping us with the sample collections.

We thank Dr. David A. Fidock and Dr. Satish K. Dhingra at Columbia University, NY, USA for assistance with zinc finger editing technique in *P. falciparum* and Dr Abdu F. Azad at University of Maryland Baltimore, MD, USA for providing the instrument for electroporation.

We would also like to show our gratitude to the all the study participants for their time and willingness to participate in the study.

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Introduction

Malaria, an infectious disease caused by protozoan parasites of the genus *Plasmodium,* is an important cause of morbidity and mortality[1]. More than half of the world's population is at risk for malaria[2]. According to World Health Organizations, in 2017, 219 million cases of malaria were reported, resulting in 435,000 deaths worldwide[3]. There are five species of *Plasmodium* that cause malaria in humans. Of these species, *P. falciparum* is responsible for most malaria mortality, including 93% of malaria deaths in Africa[3]. Globally, both malaria incidence and mortality rates have fallen. It has been estimated that malaria mortality has decreased by almost 60% from 2000 to 2015, owing in part to better diagnostics and treatment and use of insecticide-treated bed nets[4,5]. However, the emergence of drug resistance threatens recent progress and could curtail eradication efforts, if not controlled.

The emergence and spread of chloroquine-resistant parasites in the 1960s in the Greater Mekong Subregion (GMS), South America, and Africa led to the deployment of other antimalarials (e.g. sulfadoxine-pyrimethamine) to treat malaria[5–7]. However, *P. falciparum* also developed resistance to alternative therapies[8]. In the 1980s, the artemisinin derivatives were introduced, based on a semi-synthetic derivative of the compound artemisinin, which was isolated from the plant *Artemisia annua*[9]. Almost all malaria-endemic countries have now replaced resistance-compromised former first-line antimalarial drugs with artemisinin-based combination therapies[10]. Artemisinin derivatives rapidly clear parasitemia within 72-hours by mechanisms that are distinct from those of its partner drugs, which have a longer half-life[11]. In 2001, artesunate-mefloquine was implemented as the first-line treatment for uncomplicated malaria in Cambodia. By 2008, delayed parasite clearance following treatment with ACTs, began to

be reported in the western provinces, suggesting the emergence of artemisinin resistance[12,13], in addition to existing mefloquine resistance[14].

When ACTs such as artesunate-mefloquine and artemether-lumefantrine showed reduced efficacy, dihydroartemisinin-piperaquine (DHA-PPQ) became the first-line drug combination to treat artemisinin-resistant malaria in this geographic region. Piperaquine is a 4-aminoquinoline bisqinolone that targets the blood stage of the malaria parasite[15], and was used in the1980s as a monotherapy against malaria in areas of southern China[16], By 2014, reports of treatment failure with DHA-PPQ began to be reported in Vietnam[17] and Cambodia[18–21], again in the western and northern provinces, with reports of clinical treatment failure in 25%-46% of patients from 2010 to 2014 respectively[20–24], as well as reduced *in vitro* piperaquine susceptibility in parasites in this geographic region[25]. Owing to the large prevalence of DHA-PPQ treatment failure, and the corresponding decrease in the molecular marker for mefloquine resistance[26], Cambodia has now switched back to use of artesunate-mefloquine[20,22,27–29].

Significance

Although, DHA-PPQ has been replaced with artesunate-mefloquine in Cambodia, it is important to establish rigorous and coordinated efforts to investigate and characterize piperaquine resistance, as DHA-PPQ is still used in other areas of the GMS. Identification and validation of molecular markers of piperaquine resistance will provide a rapid means to measure the extent of resistance and thus guide policy decisions to combat resistance and select appropriate drugs for mass treatment or reactive case detection. Additionally, identifying which mutations are involved in resistance can provide insights into the underlying molecular mechanisms responsible for resistance. Previous studies have shown associations between *plasmepsin 2/3* gene copy number and

piperaquine resistance[14,28,30]. In a genome-wide association study of piperquine susceptibility in Cambodia, we observed that the PfCRT F145I mutation, in addition to *plasmepsin 2/3* copy number, was independently associated with piperaquine resistance, and conferred a greater degree of resistance that *plasmepsin 2/3* amplification alone[31]. Gene editing studies have recently indicated that the PfCRT F145I mutation can confer piperaquine resistance in the Dd2 *P. falciparum* strain, even in the absence of amplified *plasmepsin 2/3*[31]. In this study, we aim to further understand the contribution of PfCRT F145I to piperaquine susceptibility by using gene editing approaches to remove this mutation from Cambodian field isolates that harbor this mutation.

Objectives:

Aim 1:

To estimate piperaquine susceptibility in Cambodian field isolates carrying the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) F145I mutation. I hypothesize that parasites harboring the PfCRT F145I mutation and amplified *plasmepsin 2/3* (*pfpm2/3*) copy number will have reduced susceptibility to piperaquine, compared to control isolates without these mutations. To test this hypothesis, I will culture adapt Cambodian field isolates and genotype them to confirm the presence of the PfCRT F145I mutation and amplified *pfpm2/3* copy number using pyrosequencing and quantitative PCR (qPCR), respectively. After confirming the presence of resistance-associated mutations in the field isolates and control line, I will perform *in vitro* drug susceptibility assays ($IC_{50/90}$, LD_{50}, and Piperaquine Survival Assay (PSA)) to test *in vitro* susceptibility to piperaquine.

Aim 2:

To use gene editing to replace the PfCRT F145I mutation with the wild-type allele and estimate piperaquine susceptibility in edited lines. I hypothesize that piperaquine susceptibility will increase when the PfCRT F145I mutation is reverted to wild-type in these piperaquine-resistant parasites. To test this hypothesis, I will use zinc-finger nuclease-mediated (ZFN) gene editing to replace the PfCRT 145I mutation with PfCRT F145. Following gene editing, I will confirm the PfCRT 145 genotype and estimate *pfpm2/3* copy number in the edited field isolates. I will then assess piperaquine susceptibility in edited and non-edited isolates using piperaquine susceptibility assays.

Materials and Methods

Cambodia field isolates

Cambodian field isolates were collected by investigators at the Armed Forces Research Institute of Medical Sciences (AFRIMS) as part of a molecular and *in vitro* survey of *P. falciparum* antimalarial drug resistance in eight provinces of Cambodia. We selected three isolates collected in 2014 from the Anlong Veng site in Oddar Meanchey province that contained the PfCRT F145I mutation and amplified *plasmepsin 2/3* based on our previous genome-wide association study[31]. Cryopreserved parasites were provided by AFRIMS investigators for the purpose of culture adaptation and gene editing.

Culturing field isolates

Three cryopreserved Cambodian field isolates (asexual blood-stage parasites of *P. falciparum*) were cultured in human O+ red blood cells (RBCs) in RPMI 1640 (500ml), supplemented with 10 ug/ml gentamicin, 50 uM hypoxanthine, and 0.5% AlbumaxII

(Thermo Fisher). All cultures were maintained at ~4% hematocrit at 37ºC in an environment of 5% O_2, 5% CO_2 and 90% N_2[32].

In vitro drug susceptibility assays

To characterize the phenotypes of the three field isolates prior to gene editing, *in vitro* susceptibility to piperaquine was tested by performing a Piperaquine Survival Assay (PSA) and by comparing IC_{50}, IC_{90}, and LD_{50} values in unedited field isolates containing the mutations of interest and the Dd2 *P. falciparum* strain. IC_{50}/IC_{90} estimates the piperaquine concentration at which 50% and 90% of the parasites are inhibited, and similarly, LD_{50} estimates the concentration of piperaquine that kills 50% of the parasites. After gene editing, piperaquine susceptibility will be assessed in edited parasites by comparing PSA, IC_{50}, IC_{90}, and LD_{50} values between unedited field isolates, edited isolates (pcc1+Dd2), the parental control strain Dd2, and recombinant isogenic transfection control field isolates (pcc1 +Dd2+F145I).

To determine IC_{50}, parasites at 0.5% parasitemia and 2% hematocrit were incubated with a range of drug concentrations starting from 100 uM to 0 uM in 2-fold dilutions at 37ºC for 72 hours in 96-well plates, with gas supplied daily. Parasite growth in each well was assessed after 72 hours using Spectra Max M2/$M2^e$ (Molecular devices), which measures the DNA stained with SYBR Green I nucleic acid gel stain (Thermo Fisher Scientific). Nonlinear regression analysis was used to determine the *in vitro* IC_{50} and IC_{90} values.

Piperaquine survival assay (PSA)

The PSA (*in vitro*) was performed as described by Dura *et al*. with minor modifications, using 0-3 hours post-invasion rings from the culture-adapted field

isolates[22,33]. The synchronized cultures were obtained using MACs LD columns to isolate schizonts[34]. Schizonts were cultured for 1-3 hours to obtain 0-3 hours post-invasion rings. Parasite density and hematocrit levels were maintained at 0.5-2% (depending upon the growth of the parasites) and 2% respectively. Cultures were maintained for 48 hours at 37°C under a 5% CO_2, 5% O_2 and 90% N_2 with 200nM, 100nM, and 50nM of piperaquine tetraphosphate tetrahydrate, and included a drug-exposed culture, vehicle control, or kill control. Vehicle control parasites were cultured with 0.5% lactic acid. After 48 hours of culture, parasites were washed three times with complete medium, resuspended in complete medium (RPMI 1640, 0.5% Albumax II, hypoxanthine, and 50 ug/ml gentamicin), and cultured for another 24 hours. After 24 hours, thin blood smears were made, by fixing with methanol and staining with 10% Giemsa stain for 5 minutes. The proportion of viable parasites in both the exposed and non-exposed cultures was evaluated by counting the parasites manually under the microscope. For each culture, 20,000 erythrocytes were assessed by two independent microscopists. If there was more than 20% difference in parasitemia between the first two independent microscopists, slides were examined by a third independent microscopist. Survival rate was calculated using the formula:

$$PSA\ Survival\ Rate(\%) = \frac{No.\ of\ viable\ Parasites\ in\ the\ exposed\ culture}{No.of\ viable\ parasites\ in\ non.exposed\ culture}\ \mathbf{X\ 100}$$

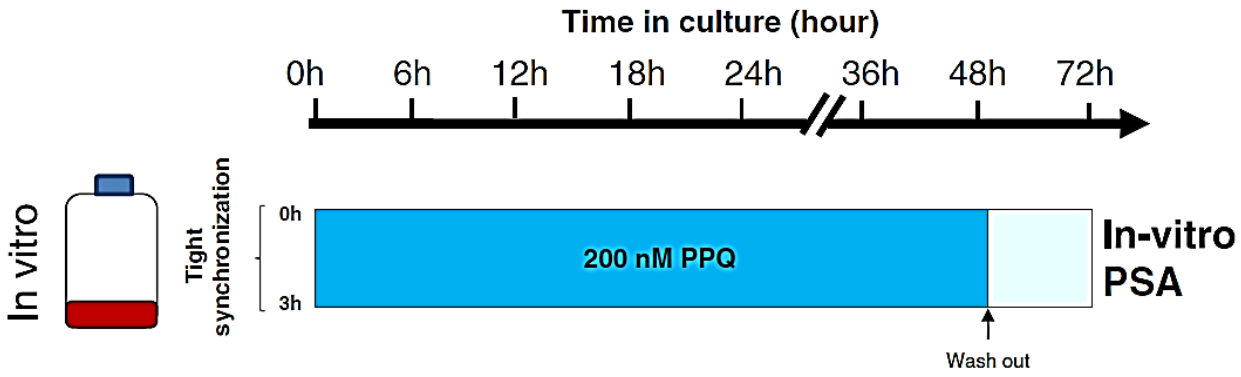


Fig. 1: In vitro piperaquine survival assay (PSA).

Synchronization of post-invasion rings and the timing of 200 nM piperaquine exposure for *in vitro* PSA performed on culture-adapted Cambodian field isolates. Survival rates were interpretable when parasites growth rates (parasite density at 72 hours/parasite density at 0hr) were >1.5%. The dark blue rectangular box represents the culture medium containing 200 nM piperaquine (exposed culture) or culture medium with 0.5% lactic acid (non-exposed culture). The light blue rectangular box represents the complete culture medium without drug for both exposed and non-exposed cultures.

LD_{50}, an alternate assessment of piperaquine susceptibility

A cytocidal assay was performed to measure LD_{50} in unedited field isolates and the parental Dd2 strain as an alternative assessment of piperaquine susceptibility. The assay was performed as mentioned in Dhingra *et al*., with minor modifications[35]. Briefly, synchronized unedited field isolates along with the parental line Dd2 were incubated at 1% starting parasitemia in a 96-well plate and 2% hematocrit at 37°C across a range of piperaquine concentrations (starting at 100 uM to 0.002 uM). Samples were run in triplicate, using a 2-fold dilution factor. After 6 hours, the drug was washed away with three rounds of washing using complete medium by centrifuging the plates at 1800 rpm for 6 minutes. The plates were again incubated at 37°C for an additional 48 hours using

drug-free complete medium. The parasitemia of the cultures in each well were estimated using a SYBR Green I nucleic acid gel stain (Thermo Fisher Scientific) and plates were read in Spectra Max M2/M2^{e} (Molecular Devices). The proportion of parasite survival (estimated as the ratio of parasitemia in drug-exposed to unexposed wells) was fitted against log-transformed drug concentrations, using Microsoft Excel.

Detection of PfCRT F145I and *plasmepsin 2* copy number

pfpm2 gene copy number in the three field isolates was estimated by quantitative PCR (qPCR). The presence of the PfCRT F145I mutation was assessed by pyrosequencing. The morphology of the three field isolates and the parental Dd2 line was compared by microscopy.

Pyrosequencing to identify PfCRT F145I

After the three field isolates were culture-adapted, genomic DNA was extracted using a QIAamp DNA Blood Mini Kit. Pyrosequencing was performed to screen for the PFCRT F145I mutation using a PyroMark Q96 MD (Qiagen) pyrosequencer. Nested-PCR was performed using HotStar Taq DNA polymerase (Qiagen) in which the reverse primer of the secondary PCR was biotinylated. Primary PCR was performed under the following conditions: 95^{o}C for 15 minutes, followed by 40 cycles of 94^{o}C for 30sec, 55^{o}C for 45 sec, and 72^{o}C for 1 minute, and final elongation at 72^{o}C for 10 minutes. Similarly, secondary PCR was performed under the following conditions: 95^{o}C for 15 minutes, followed by 25 cycles of 94^{o}C for 30sec, 55^{o}C for 45 sec, and 72^{o}C for 1 minute, and final elongation at 72^{o}C for 10 minutes. PCR products were visualized on a 2% agarose gel stained with ethidium bromide.

Streptavidin-labeled sepharose beads were bound to biotinylated secondary PCR amplicons, and sequentially washed with 70% ethanol, denaturation solution and washing buffer. PCR amplicons were then incubated with the pyrosequencing primer (listed below) and the annealing buffer. Parasites were classified as wild-type, mutant, or mixed by the single nucleotide polymorphism (SNP) at 435 nucleotide position of *pfcrt* gene and by analyzing the data in AQ mode, which provides the allele quantification that represents the relative proportion of a F145I allele in the infection. If the allele quantification of F at 145 codon of PfCRT region was greater than 90%, it was classified as wild-type, if the allele quantification of I at 145 codon of PfCRT region was greater than 90%,it was classified as mutant and if the allele quantification of both F and I at 145 codon of PfCRT region was ~50% and ~50% respectively, it was classified as mixed.

Table1: Pyrosequencing primers.

List of the external, internal, and pyrosequencing primer sequences used for the pyrosequencing of the *pfcrt* gene to screen the F145I mutation on the Cambodian field isolates.

	Primers	**Sequences**
External Primers	pfcrt145 Ext For	5'-TTTTGCTATATCCATGTTAGATGC-3'
	pfcrt145 Ext Rev	5'-CAACAATAATAACTGCTCCGAGATA-3'
Internal Primers	pfcrt145 Int For	5'-TATCCATGTTAGATGCCTGTTCAGT-3'
	pfcrt145 Int Rev	5'-Biosg-GAACAAATGATTGGATATTTCCAGTAG-3'
Pyrosequencing primer	pfcrt145_Seq	5'-TGCCTGTTCAGTCATTTTG-3'

pfpm2 copy number

A SYBR green-based quantitative PCR (qPCR) assay was used to estimate copy number of the *pfpm2* gene (PF3D7_1408000) using a Lightcycler 96 (Roche). Genomic DNA (gDNA) from culture-adapted field isolates was extracted using a Qiagen Midi kit. Primers and reagents used to perform qPCR were as described by Witkoskowi *et al*[14]. The single copy *beta-tubulin* (PF3D7_1008700) gene was used as a reference to estimate copy number of the genes of interest. The list of the primers used for qPCR and the PCR efficiencies are listed below:

Table 2: qPCR primers for *pfpm2* copy number.

List of the primers that are used in the SYBR green qPCR to determine *pfpm2* copy number.

qPCR	Primer Sequence	Sequences	Tm (°C)	Product size (bp)	Range of Melt T°C
pfpm2	*Pfpm2*_CN_F	5'-TGGTGATGCAGAAGTTGGAG-3'	59.8	79	76.8-77.2
	*Pfpm2*_CN _R	5'-TGGGACCCATAAATTAGCAGA-3'	59.4		
β-tubulin	*Pf β-tubulin*_CN_F	5'-TGATGTGCGCAAGTGATCC-3'	61.9	79	79.0-79.2
	*Pf β-tubulin*_CN_R	5'-TCCTTTGTGGACATTCTTCCTC-3'	60.5		

The master mix composition and the thermal conditions have been described previously, using 1.5ul DNA template[14]. Samples were run in duplicate along with the standard. The standard was made by mixing synthetic gene fragments (IDT) in four standards, standard 1 (1:1 molar ratio of *pfpm2* and *β -tubulin*), standard 2 (2:1 molar ratio of *pfpm2* and *β -tubulin*), standard 3 (3:1 molar ratio of *pfpm2* and *β -tubulin*), and standard 4 (4:1 molar ratio of *pfpm2* and *β -tubulin*).The lengths of *pfpm2* and of *β -tubulin* are from position 367 to 560, 193 bps (367-

aggtagttcaaatgataatatcgaattagtagatttccaaaatataatgttttatggtgatgcagaagttggagataa

ccaacaaccatttacatttattcttgatacaggatctgctaatttatgggtcccaagtgttaaatgtacaactgcaggatgtttaactaa

acatctatatgattcatctaaatc-560) and from 1183 to 1391, 208 bps(1183-

tcaacaatacagagccttaactgtgccggagttaacacaacaaatgttcgacgcaaaaaatatgatgtgcgcaagtgatccaaga

catggaagatatttaacggcatgtgctatgtttagaggaagaatgtccacaaaggaagttgacgaacaaatgttaaac

gttcaaaataaaaactcatcttattttgtcgaatggattcctcac-1391) respectively.

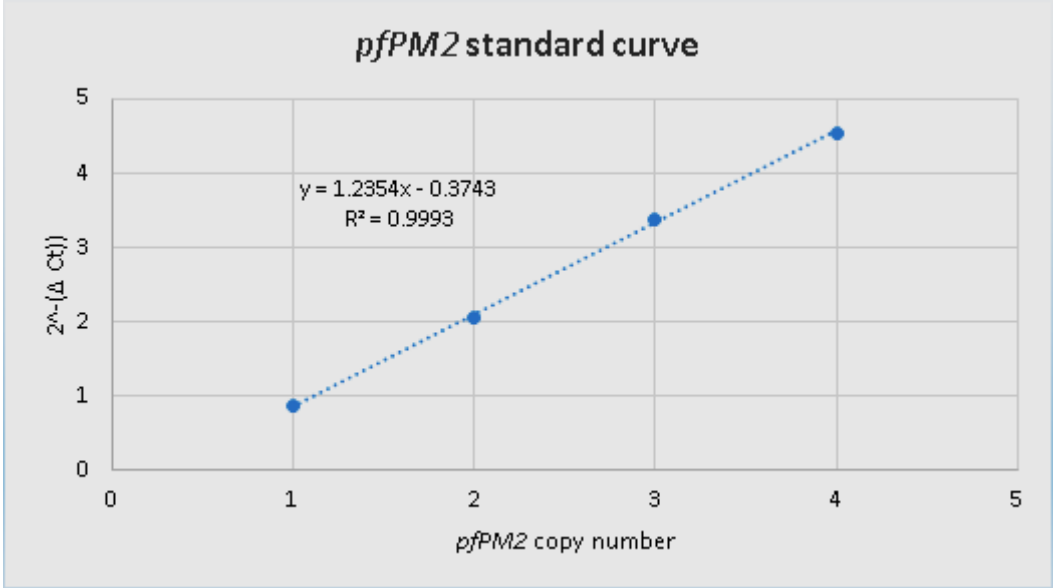
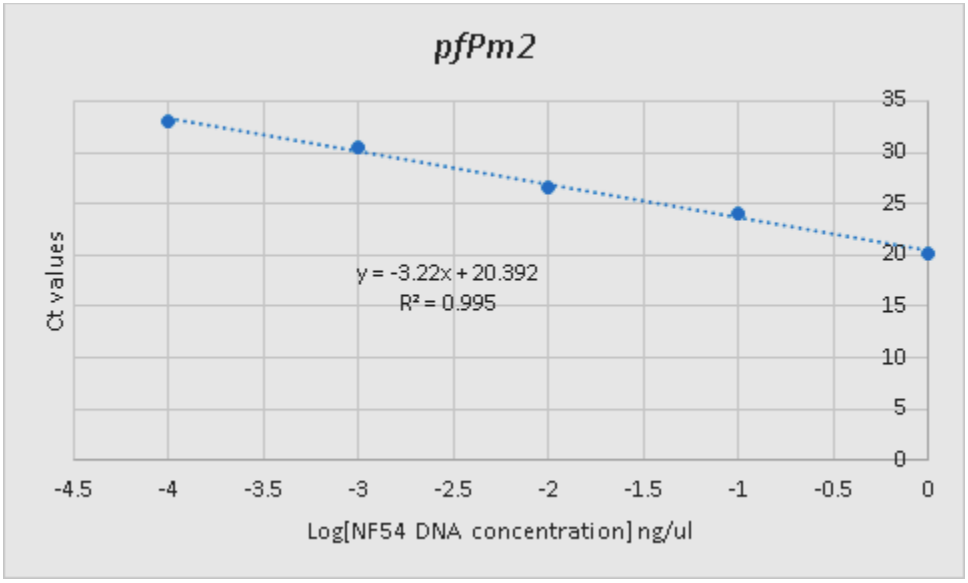


Fig 2: pfpm2 standard curve.

pfpm2 standard curve generated by using a synthetic gene fragment of pfpm2 and β -tubulin.

The NF54 line was run in each PCR as a control with a single copy of *pfpm2*. Copy number of *pfpm2* was calculated using the $2^{-\Delta ct}$ method, where $\Delta ct = C_{t\ pfpm2} - C_{t\ pf\ \beta\text{-}tubulin}$ and C_t is the qPCR threshold cycle. A copy number greater than 1.6 was used to define amplification of *pfpm*2 gene. Amplification efficiencies of both *pfpm*2 and *β -tubulin* genes were measured using a ten-fold serial dilution of NF54, and the efficiencies were very similar i.e.104% and 106% respectively.

Amplification efficiencies



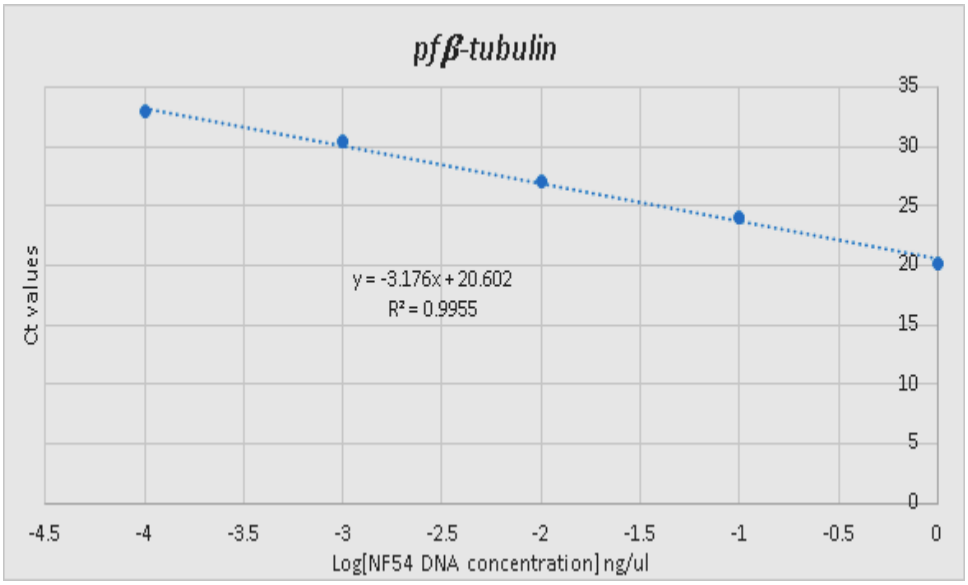


Fig 3: Amplification efficiencies of pfpm2 and β -tubulin genes.

Strategy for gene editing using ZFN

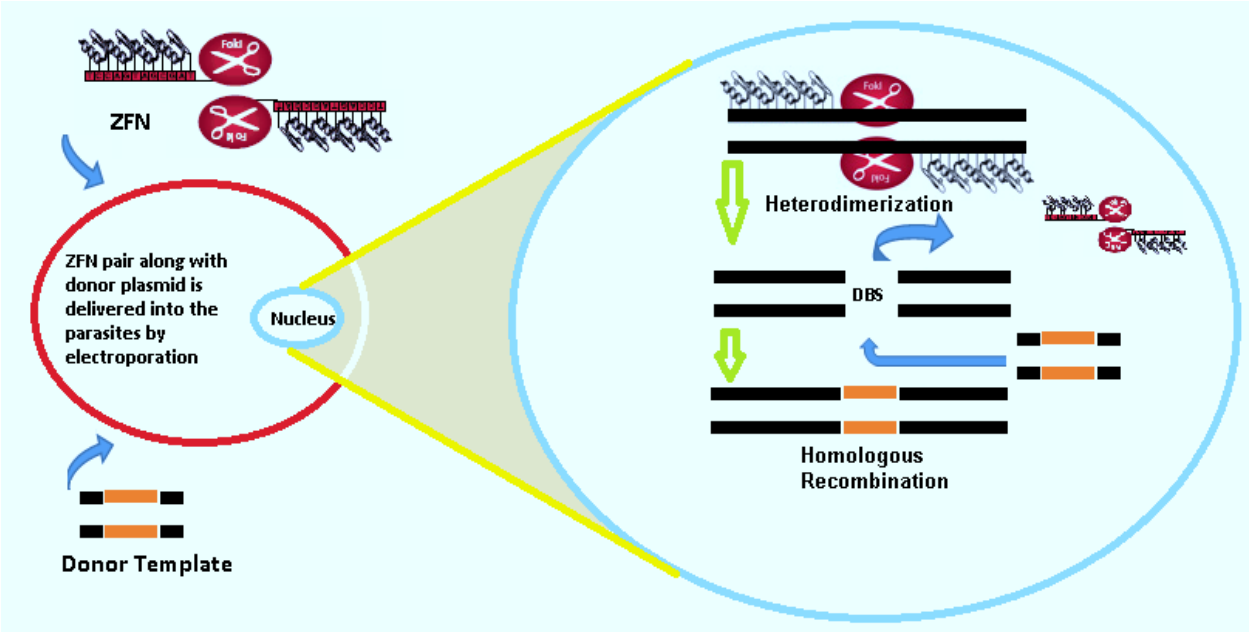


Fig 4: Schematic diagram of gene editing.

A schematic diagram showing strategy for gene editing using ZFN where the donor and ZFN-carrying plasmids are delivered into the parasites using electroporation.

Transfection of culture-adapted Cambodian field isolates was performed when cultures showed a majority of ring stages and parasitemia was greater than 5%[36]. When both the donor and ZFN-carrying plasmids are inside the nucleus of the parasites, the ZFN heterodimerizes with double-stranded DNA at a specific site and breaks the DNA. As the double-stranded DNA breaks, the donor plasmid acts as the template for homologous recombination.

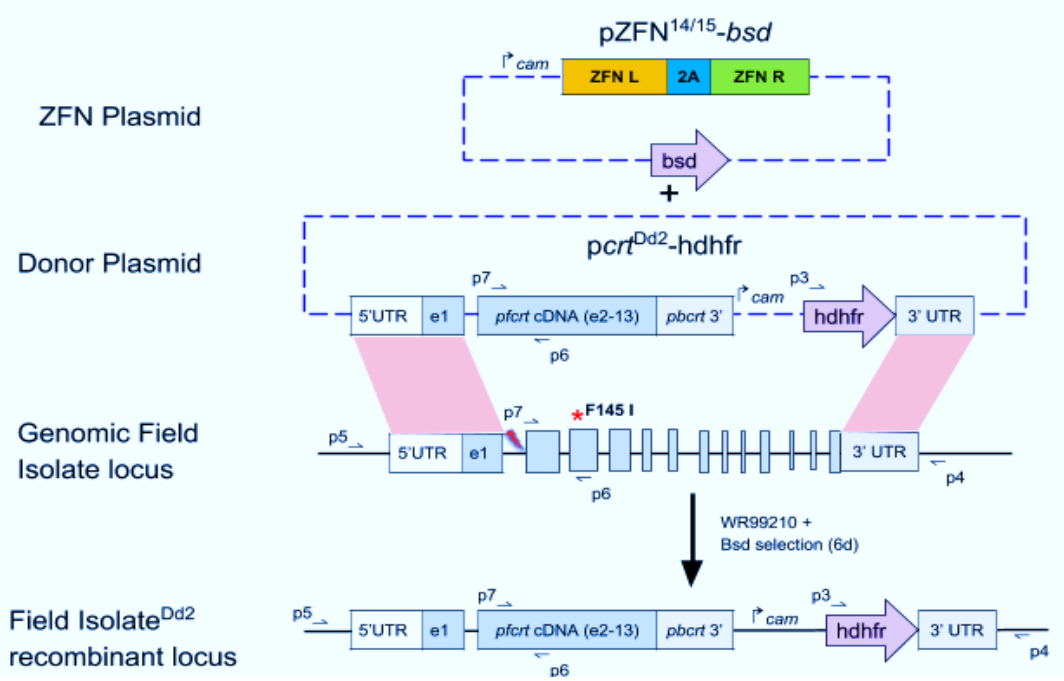


Fig 5: Schematic diagram of *pfcrt* gene editing

A schematic figure of *pfcrt* gene editing using ZFN to revert the PfCRT F145I mutation in Cambodian field isolates to the Dd2 wild-type PfCRT allele. In the figure, p*crt* stands for plasmid *crt,* pZFN stands for plasmid ZFN. Cambodian field isolates were transformed with the donor pcrt^{Dd2}-*hdhfr* plasmids, which carries the cDNA version of the Dd2 *pfcrt* allele and a human *dhfr* cassette that mediates resistance to the selection agent WR99210. Episomally enriched Cambodian field isolates were then transformed with pZFN$^{14/15}$-bsd, which is a ZFN-expressing plasmid. The *pfcrt* intron 1-exon 2 junction (shown in Figure 5 as a red thunderbolt) is targeted by 2A-linked ZFNs. The double-stranded break induced by the ZFN triggers homologous recombination, where the donor template is utilized to generate the recombinant field isolates.

Transfection of parasites via electroporation

Transfections were performed by electroporation of the ring stages of Cambodian field isolates at 5% parasitemia with 50 ug of purified circular plasmid DNA[36]. The field

isolates were first transfected with the donor plasmid and selected with 2.5nM WR99210 to enrich for episomally-transformed parasites. Parasites took ~2 months to appear in culture after the first transfection and were screened for the presence of the donor plasmid using the primers listed below. These parasites were then further transfected with ZFN plasmids (pZFN$^{14/15}$-*bsd*), when the parasites contained majority ring stages with parasitemia greater than 5% and were selected with 10mg/ml *bsd* (Thermo Fisher) for six days after the ZFN transfection. Parasites were observed by microscopy 6 to 8 weeks post electroporation and were screened for complete editing.

PCR-based screening of clones

pfcrt editing events will be confirmed using a PCR-based approach, followed by sequencing. PCR amplification will be performed on gDNA using previously published primer pairs[35]. Primers pair p6 + p7 will be used for screening the donor-transfected parasites. Similarly, primer pair p3+p4 (edited parasites, 2.5kb; wild type parasites, no product), p5+p6 (edited parasites, 1.2kb; wild type parasites, 1.4kb), and p6+p7 (edited parasites, 0.4kb; wild type parasites, 0.6 kb) will be used to screen the edited parasites. In the edited parasites, there will be removal of introns which will result in slightly shorter PCR amplicons compared to unedited parasites. The removal of the F145I mutation in the edited field isolates will also be confirmed by sequencing cDNA using primers p8 and p10. The list of primers that will be used in the screening are as follows:

Table 3: Primers for screening edited parasites.

Primers used to screen for successfully edited parasites[35].

Name	Nucleotide Sequence	Description
p3	CTCGAGATGGTTGGTTCGCTAAACTGC	hdhfr (+1-21) Xho1 Fw
p4	TTGACCCTTATATATTCCACCCA	Pfcrt 3' UTR (+1285-1308)
p5	CTTGGGCCCAAGTTGTACTGCTTCTAAGC	Pfcrt 5' UTR (-494-517) ApaI Fw
p6	CTTATCGATAAGCAGAAGAACATATTAATAGGAATACTTAATTG	Pfcrt exon 3 ClaI Rv
p7	CTTGAATTCGACCTTAACAGATGGCTCAC	Pfcrt exon 2 EcoRI Fw
p8	CCGTTAATAATAAATACACGCAG	Pfcrt 5' UTR Fw
p10	TCAAACATGACAAGGGAAATAGT	Pfcrt exon 5 Fw

Table 4: Haplotypes of parasites.

Haplotypes of the control *P. falciparum* line and field isolate 3 and desired haplotypes after gene editing of field isolate 3.

TABLE 4 Haplotypes of parental and pfcrt-modified parasites

Parasite line	Altered pfCRT haplotype	Donor plasmid	ZFN plasmid	pfCRT haplotype at listed position:												
				72	74	75	76	97	145	220	271	326	343	353	356	371
Dd2	No	None	None	C	I	E	T	H	F	S	E	S	M	G	T	I
Field Isolate 3 $^{crt\ F145I}$	No	None	None	C	I	E	T	H	I	S	E	S	M	G	T	I
Field Isolate 3 $^{Dd2\ crt}$	Yes	$pcrt^{Dd2}$-hdhfr	$pZFN^{14/15}$-bsd	C	I	E	T	H	F	S	E	S	M	G	T	I
Field Isolate 3 $^{Dd2\ crt\ F145I}$	Yes	$pcrt^{Dd2\ F145I}$-hdhfr	$pZFN^{14/15}$-bsd	C	I	E	T	H	I	S	E	S	M	G	T	I

To generate edited parasites, one field isolate was electroporated with $pcrt^{Dd2}$-h*dhfr* donor and $pZFN^{14/15}$-*bsd* ZFN-carrying plasmids that expressed *dhfr* (h*dhfr*) and blasticidin-S deaminase (*bsd*) selectable markers as shown in Fig 5. As a transfection control, the same field isolate was electroporated with $pcrt^{Dd2\ F145I}$-h*dhfr* as a donor plasmid and $pZFN^{14/15}$-*bsd* as a ZFN plasmid. Transfection controls would be the same as

unedited field isolates, except the *pfcrt* region would be shorter than in unedited field isolates owing to the plasmid excluding certain introns and would contain the h*dhfr* as a selectable marker.

Results

Distended digestive vacuoles

Culture-adapted Cambodian field isolates were observed to have distended digestive vacuoles in the trophozoite and early schizont stages compared to the lab strain Dd2.

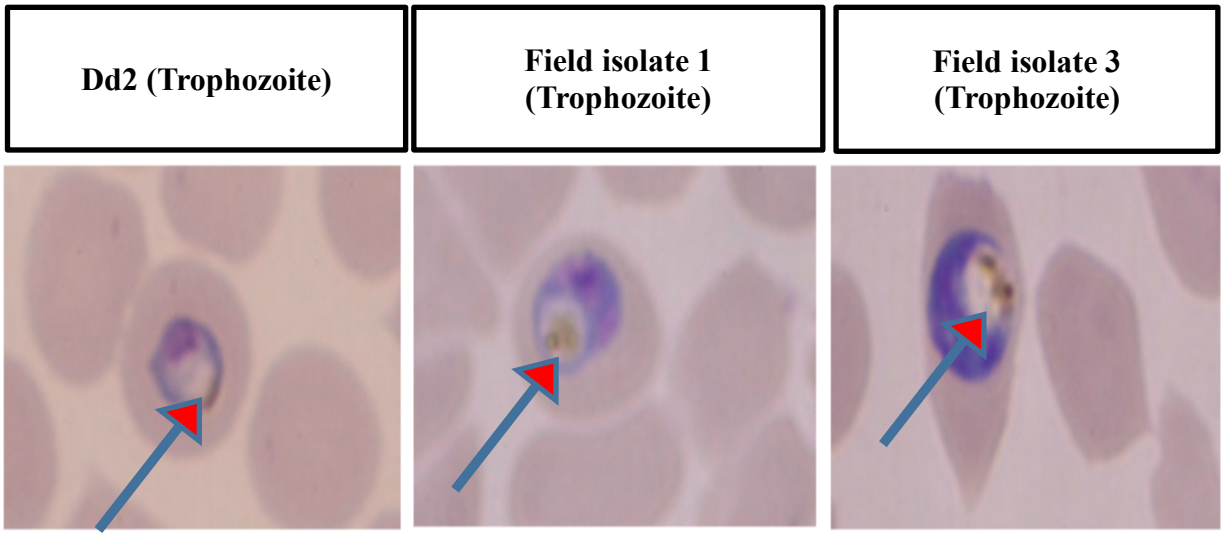


Fig 6: Morphology of parasites.

Morphology of the tropozoite stage of the lab strain Dd2 (left) and two piperaquine-resistant Cambodian field isolates (middle and right). A distinct cellular morphology was seen in the field isolates when compared with the lab strain, with Cambodian isolates having a distended digestive vacuole compared to the lab strain Dd2.

Genotypic characterization of mutations associated with piperaquine resistance

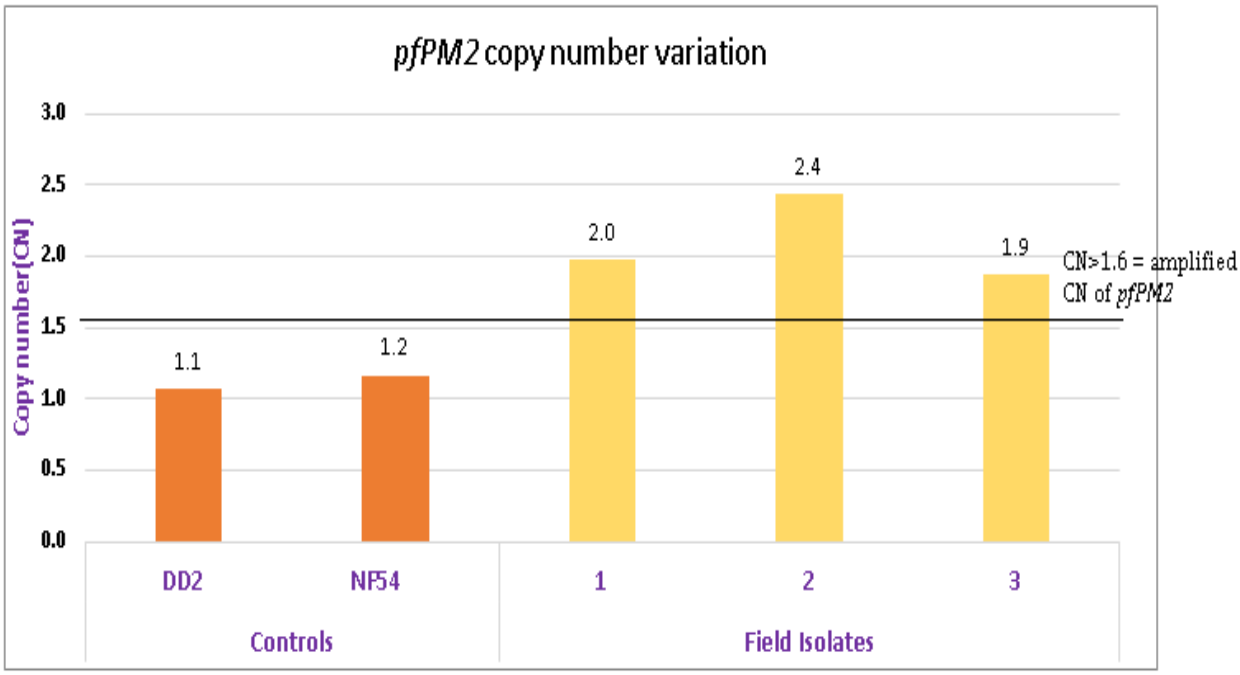


Fig 7: Copy number of *pfpm2*.

A bar graph showing the copy number variation of *pfpm2* in field isolates and lab strains Dd2 and NF54. Copy number (CN) less or equal to 1.6 is considered to be 1 and copy number greater than 1.6 is considered as 2.

Based on the SYBR green based qPCR, all the three field isolates had at least 2 copies of *pfpm2* gene, and as expected, both the lab strains Dd2 and NF54 had one copy of the *pfpm2* gene.

Pyrosequencing was used to detect the PfCRT F145I mutation. Of the three Cambodian field isolates, Field isolate 2 and 3 contained the PfCRT F145I mutations and the Field isolate 1 contained both the wild-type and mutant alleles. As expected, Dd2 did not have the PfCRT F145I mutation. Both the Field isolate 2 and 3 were selected for gene editing. The Field isolate 1 was not chosen for the gene editing since it was mixed with both wild and mutant parasites.

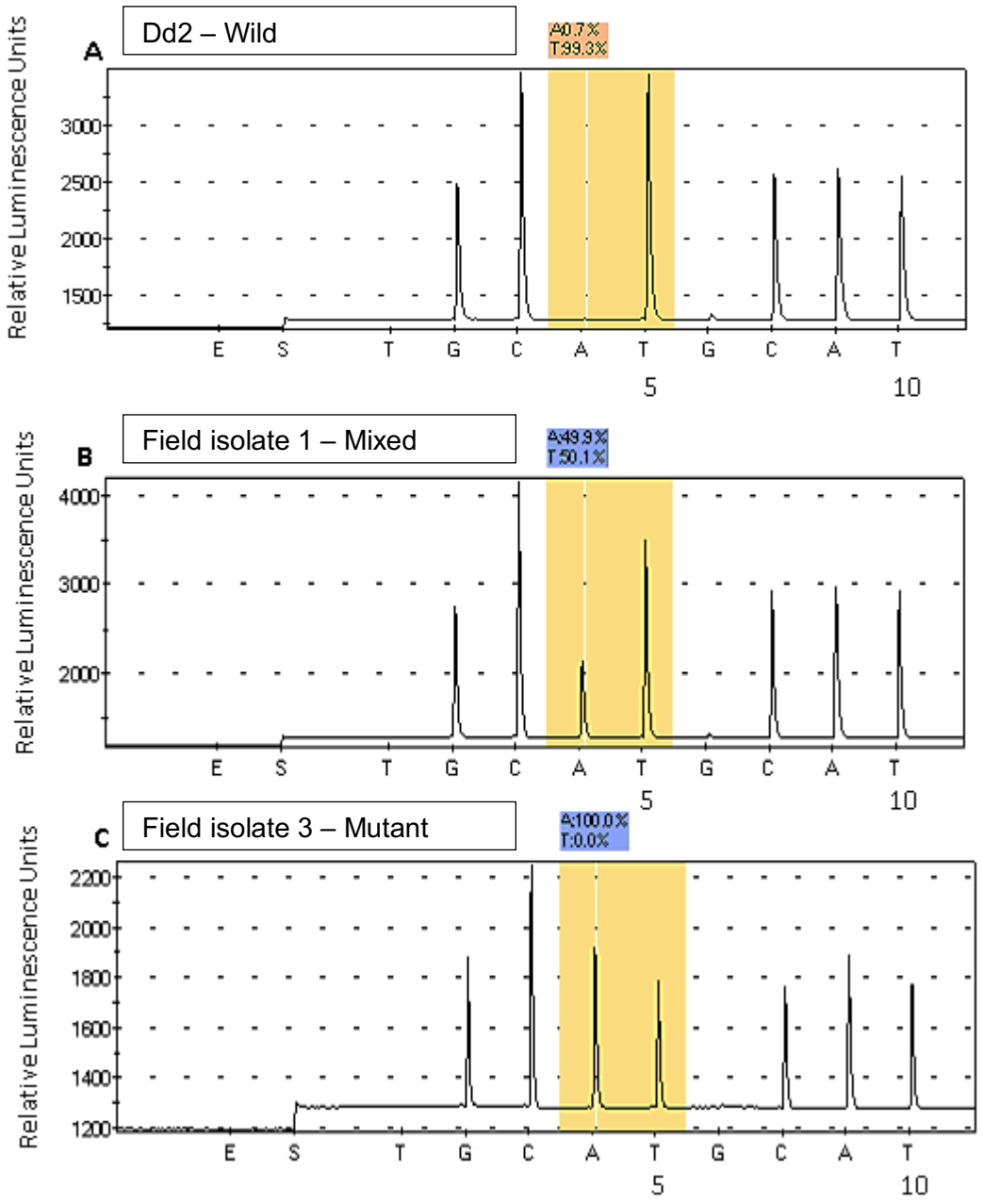


Fig 8: Pyrosequencing results.

A: Pyrogram of a piperaquine-sensitive lab strain Dd2 (positive control). The allele quantification (AQ) mode analysis shows no nucleotide change (T- 99.3% and A- 0.7%). B: Pyrogram of a piperaquine-resistant Cambodian field isolate with a mixed genotype for PfCRT F145I mutation. The allele quantification (AQ) mode analysis shows only half nucleotide change (T- 50.1% and A- 49.9%). C: Pyrogram of a piperaquine-resistant

Cambodian field isolate with a mutant genotype for PfCRT F145I. The allele quantification (AQ) mode analysis shows 100% nucleotide change (T- 100.0% and A- 0.0%)

Phenotypic characterization of Cambodian field isolates with F145I mutation and amplified *pfpm2*

Piperaquine Survival Assay (PSA)

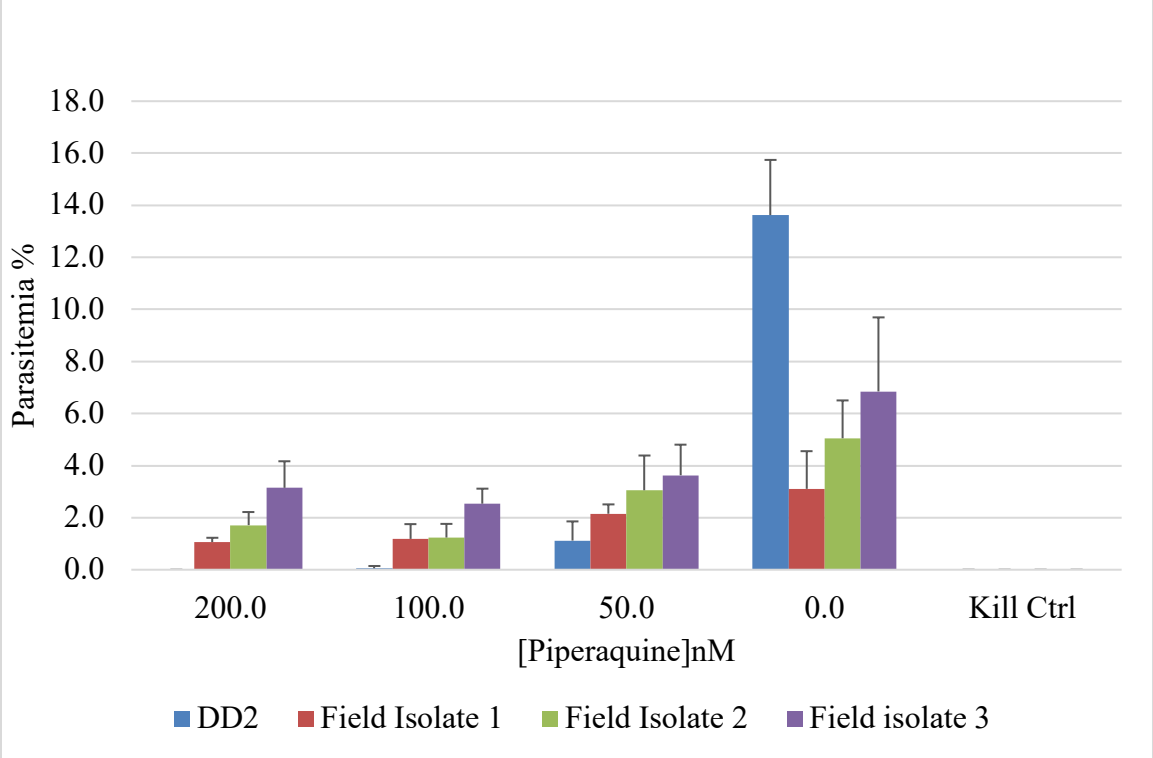


Fig 9: *In vitro* piperaquine survival assay.

In Vitro PSA to characterize the piperaquine susceptibility. The concentrations of piperaquine are in absolute concentration. The vehicle control represents the culture without any piperaquine ([0.0]nM), and the kill control represents a negative control which contains only complete medium and RBCs.

In vitro PSA was performed to characterize the phenotype of the Cambodian field isolates. Piperaquine resistance based on the PSA is defined as a parasite survival rate greater than 10%[22]. The PSA showed (Fig 9) that all three Cambodian field isolates survived in the presence of 200 nM concentration of piperaquine for 48 hours, while Dd2 was cleared completely at the same concentration of piperaquine. In addition, the survival of the field isolates is similar in the presence of different concentrations of piperaquine.

Parasites Survival rate for PSA

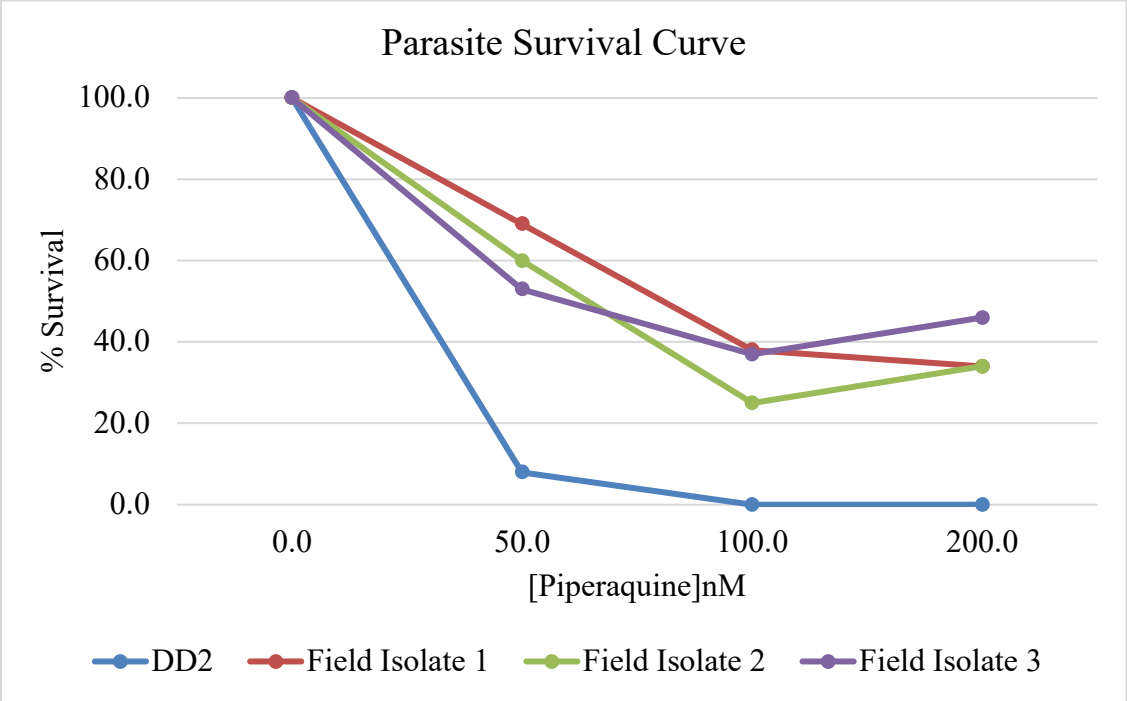


Fig 10: PSA survival curve.

PSA survival curve to determine the piperaquine resistance. The concentration of piperaquine is on the absolute concentration and the field isolate 1 has the mixed infection at the 145 loci of PfCRT region.

PSA survival rate was calculated (all three field isolates and Dd2) for each concentration of piperaquine. As shown in the Fig 10, for each concentration of the

piperaquine (i.e. [200]nM, [100]nM, and [50]nM), the survival rate of the field isolates was greater than 20% when compared with Dd2.

Validation of the successful donor plasmid transfection

All three culture-adapted field isolates had greater survival at higher concentrations of piperaquine, including the field isolate that had both mutant and wild-type alleles at the PfCRT 145 locus. Two field isolates (Field isolate 2 and Field isolate 3) that had both amplified *pfpm2* and PfCRT F145I mutation were chosen for the transfection of donor and ZFN plasmids. Two approaches for editing the field isolates were utilized. First approach, both the field isolates 2 and 3 were transfected with both the donor and ZFN plasmid simultaneously. This approach is more stressful for the parasites but if successful, often generates edited parasites in a shorter period of time. Second approach, the field isolates were first transfected with the donor plasmids, and after recovery of the donor transfected parasites, the field isolates were transfected with ZFN plasmids. This approach is less stressful for the parasites but takes a longer period of time to get the edited parasites. The first approach was not successful for either of the isolates; however, the second approach was successful for Field isolate 3 and accepted the donor plasmids (Fig 11).

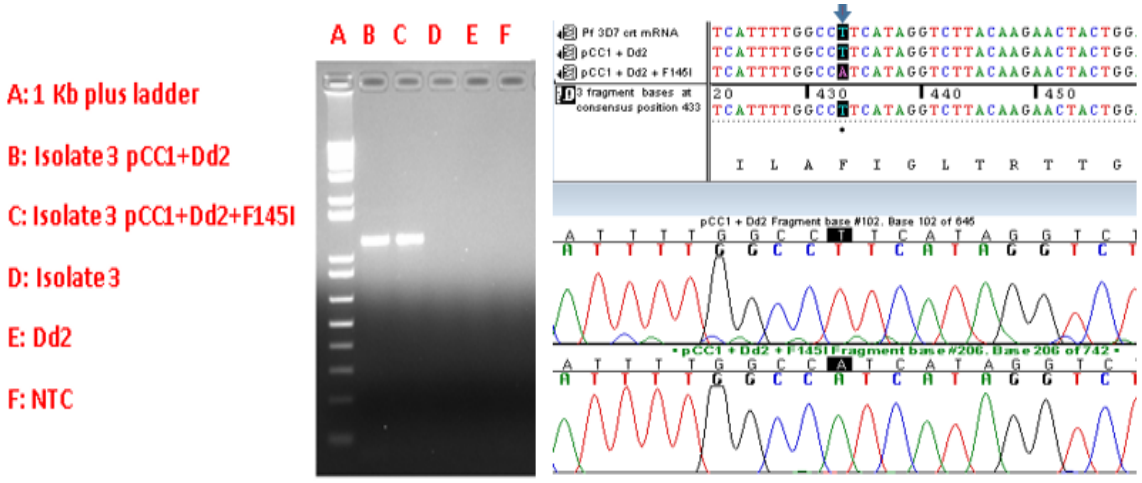


Fig 11: PCR screening

PCR based screening of the donor plasmid containing clones of Cambodian field isolate 3and the parental field isolate 3 along with Dd2 using a 2% agarose gel (left). One of the donor plasmids contains the *crt* region (pCC1+Dd2) of Dd2 and the other donor plasmid (pCC1+Dd2+F145I) contains the *crt* region of Dd2 along with the F145I allele and also known as transfection control. The transfection control helps to prove that the editing happened not by chance but with the ZFNs and donor plasmids. Chromatograms of a region of exon 2 of *crt* gene (right) obtained from Sanger sequencing of the plasmids extracted from the field isolates transfected with donor plasmids. Blue arrow shows the F145I mutation on codon 145 of PfCRT region.

Field isolated 2 and 3 were transfected on several occasions with donor plasmid and Field isolate 3 recovered the donor plasmid almost 80% of times, however, Field isolate 2 never recovered the donor plasmids. Successful transfection of donor plasmids was identified using PCR and Sanger sequencing (Fig 11 left and right). The Field isolate 3 that was transfected with donor plasmid gave the expected band (1230 bps) on a 2% agarose gel. As expected, the parental field isolate, Dd2, and the non-template control did not show a band. Sanger sequencing of the PCR product validated that the donor plasmid did not contain a mutation at codon 145 of PfCRT and the transfection control plasmid had a mutation at codon 145 of PfCRT as indicated in Fig 11 (right) by a blue arrow.

