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## **Integrative Genetic Manipulation of Plasmodium cynomolgi Reveals Multidrug Resistance-1 Y976F Associated With Increased In Vitro Susceptibility to Mefloquine**

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Running Title: PcMDR1 Y976F increases mefloquine susceptibility

**Integrative genetic manipulation of *Plasmodium cynomolgi* reveals MultiDrug Resistance-1 Y976F associated with increased *in vitro* susceptibility to mefloquine**

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**Abstract:**

The lack of a long-term *in vitro* culture method has severely restricted the study of *Plasmodium vivax*, in part because it limits genetic manipulation and reverse genetics. We used the recently optimized *P. cynomolgi* Berok *in vitro* culture model to investigate the putative *P. vivax* drug resistance marker MDR1 Y976F. Introduction of this mutation using CRISPR Cas9 increased sensitivity to mefloquine; but had no significant effect on sensitivity to chloroquine, amodiaquine, piperazine and artesunate. To our knowledge, this is the first reported use of CRISPR Cas9 in *P. cynomolgi*, and the first reported integrative genetic manipulation of this species.

Key Words: *Plasmodium cynomolgi*, *Plasmodium vivax*, Multiple Drug Resistance, Mefloquine

**Introduction:**

Chloroquine resistance (CR) was first reported in *P. vivax* in 1989, 30 years after being observed in *P. falciparum*. Chloroquine-resistant *P. vivax* is particularly prevalent in New Guinea and eastern Indonesia, but it has also emerged in or spread to many other endemic areas, including Thailand, Ethiopia, and South America<sup>1,2</sup>. Drug resistance in *P. vivax* is less well studied and understood than in *P. falciparum*, but is an equally pressing issue, as infection with multidrug-resistant parasites has been associated with increased severity and mortality, particularly in young children<sup>3</sup>.

The genetic basis of *P. vivax* CR remains elusive. Variations in two genes *crt* (chloroquine resistance transporter) and *mdr1* (multidrug resistance) incriminated for *P. falciparum* CR, have been analysed in *P. vivax* clinical isolates but yielded inconclusive results<sup>2</sup>. Moreover, although increased expression of *Pvcrt* has been clinically linked to CR, the relationship was not confirmed in *ex vivo* assays using parasites from patient samples<sup>4</sup>. One polymorphism, Y976F, in the *Pvmdr1* gene has been linked to CR in some studies but not others<sup>2,5</sup>. The unavailability of continuous *in vitro* cultivation of *P. vivax* clearly precludes and hampers efforts to validate candidate genes.

*Plasmodium cynomolgi*, a simian malaria parasite of Southeast Asian macaques, has long served as an excellent model for *P. vivax* with which it is closely related phylogenetically, the two species being nearly indistinguishable

Running Title: PcMDR1 Y976F increases mefloquine susceptibility

morphologically and biologically<sup>6</sup>. We have recently adapted one of the *P. cynomolgi* strains, Berok, to routine *in vitro* cultivation, from which a cloned line (K4A7) was derived<sup>7</sup>, thus providing parasites amenable to modern genetic manipulation that can be uniquely employed for investigation pertinent to *P. vivax*. We have applied CRISPR Cas9 to introduce the Y976F point mutation in the *Pcmdr1* gene to *P. cynomolgi* Berok to establish whether it influences drug susceptibility

## **Methods:**

### Macaque Blood

Nonhuman primate blood and serum were sourced from *Macaca fascicularis* (cynomolgus monkeys), which were maintained at the Monash Animal Research Platform, in Gippsland, Australia (MARF). Leukocytes were removed through filtration with pre-equilibrated non-woven filters, and erythrocytes were adjusted to 50% haematocrit with RPMI 1640 medium supplemented with GlutaMAX.

### Sequence alignments

*Plasmodium mdr1* sequences were sourced for homology analysis from PlasmoDB (PVX\_080100, PCYB\_101870, and PCYM\_1008800). Amino and nucleic acid sequence alignments and pairwise comparisons were completed with MegAlign Pro (DNASTAR) using MUSCLE software. Phylogenetic trees were compiled using the Kimura model with MegAlign Pro using BIONJ clustering and pairwise gap removal.

### Parasite culture

The experiments in this study used the single cell cloned line Berok (K4A7), derived from the continuous culture of *P. cynomolgi* Berok (K4)<sup>7</sup>. