Dissection of haplotype-specific drug response phenotypes in multiclonal malaria isolates

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ABSTRACT

Natural infections of *Plasmodium falciparum*, the parasite responsible for the deadliest form of human malaria, often comprise multiple parasite lineages (haplotypes). Multiclonal parasite isolates may exhibit variable phenotypes including different drug susceptibility profiles over time due to the presence of multiple haplotypes. To test this hypothesis, three *P. falciparum* Cambodian isolates IPC_3445 (MRA-1236), IPC_5202 (MRA-1240) and IPC_6403 (MRA-1285) suspected to be multiclonal were cloned by limiting dilution, and the resulting clones genotyped at 24 highly polymorphic single nucleotide polymorphisms (SNPs). Isolates harbored up to three constituent haplotypes, and exhibited significant variability ($p < 0.05$) in susceptibility to chloroquine, mefloquine, artemisinin and piperaquine as measured by half maximal drug inhibitory concentration (IC_{50}) assays and parasite survival assays, which measure viability following exposure to pharmacologically relevant concentrations of antimalarial drugs. The IC_{50} of the most abundant haplotype frequently reflected that of the uncloned parental isolate, suggesting that a single haplotype dominates the antimalarial susceptibility profile and masks the effect of minor frequency haplotypes. These results indicate that phenotypic variability in parasite isolates is often due to the presence of multiple haplotypes. Depending on intended end-use, clinical isolates should be cloned to yield single parasite lineages with well-defined phenotypes and genotypes. The availability of such standardized clonal parasite lineages through NIAID's BEI Resources program will aid research directed towards the development of diagnostics and interventions including drugs against malaria.

1. Introduction

Despite remarkable progress in reducing malaria burden over the past decade, malaria remains a major global health problem with an estimated 229 million cases and 409,000 malaria-related deaths recorded in 2019 alone (WHO, 2020). The emergence and spread of drug-resistant parasites continues to undermine malaria control and elimination efforts. Until recently, malaria parasites had developed resistance to all antimalarial drugs in clinical use with the exception of artemisinins. However, artemisinin-resistant parasites have now emerged and spread in South East Asia (Dondorp et al., 2009; Phyo et al., 2012). This raises the chilling prospect of parasite resistance spread to frontline Artemisinin-based Combination Therapies (ACTs), reminiscent of the intercontinental spread of chloroquine and sulphadoxine-pyrimethamine resistance from founder foci in SE Asia to sub-Saharan Africa (Anderson and Roper, 2005; Roper et al., 2004). This could lead to significant increases in both the number of malaria cases and malaria-related deaths as was observed with chloroquine resistance (Trape, 2001). Resistance containment efforts are currently underway to limit the spread of artemisinin-resistant parasites beyond SE Asia (Dondorp et al., 2010; WHO, 2011) and to lengthen the therapeutic lifespan of ACTs. However, the local emergence and clonal expansion of an artemisinin-resistant *Pfkelch13* R561H mutation in Rwanda, Africa (Uwimana et al., 2020) potentially undermines the premise of artemisinin resistance containment efforts. A rethink and redesign of the global artemisinin resistance containment strategy (WHO, 2011) will be critical to preserve the effectiveness of ACTs and avert a potential global malaria emergency (Woodrow and White, 2017).

In vitro antimalarial susceptibility testing plays a critical role in screening candidate antimalarials for potency (Weisman et al., 2006) as

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well as in drug resistance monitoring (Mbaisi et al., 2004; Plowe, 2003) and can help guide the rational deployment of drugs to counteract resistance. Susceptibility testing is routinely used to measure the efficacy of drugs in pre-clinical development, to help define the mechanisms of drug resistance (Ariey et al., 2014; Witkowski et al., 2013) and to measure changes in baseline antimalarial susceptibility over time and place to provide an early warning for emerging drug resistance or predict reversion of parasite susceptibility to drugs previously rendered clinically useless by resistance (Kublin et al., 2003). Although *in vivo* drug efficacy studies remain the gold standard for assessing antimalarial susceptibility, *in vitro* tests have the advantage of measuring intrinsic parasite drug sensitivity regardless of the patient's immune status, as well as pharmacokinetic and pharmacodynamic variations (WHO, 2001). In addition, results from *in vitro* drug susceptibility tests are unaffected by recurrent malaria transmission.

A variety of assays are used to measure *in vitro* antimalarial susceptibility. Classical assays include the ^{3}H-hypoxanthine Uptake Assay, which measures inhibition of parasite uptake of a radiolabeled growth precursor (Desjardins et al., 1979); the Lactate Dehydrogenase (LDH) Assay, which measures inhibition of parasite-specific LDH enzymatic activity by the antimalarial compound (Makler et al., 1993); the WHO Micro-test, which measures parasitemia and parasite maturation to schizogony upon exposure to an antimalarial compound (WHO, 2001); the double-site enzyme-linked immunodetection (DELI) assay, which detects antibodies against parasite-specific antigens (Noedl et al., 2005) and fluorescence-based assays including those using DNA-intercalating and fluorescing dyes such as PicoGreen® (Corbett et al., 2004) and SYBR® Green I (Hartwig et al., 2013; Johnson et al., 2007) to measure inhibition of parasite DNA replication. Recently, parasite viability assays designed to better mimic the stage-specific *in vivo* pharmacological activity of certain antimalarial compounds have been developed. These include the Ring-stage Survival Assay (RSA) and the Piperaquine Survival Assay (PSA), which measure parasite susceptibility to artemisinin (Witkowski et al., 2013) and piperaquine (Duru et al., 2015), respectively, by determining the proportion of viable parasites following exposure to these compounds. Regardless of assay read-out, all *in vitro* antimalarial susceptibility assays involve incubating malaria parasites at a defined parasitemia and hematocrit to known concentrations of an antimalarial test compound to determine growth inhibition. Because natural malaria isolates often comprise multiple parasite lineages (Fola et al., 2017; Nkhoma et al., 2012) which may vary in drug susceptibility and/or growth rates, *in vitro* drug sensitivity testing of such isolates may yield different susceptibility patterns on independent occasions. The *in vitro* susceptibility profile of a multiclonal isolate may vary markedly depending on the abundance of each co-infecting parasite lineage (haplotype) at the time the assay is performed. Analysis of *in vitro* antimalarial susceptibility profiles of artificial parasite mixtures comprising different proportions of drug-sensitive and drug-resistant laboratory lines renders some support for this notion (Liu et al., 2008). The *in vitro* susceptibility profile of each parasite mixture was shown to vary according to the relative abundance (proportion) of each parasite line within the mixture (Liu et al., 2008). It is very likely that the same phenomenon occurs in naturally occurring mixtures of malaria parasite lineages.

To investigate whether significant variation in independent estimates of *in vitro* antimalarial susceptibility for a carefully selected set of malaria isolates is due to the presence of multiple parasite lineages with differing levels of drug susceptibility, we studied three natural isolates from Cambodia. We examined whether these isolates contain multiple parasite haplotypes, identified their component parasite haplotypes and measured haplotype-specific drug response phenotypes. The data generated provide insights into how co-infecting parasite haplotypes contribute to the drug susceptibility profile of a parasite isolate. This data is provided through BEI Resources as part of the characterization and authentication for each of these isolates.

2. Materials and methods

2.1. Selection and in vitro culture of Plasmodium falciparum isolates

Three *Plasmodium falciparum* isolates IPC_3445 (MRA-1236), IPC_5202 (MRA-1240) and IPC_6403 (MRA-1285) (Supplementary Material, Table S1) were selected for this study because they were suspected to contain multiple parasite lineages. Two additional isolates, IPC_6261 (MRA-1284) and IPC_6403 (MRA-1286), were included in the study as controls for measuring *in vitro* piperaquine susceptibility. The five clinical isolates were sampled from Cambodia between 2010 and 2013, and have variable susceptibility to artemisinin and piperaquine. Isolates were obtained from BEI Resources (https://www.beiresources.org) as deposited by Dr. Didier Ménard of the Institut Pasteur du Cambodge.

Isolates were culture-adapted in O+ human erythrocytes using RPMI 1640 media (Gibco; Cat # 21870-084) supplemented with 4 μg/mL Gentamicin (Gibco; Cat # 15750-060), 0.21% Sodium Bicarbonate (Gibco; Cat # 21870-084), 22 mM HEPES buffer (Gibco; Cat # 15630-080), 0.18 mM Hypoxanthine (Sigma; Cat #H9636), 0.18% Glucose (Sigma; Cat #G7021), 1.77 mM L-Glutamine (Gibco; Cat # 25030-149) and 8% human serum under standard conditions of *in vitro P. falciparum* culture.

2.2. Confirmation of the multiclonal nature of isolates

To determine whether the three isolates IPC_3445 (MRA-1236), IPC_5202 (MRA-1240) and IPC_6403 (MRA-1285) contain multiple parasite haplotypes, parasite DNA was extracted from each isolate using DNA Blood Mini Kits (Qiagen, USA) and genotyped at 24 highly polymorphic SNPs as described previously (Daniels et al., 2008). Briefly, 20 μL of a master mix comprising 12.5 μL of the TaqMan Universal PCR Master Mix (Applied Biosystems; Cat # 4324018), 6.875 μL of nuclease-free water and 0.625 μL of the 40× pre-formulated Taqman SNP assay (Applied Biosystems; Cat # 4332077) per reaction was added to each well of a 96-well real-time PCR plate pre-loaded with 5 μL (50 ng) of each parasite DNA sample. Parasite DNA samples from *P. falciparum* laboratory lines 3D7 (MRA-102), DD2 (MRA-150), NF54 (MRA-1000), HB3 (MRA-155) and V1S (MRA-176) were genotyped alongside the clinical isolates as positive controls. Samples were amplified using the CFX96 real-time PCR instrument (Bio-Rad, USA), and results analyzed using the Bio-Rad's Allelic Discrimination Software. Because blood-stage malaria parasites are haploid, clonal isolates are expected to harbor only one allele at each locus. However, for purposes of this project, isolates were deemed multiclonal if they had multiple alleles at ≥2 loci. We used such a stringent threshold because on this genotyping platform, one random SNP occasionally yields a heterozygous base call even in well-characterized laboratory clonal lines such as 3D7 and DD2 (Nkhoma et al., 2018).

2.3. Isolation of single parasite lineages from confirmed multiclonal isolates

Because direct genotyping or sequencing cannot accurately identify parasite haplotypes present in a multiclonal malaria isolate, singly-infected erythrocytes were isolated from each isolate by limiting dilution (Nkhoma et al., 2012; Rosario, 1981) and genotyped at 24 polymorphic SNPs to resolve constituent parasite haplotypes. To maximize the probability of isolating erythrocytes infected by exactly one parasite, parasite-infected erythrocytes were seeded in the 96-well plate at a dilution approximating 0.25 parasites per well. Briefly, 100 μL of a parasite culture containing ~2.5 parasitized erythrocytes per mL of RPMI 1640 media and 100 μL of uninfected red blood cells at 2% hematocrit were inoculated into each well of a sterile 96-well plate. The plate was incubated at 37 °C in a sealed chamber supplied with a gas mixture containing 90% N_2, 5% O_2 and 5% CO_2. The media in the

cloning plate was changed once every three days during the first 12 days of cloning and once every two days for the next 9 days. On day 21, parasite-positive wells were identified by detecting parasite DNA using a fluorescent DNA-labelling dye, SYBR® Green I (Molecular Probes, USA). To achieve this, 100 μL of cells resuspended from each well of the cloning plate were transferred to a new 96-well detection plate followed by the addition of 100 μL of a Lysis Buffer/SYBR® Green I solution (0.2 μL of 10,000× SYBR® Green 1 solution with 1000 μL of Lysis buffer containing 20 mM Tris at a pH of 7.5, 5 mM EDTA, 0.008% w/v Saponin and 0.08% v/v Triton X-100). The detection plate was incubated for 45 min in the dark at room temperature and fluorescence was measured using a SpectraMax microplate reader (Molecular Devices, USA) at excitation and emission wavelengths of 490 and 540 nm, respectively. Wells containing parasitized cells were identified as those with elevated fluorescence intensity relative to the background and were confirmed by microscopic examination of thin smears prepared from resuspended cells. Parasites from each positive well were expanded in 75 cm^2 culture flasks to produce enough material for genomic DNA extraction, *in vitro* antimalarial susceptibility testing and for cryopreservation.

Throughout this manuscript, we refer to parasites obtained from individual wells of a 96-well plate through limiting dilution cloning of each parasite isolate as "parasite clones". In contrast, the term "parasite haplotype" or "clonal parasite lineage" is used to denote parasites that are proven (through genotyping of 24 highly-polymorphic SNPs) to derive from a single progenitor cell and carrying a set of alleles inherited together as a unit.

2.4. Genotyping of parasite clones and identification of single parasite lineages including their relationships

To identify parasite haplotypes in each isolate, DNA from each parasite clone was genotyped at 24 highly-polymorphic single nucleotide polymorphisms (SNPs) as described previously (Daniels et al., 2008). SNP data for parasite clones deemed clonal (with less than two heterozygous loci) were used to construct a phylogenetic tree to identify parasite haplotypes present in each isolate and their relationship to one another. The tree was generated using the Phylogeny Inference Package (PHYLIP) (http://evolution.genetics.washington.edu/phylip.html) based on the genetic distance metric 1-ps, where *ps* is the proportion of alleles shared between two parasite clones. Parasite clones were considered genetically distinct if they were divergent at ≥2 loci. Where they differed at just one SNP, they were deemed identical and collapsed into a single parasite haplotype.

To determine relatedness between parasite haplotypes, we simulated allele-sharing (*ps*) expected of unrelated parasites, half-siblings (sibs), full-sibs and parasites derived from mating between full-sib parasites as described previously (Anderson et al., 2010; Nkhoma et al., 2012). Simulations were conducted using population-level allele frequencies for each of the 24 SNPs estimated from whole genome sequences of Cambodian parasites (https://plasmodb.org/plasmo/). To assign the order of relatedness to any pair of parasite haplotypes, we fitted their observed *ps* into the simulated *ps* distribution expected of parasites in different relatedness categories. Due to some overlap in *ps* distributions, we used the upper confidence limit for the *ps* distribution of unrelated parasites to distinguish between related and unrelated parasites. Similarly, the upper confidence limit for the full-sib *ps* distribution was used as a cut-off for distinguishing full-sib parasites from parasites that are more related than full-sib parasites.

To examine the resolution power of the SNP assay for Cambodian parasites, we used a loci-resampling approach as implemented in Gen-Clone version 2.0 (Arnaud-Haond and Belkhir, 2007), and determined the maximal parasite diversity observed when different numbers of randomly selected SNPs are genotyped.

2.5. Standard in vitro antimalarial susceptibility testing

In vitro antimalarial susceptibility of three multiclonal isolates and their component parasite haplotypes were measured against chloroquine (CQ), mefloquine (MFQ) and piperaquine (PPQ) using a standardized SYBR® Green Antimalarial Assay (Hartwig et al., 2013). CQ and MFQ were both purchased from Sigma Aldrich while PPQ was obtained from the Worldwide Antimalarial Resistance Network (https://www.wwarn.org/). Drugs were prepared as 10 mM stock solutions in distilled water (CQ) or 70% ethanol (MFQ and PPQ) and were filter-sterilized. Drug assays were run in duplicate at 0.5% parasitemia and 1.5% hematocrit in 96-well plates and were incubated at 37 °C for 72 h. Each drug assay run included at least one of the three clonal *P. falciparum* laboratory lines 3D7 (MRA-102), DD2 (MRA-150) or K1 (MRA-159) as a control. Following the incubation step, parasites were lysed by freeze-thaw, transferred to a new 96-well detection plate and then labeled with a DNA-fluorescent dye SYBR® Green I (Molecular Probes, USA). The detection plate was incubated in the dark for 45 min and fluorescence was measured at excitation and emission wavelengths of 490 and 540 nm respectively using the SpectraMax fluorescence microplate reader (Molecular Devices, USA). Dose-response curves and IC$_{50}$ values were determined from log-transformed fluorescence counts at each serial dilution of the antimalarial drug using GraphPad Prism v7.0 (GraphPad, USA). An unpaired *t*-test was used to assess whether any two IC$_{50}$ values are significantly different. Mean IC$_{50}$s for ≥3 independent assays were compared using one-way analysis of variance (One-way ANOVA). Both statistical tests were implemented in GraphPad Prism v7.0 with the level of significance set at p < 0.05.

2.6. In vitro antimalarial susceptibility testing using parasite survival assays

Because standard IC$_{50}$ assays fail to adequately capture *in vitro* parasite susceptibility to artemisinins (Dondorp et al., 2009) and PPQ (Duru et al., 2015), ring-stage survival assays (RSAs) and PSAs were performed to determine parasite response to dihydroartemisinin (DHA) and PPQ respectively (Witkowski et al., 2013; Duru et al., 2015). Highly synchronized ring-stage parasites were exposed to a high dose of the antimalarial drug (700 nM DHA for 6 h and 200 nM PPQ for 48 h) in 48-well plates under standard *in vitro* culture conditions. Drug was removed, and parasites allowed to recover in normal growth media for another 66 h in the case of DHA-exposed parasites and 24 h for the PPQ-exposed parasites. Parasite viability, expressed as percent parasite survival, was determined by microscopic examination and counting of viable parasites in the drug-treated wells versus the untreated to which no drug was added. Parasites were considered resistant to DHA or PPQ if they showed a survival rate of ≥10%.

2.7. Sequencing and genotyping of antimalarial resistance loci

To resolve whether significant variability in antimalarial drug response between any two parasite samples is due to variation in the spectrum of resistance-conferring mutations they harbor, we performed genotyping and amplicon sequencing of genes known to confer antimalarial resistance. We examined the presence of both single nucleotide polymorphisms (SNPs) and copy number mutations (gene amplifications) known to mediate parasite resistance to chloroquine, mefloquine, piperaquine and artemisinins.

2.7.1. Detection of SNPs conferring resistance to CQ, artemisinins and MFQ

CQ resistance-mediating point mutations in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) (Fidock et al., 2000) were detected by Sanger sequencing. Briefly, genomic DNA from malaria isolates and parasite haplotypes cloned from three of the isolates was amplified using five primer sets spanning the entire *pfcrt* gene as described previously (Chen et al., 2003). PCR amplicons were purified using a PCR

purification kit (Qiagen; Cat # 28104) and sequenced on the ABI 3500 capillary sequencer (Illumina, USA). Similarly, point mutations in the *P. falciparum* multi-drug resistance gene (*pfmdr-1*), which mediate resistance to a variety of aminoquinoline and aryl-alcohol drugs including MFQ and CQ (Duraisingh et al., 2000a, 2000b; Sidhu et al., 2005; Veiga et al., 2016), were detected by Sanger sequencing of the ~4.2 kb *pfmdr-1* amplicon (Supplementary Material, Table S2). SNPs in the *K13* gene, which are known to mediate parasite resistance to artemisinins (Anderson et al., 2016; Ariey et al., 2014), were detected by Sanger sequencing as described previously (Ariey et al., 2014). Sequence data were analyzed and visualized using DNASTAR Lasergene (http s://www.dnastar.com).

2.7.2. Detection of copy number mutations associated with MFQ and PPQ resistance

We used the $\Delta\Delta C_T$ method (Price et al., 2004) to determine copy number (CN) of *pfmdr-1* and *plasmepsin II* (*pfmp-2*) genes implicated in MFQ and PPQ resistance respectively (Price et al., 2004; Witkowski et al., 2017). To measure *pfmdr*-1 CN, we amplified both *pfmdr-1* (target) and β-*tubulin* (single copy gene; endogenous control) in the same wells of a 96-well PCR plate (Bio-Rad; Cat # MLL9601) by real-time PCR using gene-specific primers and distinct fluorescently-labeled probes for each gene (Supplementary Material, Table S2). Similarly, *pfmp2* CN was determined using a multiplex real-time PCR assay targeting both the *pfmp-2* gene (target) and *serine tRNA-ligase* gene (single copy gene). Each sample was analyzed in triplicate with the input DNA normalized to 75 ng. Briefly, we prepared a master mix comprising 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems; Cat # 4304437), 6.250 μL of nuclease-free water, 0.625 μL of 40× pre-formulated *pfmdr-1* or *pfmp-2* gene expression assay (Applied Biosystems; Cat # 4304437) and 0.625 μL of the 40× pre-formulated β-*tubulin* or *serine tRNA-ligase* gene expression assay (Applied Biosystems; Cat # 4304437) for each reaction. The master mix (20 μL) was aliquoted into each well of a 96-well PCR plate pre-loaded with 5 μL (75 ng) of each parasite DNA sample. Samples were amplified using the CFX96 real-time PCR instrument, and results analyzed using the gene expression module. *Pfmdr*-1 CN was normalized to the single copy β-*tubulin* gene and a calibrator sample, 3D7 (MRA-102), which carries single copies of both *pfmdr-1* and β-*tubulin*. In parallel with the samples, we also analyzed parasite DNA from a clonal laboratory line DD2 (MRA-150), which carries multiple *pfmdr-1* copies, as a control. Similarly, alongside test samples, we amplified parasite DNA from the isolate IPC_6261 (MRA-1284), which carries multiple *pfmp-2* copies, as a control for the *pfmp-2* CN assay. In the final determination of CN, all CN estimates were rounded to the nearest integer.

3. Results

3.1. Independent tests for suspected multiclonal isolates yield different antimalarial susceptibility profiles

We observed significant variability in absolute CQ susceptibility of suspected multiclonal isolates when each isolate was assayed on independent days (Fig. 1A). Clonal laboratory parasite lines such as K1 (MRA-159) showed no such variation (Fig. 1B). SNP genotyping confirmed that multiple parasite lineages are present in isolates suspected to be multiclonal (Supplementary Material, Figure S1 and Table S3).

3.2. Limiting dilution cloning of multiclonal isolates and SNP genotyping of parasite clones

Limiting dilution of isolates IPC_3445 (MRA-1236), IPC_5202 (MRA-1240) and IPC_6403 (MRA-1285) yielded 23, 28 and 51 parasite clones respectively against a total of 96 wells seeded for IPC_3445 and IPC_5202 and 192 wells (two 96-well plates) for IPC_6403. Assuming a Poisson distribution of parasites in the 96-well cloning plate (Nkhoma
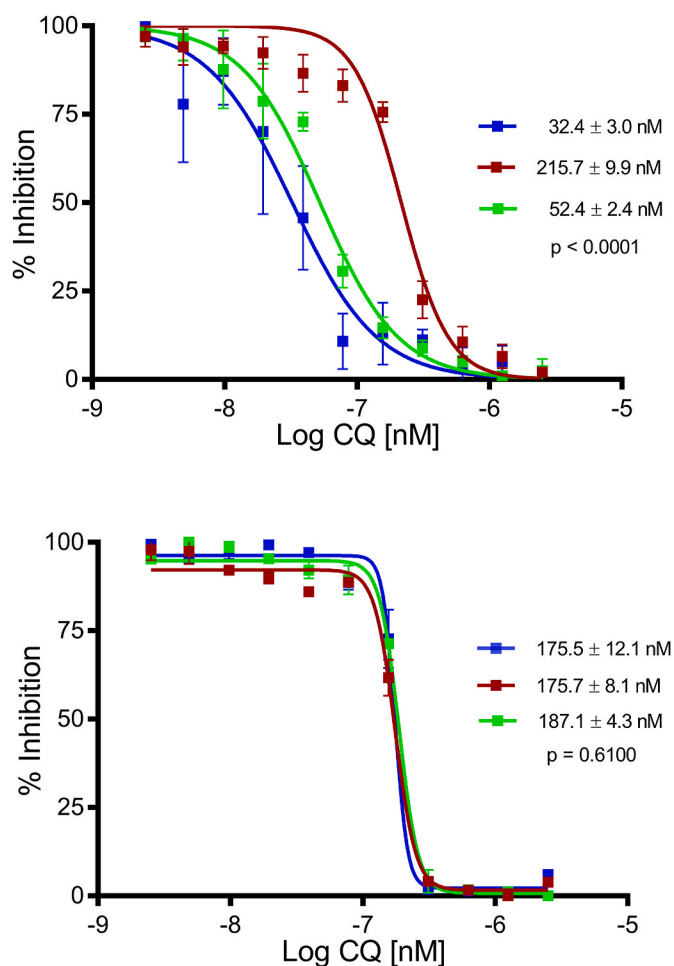

Fig. 1. Independent drug susceptibility assays for suspected multiclonal isolates yield significantly different profiles. Chloroquine (CQ) dose-response curves for *P. falciparum* IPC_5202 (MRA-1240), a Cambodian isolate suspected to harbor multiple parasite lineages (*Panel A*) and those of *P. falciparum* K1 (MRA-159), a clonal laboratory line (*Panel B*). Colored curves on each plot show results from three independent CQ IC_{50} assays. While IC_{50} values for the isolate IPC_5202 vary significantly between independent tests (One-way ANOVA; p < 0.0001), those for K1 do not. We hypothesized that the observed variability in CQ susceptibility between independent tests for IPC_5202 results from assaying multiple parasite lineages (haplotypes) differing in drug susceptibility.

et al., 2018), 22 parasite-positive wells are expected per plate of which 19 are seeded with exactly one parasitized red blood cell. Genotyping using the 24-SNP Molecular Barcode Assay (Fig. 2) showed that 18 of 23, 25 of 28 and 39 of 51 parasite clones isolated from IPC_3445, IPC_5202 and IPC_6403 each had one allele per locus (Supplementary Material, Table S3) indicative of a single parasite lineage. Five, three and 12 parasite clones from IPC_3445, IPC_5202 and IPC_6403 respectively, had multiple alleles at ≥2 loci (Supplementary Material, Table S3). As these clones were deemed to consist of mixed haplotypes (non-clonal), they were excluded from further genetic and phenotypic analyses in the study. In addition, we excluded data for the A/T SNP on chromosome 10 and position 00008237618 of the *P. falciparum* genome because it performed poorly on this set of Cambodian isolates with high levels of missing data (>60%) even after multiple genotyping attempts (Supplementary Material, Table S3). By resampling different numbers of SNPs and determining how much of the maximal parasite diversity is captured, we demonstrate that the SNP assay used has adequate resolution power for discriminating Cambodian parasites (Supplementary Material, Figure S2).
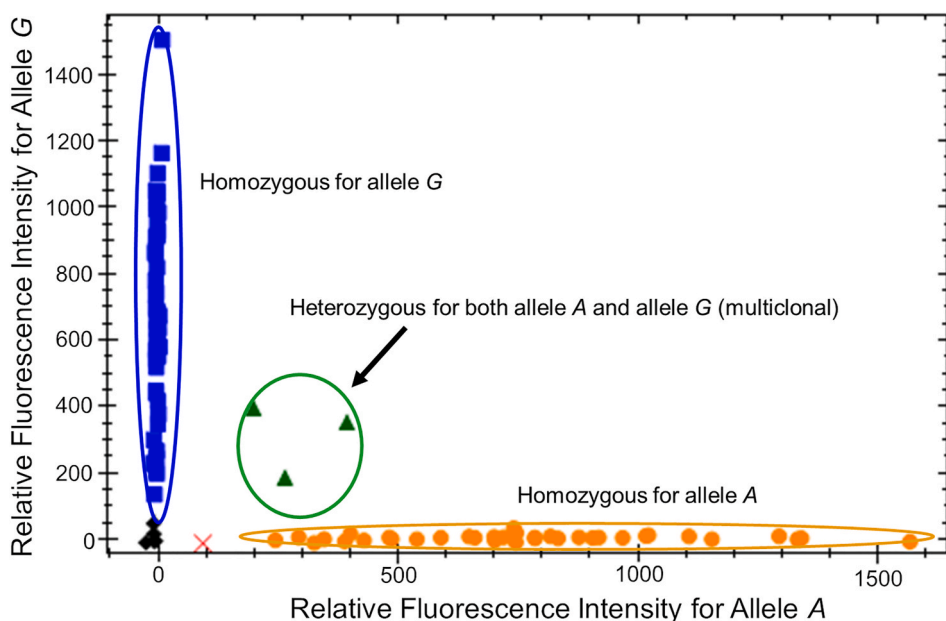
Fig. 2. SNP genotyping of parasite clones isolated by limiting dilution. Shown here is an example Allelic Discrimination Plot with genotyping results for the A/G SNP located on chromosome 11 and position 000117114 of the *P. falciparum* genome. Each SNP site is interrogated using a set of allele-specific primers that span the SNP locus and two allele-specific probes each labeled with a distinct fluorescent dye (FAM or VIC). Each dot on the plot represents a genotype call for a single sample. Parasite clones in the orange cluster along the horizontal axis are homozygous for allele *A* while those shown in the blue cluster along the vertical axis are homozygous for allele *G*. The green cluster in the middle of the plot contains parasite clones that are still heterozygous (multiclonal) following limiting dilution. The letter "X" within the plot represents a genotyping failure while black dots are no template controls. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Extensively studied multiclonal isolates harbor up to three parasite haplotypes

Phylogenetic analysis of SNP data for parasite clones considered clonal revealed that IPC_3445 (MRA-1236), IPC_5202 (MRA-1240) and IPC_6403 (MRA-1285) each contained two to three parasite haplotypes (Fig. 3A). Parasite haplotypes from the same isolates were predominantly related (Fig. 3B), sharing 90.7% of alleles (range: 87.0–91.3%) within isolates compared with 57.3% (range: 30.4–73.9%) between isolates. The difference in relatedness between parasite haplotypes cloned from the same and different isolates cannot be explained by chance (Permutation analysis; p < 0.00001). By simulating allele-sharing expected of parasites that are related to different degrees (Fig. 3C), we demonstrate that all parasite haplotypes cloned from the three different isolates exhibit a level of allele-sharing expected of unrelated parasites (Supplementary Material, Table S4). In contrast, only 14.3% of parasite haplotypes from the same isolates are unrelated while 85.7% are related at the half-sib level or greater (Supplementary Material, Table S4).

3.4. Parasite isolates and their component haplotypes show a range of antimalarial susceptibility profiles

We observed significant variation in parasite susceptibility to CQ, MFQ and PPQ (Table 1). Isolates IPC_3445 (MRA-1236), IPC_5202 (MRA-1240) and IPC_6403 (MRA-1285) and their constituent parasite haplotypes were either sensitive (IC$_{50}$ < 30 nM), moderately resistant (IC$_{50}$ > 30 nM but < 100 nM) or fully resistant to CQ (IC$_{50}$ ≥ 100 nM). However, their absolute levels of susceptibility were significantly different (Supplementary Material, Table S5). Of the two IPC_3445 (MRA-1236) component haplotypes isolated, MRA1236-hap1 was CQ-sensitive while MRA1236-hap2 showed moderate CQ resistance. Similarly, two parasite haplotypes cloned from IPC_5202 (MRA-1240) were resistant to MFQ (MRA1240-hap2 and MRA1240-hap3) while the third haplotype (MRA1240-hap1) demonstrated a mefloquine-sensitive response (IC$_{50}$ < 30 nM). None of the three extensively studied isolates and their component parasite haplotypes were resistant to PPQ based on the threshold IC$_{50}$ of ≥90 nM thought to indicate resistance (Amato et al., 2017; Robert et al., 2019). Nonetheless, absolute PPQ IC$_{50}$ values differed significantly between and within isolates (Table 1; Supplementary Material, Table S5). Another striking feature of these

data is that the IC$_{50}$ of the most abundant parasite haplotype within each isolate reflects that of the uncloned isolate. Based on empirical estimates of the number of parasite clones obtained by limiting dilution, MRA1236-hap1, MRA1240-hap1 and MRA1285-hap1 were the most abundant parasite haplotypes in isolates IPC_3445 (MRA-1236), IPC_5202 (MRA-1240) and IPC_6403 (MRA-1285) respectively (Fig. 3A). CQ, MFQ and PPQ IC$_{50}$s for each of these parasite haplotypes reflected those of parental isolates from which they were cloned (Table 1).

3.5. Co-infecting parasite haplotypes show similar parasite survival rates following drug exposure

RSAs, which measure parasite viability following a specific dose-time exposure to DHA, showed similar survival rates for parasite haplotypes from the same isolate (Fig. 4A). Nonetheless, there was significant variation in parasite survival rates between different malaria isolates with some isolates exhibiting a parasite survival rate of <10% while others demonstrating a survival rate of ≥10% (Fig. 4A) indicative of artemisinin sensitivity and resistance respectively (Witkowski et al., 2013). We also observed significant variation in artemisinin resistance levels between the different malaria isolates with isolates IPC_3445 (MRA-1236), IPC_6261 (MRA-1284), IPC_6403 (MRA-1285) and IPC_6293 (MRA-1286) showing a parasite survival rate between 10 and 30% and the isolate IPC_5202 (MRA-1240) exhibiting a parasite survival rate as high as 50%. Similar data were obtained from PSAs, which measure parasite viability following exposure to a pharmacologically relevant concentration of PPQ (Fig. 4B). While isolates IPC_3445 (MRA-1236), IPC_5202 (MRA-1240) and IPC_6403 (MRA-1285) and their component haplotypes showed a parasite survival rate of <10% predictive of PPQ sensitivity, isolates IPC_6261 (MRA-1284) and IPC_6293 (MRA-1286) had a parasite survival rate of ≥10%, which has been recently proposed as the threshold for *in vitro* PPQ resistance (Duru et al., 2015).

3.6. Parasites with differential drug susceptibility often harbor different sets of drug resistance mutations

Sequencing of the CQ-resistance *pfcrt* gene demonstrated that each of the isolates IPC_3445 (MRA-1236), IPC_5202 (MRA-1240) and IPC_6403 (MRA-1285) including their constituent parasite haplotypes harbored a
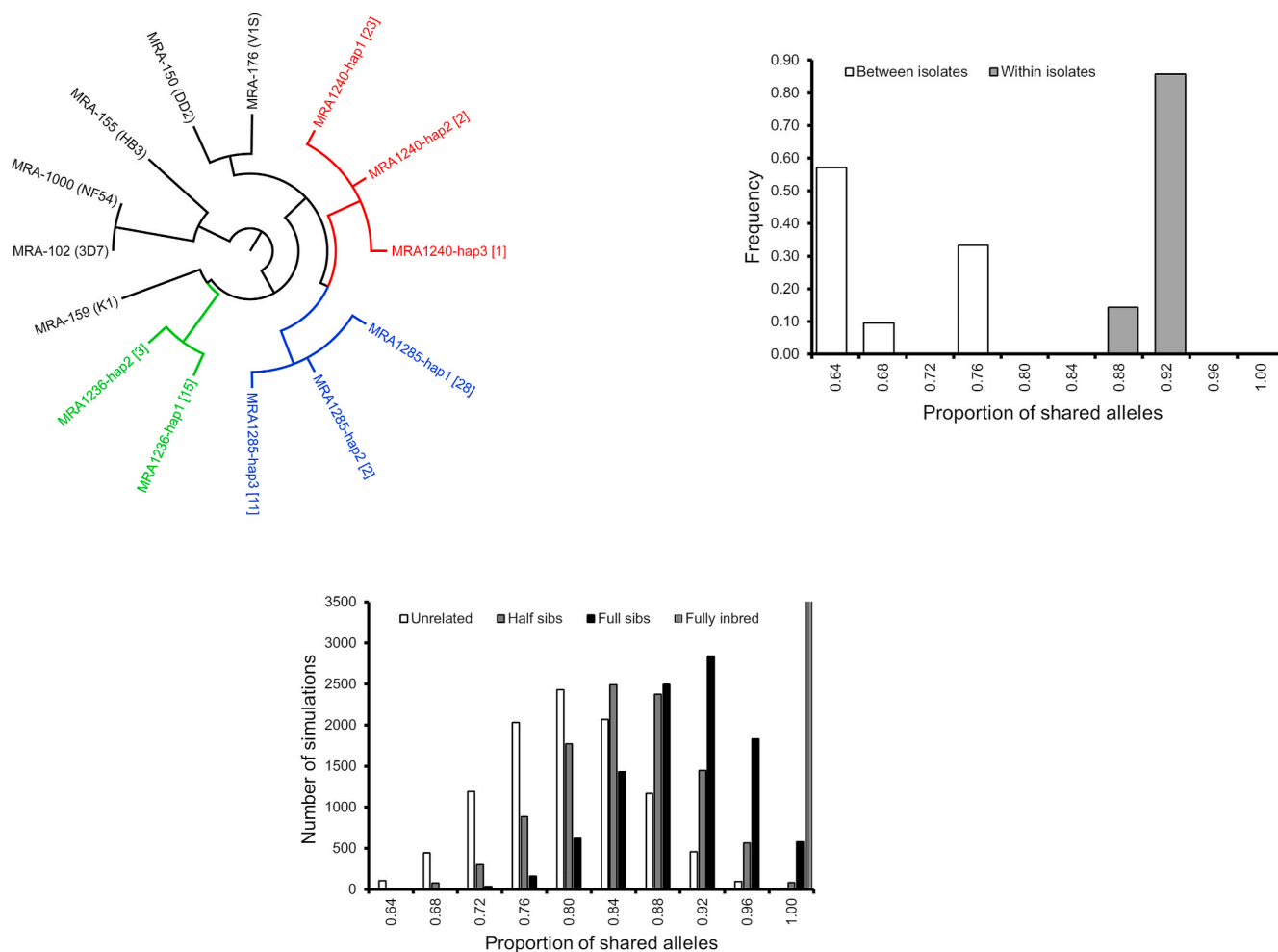
Fig. 3. Parasite diversity and relatedness within isolates. *Panel A* is a phylogenetic tree showing relationships between parasite haplotypes cloned from the same or different isolates. Haplotypes from the same isolate are shown using the same color scheme in red (IPC_5202/MRA-1240 haplotypes), blue (IPC_6403/MRA-1285 haplotypes) or green (IPC_3445/MRA-1236 haplotypes) while those in black are laboratory control lines. Each square bracket contains the number of parasite clones bearing a particular haplotype. *Panel B* shows observed pairwise allele-sharing (*ps*) between parasite haplotypes from the same or different isolates. Parasite haplotypes within isolates tend to be related, sharing more alleles than those from different isolates. *Panel C* shows simulated *ps* distributions expected of parasites in different classes of relatedness. Simulated *ps* distributions allow assessment of the degree of relatedness between parasite pairs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mutated PfCRT comprising amino acids cysteine, isoleucine, glutamic acid, threonine, histidine, serine, glutamic acid, serine, threonine and isoleucine at PfCRT codons 72, 74, 75, 76, 97, 220, 271, 326, 356 and 371 respectively (Table 2). In addition, MRA1236-hap2 had a rare PfCRT I358L polymorphism. Another important feature of these data is the identification of a recently arisen PfCRT mutation resulting in the substitution of the wild-type amino acid histidine (H) for tyrosine (Y) at codon 97. This mutation was only found in isolates IPC_6261 (MRA-1284) and IPC_6293 (MRA-1286). Interestingly, these are the only Cambodian parasites that showed elevated parasite survival rates following PPQ exposure (Fig. 4B). Sequence variation between parasite isolates was also observed at PfMDR-1 codon 184 with some isolates harboring the amino acid phenylalanine (F) in place of the wild-type tyrosine (Y) residue (Table 2). However, there was no *pfmdr-1* sequence variation between parasite haplotypes from the same isolate. Except for isolates IPC_6261 (MRA-1284) and IPC_6293 (MRA-1286) and laboratory control parasites 3D7 (MRA-102) and K1 (MRA-159), all Cambodian parasites analyzed in this study had multiple copies of the *pfmdr-1* gene implicated in mefloquine resistance (Table 2). Sequencing of Cambodian parasites at the artemisinin resistance-associated K13 locus (Ariey et al., 2014) revealed variation at codons 539 and 580 between isolates with no variation observed between parasite

haplotypes cloned from the same isolate (Table 2). All the Cambodian parasites studied had either the C580Y or the R539T mutation in *K13*. In addition, in addition to harboring the C580Y K13 mutation and the H97Y PfCRT mutation, isolates IPC_6261 (MRA-1284) and IPC_6293 (MRA-1286) carried multiple copies of the *plasmepsin II* (*pmp2*) gene implicated in piperaquine resistance (Witkowski et al., 2017).

4. Discussion

In this study, we hypothesized that significant variation in independently measured IC$_{50}$s for a carefully selected set of naturally occurring *P. falciparum* isolates derives from the presence of multiple parasite haplotypes with differing levels of drug susceptibility. Our data demonstrate that isolates IPC_3445 (MRA-1236), IPC_5202 (MRA-1240) and IPC_6403 (MRA-1285) each carry multiple related parasite haplotypes, often differing in absolute antimalarial susceptibility. Variability in drug response is more pronounced between isolates than between parasite haplotypes from the same isolate. This variation in antimalarial drug susceptibility is associated with specific sets of resistance-conferring mutations borne by parasites.

Table 1

Half maximal inhibitory concentrations (IC$_{50}$s) of clinical isolates, their constituent parasite haplotypes and laboratory control lines.

IC$_{50}$s are shown as the mean ± standard error of the mean (SEM) for at least three independent assays. Previously defined thresholds for chloroquine susceptibility are: IC$_{50}$ < 30 nM (sensitive), IC$_{50}$ between 30 and 100 nM (moderately resistant) and IC$_{50}$ ≥ 100 nM (resistant). Those for mefloquine susceptibility are: IC$_{50}$ < 30 nM (sensitive) and IC$_{50}$ > 30 nM (resistant) while an IC$_{50}$ ≥ 90 nM has been proposed as the threshold for *in vitro* PPQ resistance. Data show significant variation in absolute CQ, MFQ and PPQ IC$_{50}$s between and within the different isolates.

| Parasite ID	IC$_{50}$ (Mean ± SEM)		
	Chloroquine	Mefloquine	Piperaquine
3D7 (MRA102)	9.8 ± 1.7	21.3 ± 3.9	25.5 ± 8.7
DD2 (MRA150)	73.4 ± 7.4	13.6 ± 2.5	22.3 ± 4.6
K1 (MRA159)	179.4 ± 3.8	6.9 ± 0.3	33.6 ± 3.1
IPC_3445 (MRA1236)	26.0 ± 5.3	38.7 ± 8.7	21.6 ± 5.7
MRA1236-hap1	26.7 ± 1.8	35.9 ± 3.3	37.8 ± 1.7
MRA1236-hap2	38.5 ± 2.7	52.3 ± 8.5	52.7 ± 3.6
IPC_5202 (MRA1240)	100.2 ± 58.1	36.0 ± 17.2	28.6 ± 14.6
MRA1240-hap1	66.8 ± 10.8	26.9 ± 1.9	14.9 ± 1.4
MRA1240-hap2	78.7 ± 7.3	42.4 ± 2.9	35.2 ± 4.1
MRA1240-hap3	100.1 ± 9.2	79.2 ± 11.0	48.3 ± 10.1
IPC_6403 (MRA1285)	77.8 ± 35.3	15.9 ± 1.1	15.8 ± 5.5
MRA1285-hap1	78.4 ± 1.8	24.0 ± 2.2	17.9 ± 0.1
MRA1285-hap2	97.5 ± 4.5	25.4 ± 1.2	16.3 ± 0.4
MRA1285-hap3	31.9 ± 1.5	17.9 ± 1.2	17.4 ± 1.0
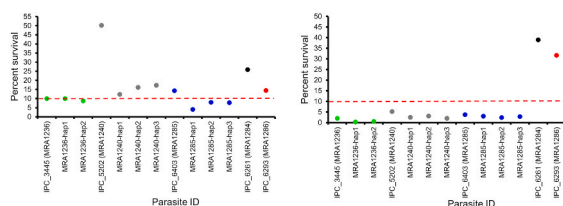
Fig. 4. Parasite survival rates following exposure to Dihydroartemisinin and Piperaquine. *Panel A* shows the percentage of viable parasites after exposure to DHA as estimated by the Ring-stage Survival Assay. *Panel B* shows parasite viability following PPQ exposure as determined by the Piperaquine Survival Assay. Each dot on either plot shows the survival rate for each parasite sample (horizontal axis). Each isolate and its component parasite haplotypes are shown using the same color scheme. The dotted line on each plot represents a previously defined threshold for categorizing parasites as either drug-susceptible (percent survival rate <10%) or drug-resistant (percent survival rate ≥ 10%). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.1. Genetic diversity of parasites in naturally occurring P. falciparum isolates

Multiple parasite haplotypes were observed in each of the three isolates initially suspected to be multiclonal (Fig. 3A). The number of parasite haplotypes observed per isolate (two to three) may be an underestimate of the total number of parasite lineages within the initial isolate. This is primarily because parasite haplotypes that replicate poorly in culture may be lost or competitively suppressed, leading to incomplete capture of parasite lineages from the initial isolate. Inherently, not all parasite-positive wells will contain clonal parasite populations following limiting dilution. Genotyping of parasites from such wells yields heterozygous (mixed) base calls at some loci (Fig. 2) indicating that they are still comprised of multiple lineages. The number of wells containing non-clonal parasite populations after limiting dilution (five, three and 12 for IPC_3445, IPC_5202 and IPC_6403 respectively) is consistent with expectations from the Poisson distribution of parasites in a 96-well cloning plate (Nkhoma et al., 2018). We observed that parasite haplotypes from the same isolate are predominantly related and show greater allele-sharing than parasite haplotypes from different isolates

(Fig. 3B). By simulating allele-sharing expected of parasites in different classes of relatedness (Fig. 3C), we found that all parasite haplotypes from different isolates are unrelated while 85.7% of parasite haplotypes from the same isolates are related at the half-sib level or greater (Supplementary Material, Table S4). These findings indicate that parasite haplotypes found in individual isolates predominantly arose from single mosquito inoculation of meiotic products of recombination into individual human hosts. These data are not unexpected, given the low and decreasing malaria transmission levels in Cambodia (Maude et al., 2014). They are consistent with the observation that parasite co-transmission from single mosquito bites plays a more pronounced role in generating within-isolate parasite diversity than inoculation of genetically diverse parasites from multiple mosquito bites (Nair et al., 2014; Nkhoma et al., 2012, 2018, 2020).

4.2. Variation in absolute drug susceptibility between co-infecting parasite haplotypes

Although drug susceptibility profiles of co-infecting parasite haplotypes often fell within one of the three broad categories of susceptibility (sensitive, moderately resistant or fully resistant), there was significant variation in absolute IC$_{50}$ values within each of these groupings. In the first case of variable drug susceptibility, both drug-sensitive and drug-resistant parasite haplotypes were found in the same isolate. For example, the isolate IPC_5202 (MRA-1240) harbored both the MFQ-sensitive parasite haplotype MRA1240-hap1, and two MFQ-resistant haplotypes, MRA1240-hap2 and MRA1240-hap3. Interaction between drug-sensitive and drug-resistant parasites co-infecting the same host can drive the evolution of drug resistance in a variety of ways. First, within-host competition between drug-sensitive and resistant haplotypes can lead to competitive release of drug resistance where a drug-sensitive haplotype is killed by drug treatment, but the drug-resistant haplotype is selected, resulting in intensification of resistance (Wargo et al., 2007). Second, the presence of both drug-susceptible and drug-resistant parasite haplotypes within an isolate may allow the former to expand at the expense of the latter when drug pressure is reduced or eliminated. This is often the case because drug resistance-conferring mutations tend to be detrimental to parasite fitness in the absence of drug pressure. For example, the cessation of CQ use in Malawi led to the loss of CQ-resistant parasites (Kublin et al., 2003) and full restoration of drug efficacy (Laufer et al., 2006). The loss of CQ resistance (CQR) was attributed to reduced fitness of CQ-resistant parasites in the absence of CQ pressure (Laufer et al., 2010). We might expect the loss of drug resistance through this mechanism to be more pronounced and proceed faster in parasite populations with large proportions of multiclonal infections. This is because multiclonal infections comprising both drug-sensitive and resistant parasites provide an opportunity for within-host competition, promoting the expansion of drug-susceptible parasites at the expense of the less fit resistant ones. Third, the presence of both drug-susceptible and resistant parasites within the same isolate can either lead to the creation or breakdown of allelic combinations that mediate antimalarial drug resistance (Talisuna et al., 2003). In the second case of variable drug susceptibility, parasite haplotypes from the same isolate fell into one of three broad categories of susceptibility but their absolute CQ, MFQ and PPQ IC$_{50}$s differed significantly (Table 1; Supplementary Material, Table S5). This variation in IC$_{50}$ may be associated with specific sets of resistance-conferring mutations that these parasite haplotypes carry, a hypothesis directly tested by this study. As co-infecting parasite haplotypes from two of the three extensively studied isolates were invariant at known drug-resistance loci including *pfcrt* and *pfmdr-1* (Table 2), it is possible that other, yet unidentified, genetic variants contribute to variation in absolute CQ IC$_{50}$ values. Technology now exists to efficiently produce genetic crosses between drug-susceptible and drug-resistant parasite strains in a humanized mouse (Vaughan et al., 2015). Coupling this technology with whole genome sequencing and rapid screening of large

Table 2
Sequence polymorphisms in PfCRT, PfMDR-1 and K13, and copy number of *mdr-1* and *pmp2* genes.

Parasite ID	PfCRT											PfMDR-1					K13		Copy #	
	72	74	75	76	97	220	271	326	356	358	371	86	184	1034	1042	1246	539	580	*mdr-1*	*pmp2*
3D7 (MRA-102)	C	M	N	K	H	A	Q	N	I	I	R	N	Y	S	N	D	R	C	1	1
DD2 (MRA-150)	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	I	**I**	**F**	Y	S	N	D	R	C	**3**	1
K1 (MRA-159)	C	**I**	**E**	**T**	H	**S**	**E**	**S**	I	I	**I**	**F**	Y	S	N	D	R	C	1	1
IPC_3445 (MRA1236)	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	I	**I**	N	Y	S	N	D	R	**Y**	**2**	1
MRA1236-hap1	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	I	**I**	N	Y	S	N	D	R	**Y**	**2**	1
MRA1236-hap2	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	**L**	**I**	N	Y	S	N	D	R	**Y**	**2**	1
IPC_5202 (MRA1240)	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	I	**I**	N	**F**	S	N	D	**T**	C	**2**	1
MRA1240-hap1	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	I	**I**	N	**F**	S	N	D	**T**	C	**3**	1
MRA1240-hap2	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	I	**I**	N	**F**	S	N	D	**T**	C	**2**	1
MRA1240-hap3	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	I	**I**	N	**F**	S	N	D	**T**	C	**3**	1
IPC_6403 (MRA1285)	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	I	**I**	N	**F**	S	N	D	R	**Y**	**2**	1
MRA1285-hap1	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	I	**I**	N	**F**	S	N	D	R	**Y**	**2**	1
MRA1285-hap2	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	I	**I**	N	**F**	S	N	D	R	**Y**	**2**	1
MRA1285-hap3	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	I	**I**	N	**F**	S	N	D	R	**Y**	**2**	1
IPC_6261 (MRA1284)	C	**I**	**E**	**T**	**Y**	**S**	**E**	**S**	**T**	I	**I**	N	Y	S	N	D	R	**Y**	1	**3**
IPC_6293 (MRA1286)	C	**I**	**E**	**T**	**Y**	**S**	**E**	**S**	**T**	I	**I**	N	Y	S	N	D	R	**Y**	1	**2**

Numbers below each gene name are amino acid positions within the gene product. Letters are abbreviations for the different amino acids for example, "C" for "Cysteine". Amino acids shown in bold denote point mutations relative to the wild-type 3D7 (MRA-102) control. Copy # is the estimated number of copies of the gene. Where this is shown in bold, it means the gene in that sample is amplified relative to MRA-102, which has a single gene copy.

chemical libraries for both antimalarial potency and cytotoxicity (Mott et al., 2015) may help uncover additional genetic variants that influence absolute antimalarial drug susceptibility levels. Another important feature observed with these data is that an IC$_{50}$ of one of the co-infecting parasite haplotypes always reflected that of the uncloned parental isolate. It suggests that the most abundant haplotype at the time of the assay dominates the antimalarial susceptibility profile, masking the effect of minority frequency haplotypes. We observed that mean IC$_{50}$ values for multiclonal isolates tend to have large standard errors (Table 1). This is largely due to averaging IC$_{50}$ datapoints that are markedly different, and in most cases spanning a range of drug susceptibility profiles from sensitivity to resistance.

4.3. Relationship between absolute drug susceptibility and the presence of drug resistance polymorphisms

Except for MRA1236-hap1, all Cambodian parasites examined in this study were moderately or fully resistant to chloroquine based on previously defined IC$_{50}$ thresholds (Table 1) and carried the K76T *pfcrt* mutation (Table 2), which is considered the key determinant of CQR (Djimde et al., 2001; Fidock et al., 2000). The K76T mutation existed in a classical SE Asian CQR genetic background (Fidock et al., 2000) except in parasite isolates IPC_6261 (MRA-1284) and IPC_6293 (MRA-1286), which bore the amino acid tyrosine (Y) in place of histidine (H) at PfCRT codon 97. The H97Y *pfcrt* mutation is an emerging polymorphism in the Greater Mekong Region (Hamilton et al., 2019) and has been shown to confer PPQ resistance in allele-exchange experiments (Ross et al., 2018). In this study, parasites harboring the H97Y *pfcrt* mutation showed elevated parasite survival rates in standard PSAs (Fig. 4B). This observation reinforces the notion that the H97Y *pfcrt* mutation could be a suitable molecular marker for PPQ resistance. We identified a rare isoleucine (I) to leucine (L) polymorphism at PfCRT codon 358 in MRA1236-hap2. This polymorphism was absent in the uncloned parental isolate, IPC_3445 (MRA-1236), suggesting that it existed at too low a frequency to be detected but clonally expanded in the parasite lineage. It is currently unknown whether this mutation has a direct functional role in antimalarial drug resistance.

We observed that a parasite lineage MRA1236-hap1 exhibits CQ sensitivity (IC$_{50}$ < 30 nM) but has the CQR PfCRT haplotype. Polymorphisms in *pfmdr-1* and elsewhere within *pfcrt* are known to modulate the level of CQR in CQ-resistant parasites (Duraisingh et al., 2000a, 2000b; Jiang et al., 2008; Sidhu et al., 2005; Veiga et al., 2016) and may even abrogate CQR in parasites carrying a CQR PfCRT haplotype

(Johnson et al., 2004). While none of the previously identified CQR-annulling *pfcrt* mutations including the S163R mutation (Johnson et al., 2004) were found in MRA1236-hap1 and all other parasites studied, examination of full-length *pfmdr-1* revealed that isolates IPC_5202 (MRA-1240) and IPC_6403 (MRA-1285) plus their constituent parasite haplotypes bore the Y184F *pfmdr-1* mutation (Table 2). This mutation was previously shown to have minimal impact on parasite susceptibility to CQ and MFQ (Veiga et al., 2016). Nonetheless, in a parasite genetic background containing asparagine (N) at PfMDR-1 codon 86 as was observed in this study (Table 2), the Y184F *pfmdr-1* mutation was shown to decrease parasite susceptibility to PPQ (Veiga et al., 2016). In examining how a clonal parasite lineage such as MRA1236-hap1 might exhibit CQ sensitivity (IC$_{50}$ < 30 nM) but harbor the CQR *pfcrt* haplotype and no resistance-modulating mutations in *pfmdr-1*, it is worth considering the importance of parasite genetic background in assessing how malaria parasites might respond to drugs. Compensatory mutations are known to reduce the fitness cost of drug resistance without jeopardizing resistance itself (Levin et al., 2000; Maisnier-Patin and Andersson, 2004; Vincent et al., 2018). These mutations do not necessarily need to occur within the drug-resistance gene itself. For example, amplification of the GTP cyclohydrolase gene 1 (gch1) compensates for the loss of parasite fitness due to accumulation of high-level antifolate resistance mutations in the *dihydrofolate reductase* (*dhfr*) and *dihydropteroate synthase* (*dhps*) genes (Heinberg et al., 2013; Nair et al., 2008). Although gch1, *dhfr* and *dhps* all reside in the folate biosynthesis pathway, this copy number polymorphism occurs within the *gch1* gene that has no known antifolate resistance-conferring point mutations. Because we primarily focused on genes known to play a major role in CQR, we cannot rule out the possibility that other, yet unidentified, genetic variants reduce the level of CQR in MRA1236-hap1.

All Cambodian parasites analyzed in this study (n = 11) had multiple *pfmdr-1* copies but only 55% (n = 6) were resistant to MFQ (IC$_{50}$ > 30 nM). By virtue of harboring multiple *pfmdr-1* copies, all these parasites would have been expected to exhibit MFQ resistance (Price et al., 2004). However, this was not the case. All five MFQ-sensitive and multi-copy *pfmdr-1* parasites carried the Y184F PfMDR-1 mutation (Table 2). Although this mutation appears to have limited direct impact on MFQ resistance (Veiga et al., 2016), it could play a compensatory role, offsetting some of the fitness burden associated with *pfmdr-1* amplification, and reducing overall MFQ resistance levels. All Cambodian isolates examined in this study carried either the C580Y or R539T *K13* polymorphism implicated in artemisinin resistance and had a parasite

survival rate of ≥10% indicative of artemisinin resistance (Anderson et al., 2016; Ariey et al., 2014; Witkowski et al., 2013). Each parasite isolate and its component parasite haplotypes had the same *K13* polymorphism. Neither of the IPC_5202 (MRA-1240) component parasite haplotypes showed a parasite survival rate reflecting that of the uncloned parental isolate. As parasites that grow poorly in culture may be lost during dilution cloning, it is conceivable that parasite haplotypes with survival rates as high as that of IPC_5202 (MRA-1240) were not sampled during cloning. Drug treatment of isolates comprising parasite haplotypes with differential parasite survival rates may preferentially select for the haplotype with the highest survival rate, leading to its identification (Mideo et al., 2016). Only isolates with elevated parasite survival rates following PPQ exposure (Fig. 4B) had multiple copies of *pmp2* and the emerging H97Y *pfcrt* mutation (Table 2). The H97Y *pfcrt* mutation has been shown to confer PPQ resistance in the presence or absence of multicopy *pmp2* in allelic-exchange experiments (Veiga et al., 2016). In contrast, altering the number of *pmp2* copies in the parasite genome does not influence PPQ sensitivity (Veiga et al., 2016). It, therefore, appears that while the H97Y *pfcrt* mutation is a critical determinant of PPQ resistance, the role of multicopy *pmp2* in mediating PPQ resistance is merely compensatory (Veiga et al., 2016). We observed that co-infecting parasite haplotypes tend to be related (Fig. 3) and harbor the same set of polymorphisms at drug resistance loci including *pfcrt*, *pfmdr-1* and *K13* (Table 2). By sharing the same set of antimalarial resistance-conferring mutations and excluding non-relatives from their niche, co-infecting parasite haplotypes may be able to reduce within-host competition and enhance cooperative interactions, which are beneficial to their survival.

5. Conclusion

In summary, our findings demonstrate that malaria isolates often comprise multiple parasite lineages with differing levels of antimalarial susceptibility. *In vitro* susceptibility testing of such isolates may yield different susceptibility patterns on independent occasions. The presence of multiple parasite lineages within an isolate can also interfere with the genotyping and sequencing undertaken to ascertain genetic identity. Depending on intended end-use, multiclonal isolates should be cloned to yield single parasite lineages with well-defined phenotypes and genotypes. In particular, we recommend cloning of clinical isolates prior to use for *in vitro* drug susceptibility studies for screening candidate antimalarials. The practice of using standardized clonal parasite lineages in drug susceptibility screening should minimize variability in drug response across independent assays. Clonal parasite lineages generated in this study have well-defined *in vitro* antimalarial susceptibility phenotypes and genotypes at major antimalarial resistance loci including K13, PfCRT and PfMDR-1. This makes them an extremely valuable resource as potential controls for screening candidate antimalarials and for drug resistance studies. These parasite lines will soon be made available to the malaria research community through the MR4 collection of NIAID's BEI Resources Program (https://www.beiresources.org).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.03.001.

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