***In vitro* IC_{50} profile of the Cambodian field isolate and its clones for piperaquine**

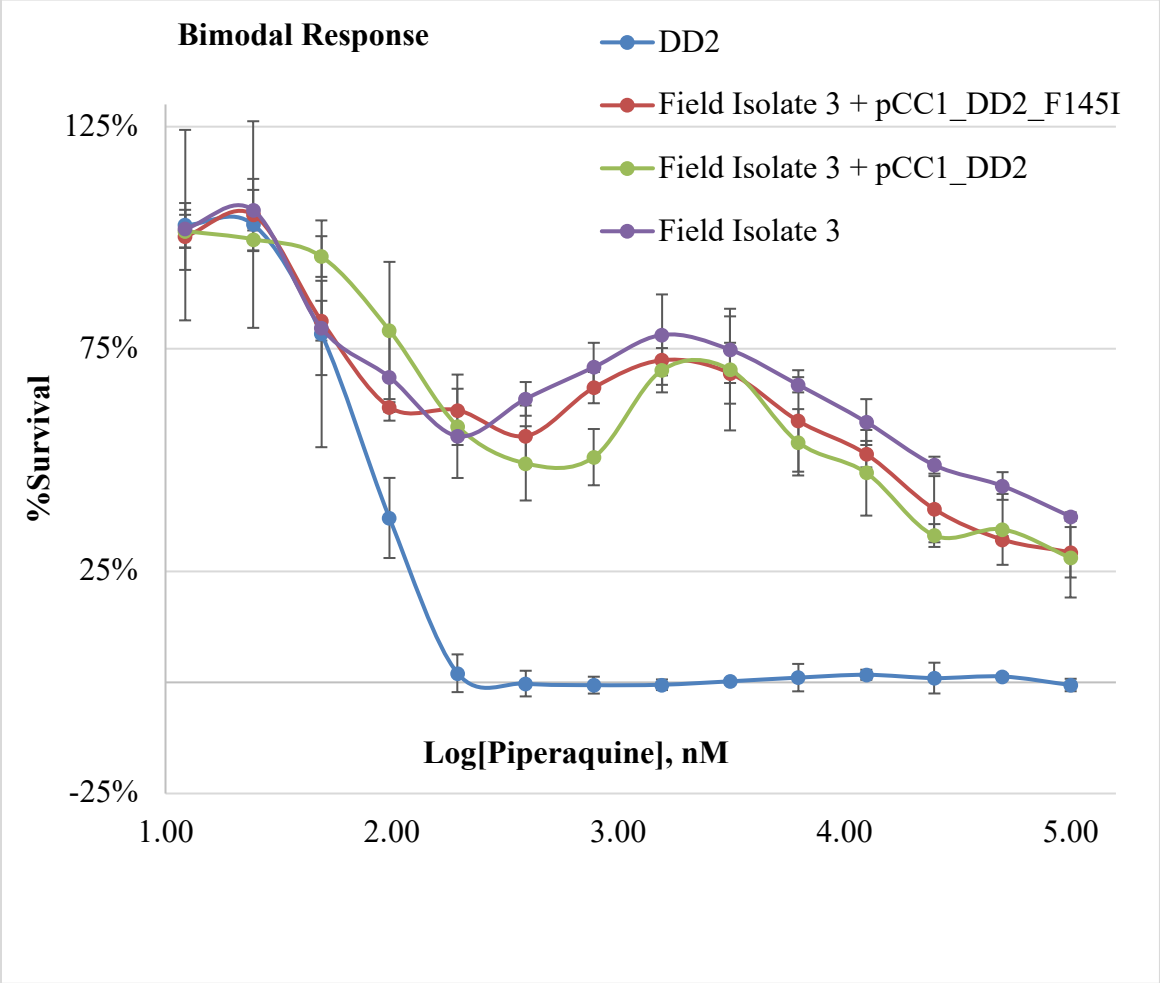


Fig 12: *In vitro* IC_{50}.

Survival curve of field isolate generated by exposing in a range of piperaquine concentration for 72 hours. The blue line represents Dd2 and rest of the lines i.e. purple, green and red represent the parental field isolate, parental field isolate with donor plasmid, and parental field isolate with donor plasmid with F145I mutation.

After the transfection of donor plasmid on the Field isolate 3, IC_{50} and IC_{90} curves were generated by exposing the Field isolate 3 along with its clones that had donor plasmids (pCC1_Dd2 and pCC1_Dd2_F145I) and Dd2 with a serial dilution of

piperaquine concentrations for 72 hours. IC_{50} and IC_{90} were generated for both the parental Field isolate 3 and its clones with donor plasmids to see if the transfected donor plasmids would result in a phenotypic change. The parasites were mostly synchronized in the ring stage prior to the assay being performed. Bimodal response curves made estimation of the IC_{50} and IC_{90} difficult. These bimodal response curves are similar to those observed in other studies; however unlike previous studies, 100% clearance of parasites was not observed at a 5-log concentration (100 uM)[37] of piperaquine. The bimodal response curves for the parental field isolates and its clones with donor plasmids were almost identical (Fig 12) indicating that the donor plasmids did not have a significant effect on the drug response.

***In vitro* LD_{50} profile of the field isolate and its clones with donor plasmids and Dd2**

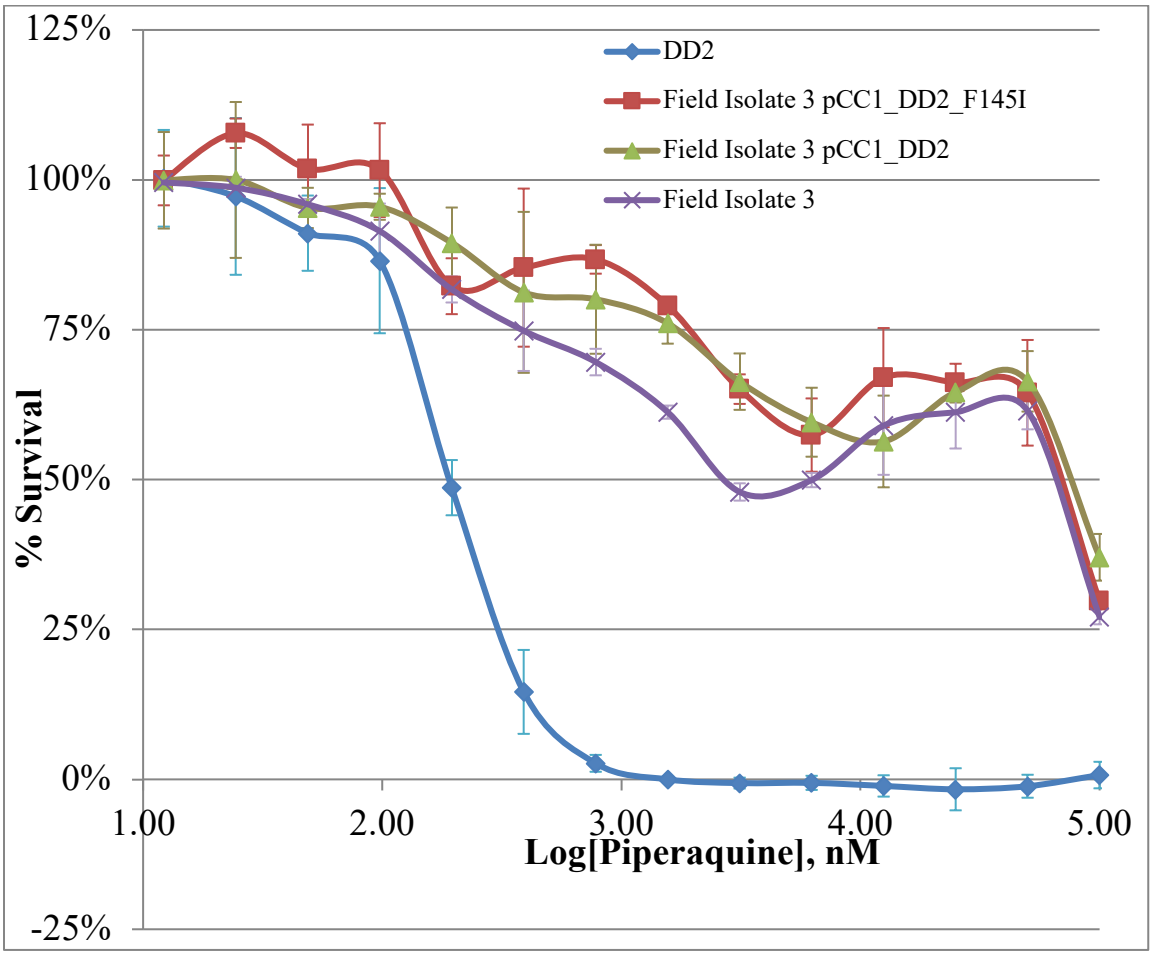


Fig 13: *In vitro* LD_{50}.

Cytocidal assay done by exposing the Field isolate 3 and its clones with donor plasmids and Dd2 in a range of piperaquine concentrations (2-fold dilution, starting from 100,000 nM) for 6 hours and in a drug free medium for another 48 hours to test the piperaquine cytocidal potency.

Piperaquine cytocidal potency was tested by performing LD_{50} where the Field isolate 3 along with its clones with donor plasmids (pCC1_Dd2 and pCC1_Dd2_F145I) and Dd2 were exposed in a range of piperaquine concentrations for 6 hours and cultured for another 48 hours in drug free complete medium. As shown in Fig 13, the Field isolate

3 along with its donor plasmid-transfected clones (pCC1_Dd2 and pCC1_Dd2_F145I) were not cleared even at a 5-log concentration (100 uM) of piperaquine.

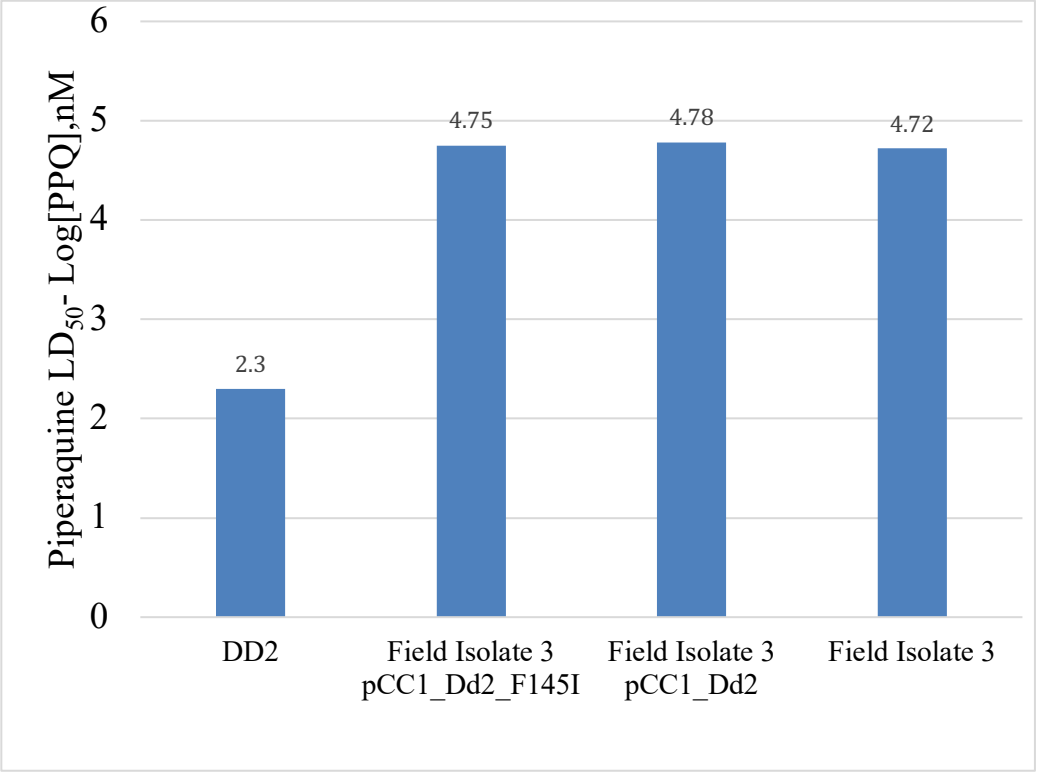


Fig 14: Piperaquine cytocidal potency.

Piperaquine cytocidal potency between Parental Field isolate 3, Field isolate 3 with pCC1_Dd2 plasmid, Field isolate 3 with pCC1_Dd2_F145I plasmid and Dd2 was determined using LD_{50} value.

LD_{50} values of Dd2, Field isolate 3 and its clones with donor plasmids (pCC1_Dd2 and pCC1_Dd2_F145I) were calculated by plotting the percentage of survival against log-transformed piperaquine concentrations using untreated Field isolate 3 as the 100% survival (Fig: 13). LD_{50} level of Field isolate 3 along with its donor transfected clones was more than two-fold greater than the control Dd2 lab strain (Fig 14). As shown in Fig 13, the Field isolate 3 along with its donor plasmid-transfected

clones (pCC1_Dd2 and pCC1_Dd2_F145I) were not cleared even at a 5-log concentration (100 uM) of piperaquine.

Drastic change in the IC_{50} for WR99210 between parental and edited field isolate

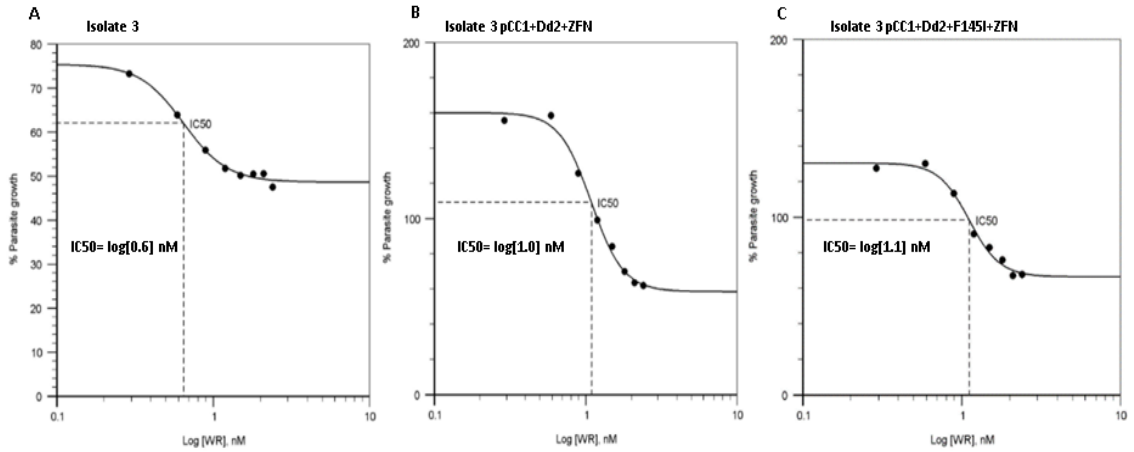


Fig 15: IC_{50} of parental and edited isolate.

Comparing the IC_{50} value for WR drug of the Cambodian field isolate 3 before and after gene editing. A. represents the IC_{50} for parental field isolate (unedited), B. represents the donor and ZFN transfected clone of the field isolate (edited) and C. represents the donor with F145I mutation and ZFN transfected clone of the field isolate (transfection control).

After successful transfection of donor plasmids in the Cambodian field isolate 3 harboring both the PfCRT F145I mutation and amplified *pfpm2*, the ZFN plasmid was transfected. The parasites recovered after ZFN transfection but when they were screened using PCR to validate successful gene editing, they did not show the expected band size, indicating that these isolates were not edited. However, a traditional IC_{50} drug assay was performed in both edited and unedited field isolates with WR99210, a drug that enriches for episomally-edited parasites, and showed a drastic difference in the IC_{50} value between the edited and unedited parasites (Fig 15), suggesting that the isolates have been edited.

Discussion

Antimalarial drug resistance poses a threat to malaria control and elimination efforts in malaria endemic regions where resistance is prevalent. Recently, Cambodia, an epicenter of antimalarial drug resistance, and its neighboring countries have been threatened by the declining efficacy of ACTs, more specifically, dihydroartemisinin-piperaquine (DHA-PPQ). DHA-PPQ was the first-line treatment for uncomplicated malaria in Cambodia, but increased treatment failures resulted in a change back to artesunate-mefloquine as the first-line treatment[22]. As DHA-PPQ is still being used in other parts of Southeast Asia, as well as other malaria endemic countries[38], it is very important to understand and monitor multi-drug resistance in the Southeast Asia.

To validate the role of PfCRT F145I in piperaquine resistance, three Cambodian field isolates containing this mutation were cultured adapted and genotypically and phenotypically characterized before and after gene editing. We confirmed that these field isolates had both amplified *pfpm2* gene copy number and the PfCRT F145I mutation and had reduced susceptibility to piperaquine. In contrast to the Dd2 strain, which showed no survival in a PSA, all three field isolates showed high survival (> 30%) (Fig 10) when exposed to piperaquine. Our prior association study has shown that parasites with both amplified *pfpm2* and PfCRT F145I had significantly greater piperaquine IC90 compared to parasites with only amplified *pfpm2* or single copy *pfpm2*[31]. A subsequent gene editing study showed that PfCRT F145I, as well as other PfCRT mutations, could confer piperaquine resistance when introduced into the Dd2 strain containing a single copy of *pfpm2*, with the F145I mutation conferring the highest level of piperaquine resistance[39] At this stage of the project, we do not have evidence of successfully edited parasites where PfCRT F145I has been replaced with the wild-type allele; however, once edited

parasites are generated we will be able to assess the effect of removal of F145I on both parasite susceptibility to piperaquine and on maintenance of amplified *pfpm2*. As seen in a previous study where PfCRT H97Y and M343L were replaced with wild-type alleles, it is possible that the Cambodian field isolate may revert to a single copy of *pfpm2* after successful replacement of F145I [39]. It has been suggested that PfCRT variants may have a fitness cost to the parasite, and amplification of *pfpm2* may play a compensatory role in reducing this fitness cost[39,40]. When the PfCRT variant is removed, amplification of *pfpm2* may no longer be as beneficial to the parasite, resulting in loss of additional copies of *pfpm2*.

All the Cambodian field isolates in this study displayed a distinct morphological trait of having a swollen and translucent digestive vacuole in the late trophozoite and schizont stages (Fig 6). Prior studies also have reported swollen digestive vacuoles in Dd2 parasites edited to contain PfCRT mutations including F145I, G353V, C101F, and L272F[35,39,41]. Although, a previous study suggested that the having multiple copies of *pfpm2* along with PfCRT variants ameliorates the enlarged digestive vacuoles, our study showed parasites with enlarged vacuoles in parasites with both mutations. It has been suggested that the enlarged digestive vacuole might be a result of accumulation of natural substances within the digestive vacuole, stemming from the PfCRT mutations interfering with the transportation of substances in and out of the digestive vacuole[41,42], and that this may result in reduced parasite fitness[39]. After successful editing to replace F145I, we will compare parasite fitness between the unedited and edited parasites.

A traditional IC_{50} drug assay performed in both edited and unedited field isolates using the drug WR99210 showed a drastic difference in the IC_{50} value between the edited and unedited parasites (Fig 15) but did not show the expected band on an agarose gel

when a PCR was performed to confirm successful editing. This observed phenomenon might be due to the integration of the donor plasmid in a non-specific location of the parasite genome (i.e. other than the PfCRT region) or perhaps a low frequency of edited parasites in comparison to the unedited parasites. We are currently exposing the edited parasites to ~ 400 nM of md-Chloroquine to enrich for edited parasites, since a recent study has shown that parasites with PfCRT F145I mutation on a Dd2 haplotype had an IC_{50} of 200 nM for md-Chloroquine[39]. All parasites disappeared in 4-5 days after exposure to 400 nM of md-Chloroquine. I am currently waiting for the edited parasites to revive.

Future Directions

Three Cambodian field isolates containing the PfCRT F145I mutation were cultured adapted and underwent phenotypic and genotypic characterization to confirm piperaquine resistance. Of the three field isolates, one was successfully transfected with the donor plasmid (pcc1+Dd2) and transfection control (pcc1+Dd2+F145I). The field isolate that was successfully transfected with donor plasmids has undergone ZFN plasmid transfection, but to date we do not have evidence of successful editing. If the isolate is not edited, I will electroporate the Field isolate 3 that has donor plasmids (pcc1+Dd2 and pcc1+Dd2+F145I) with the ZFN plasmid again. After successful gene editing of the isolates, I will characterize both phenotype and genotype of the edited parasites and compare with the unedited parental line, transfection control, and Dd2. I will screen for *pfpm*2 amplification and PfCRT F145I throughout passage of the field isolates (both edited and unedited) to validate if long term culturing of field isolates brings any changes.

I will also perform whole genome sequencing of both the edited parasites and unedited parasites that underwent ZFN transfection for comparison with the whole genome sequence of the parental isolate, this will allow identification of other changes in the parasite genome that may have occurred off target.

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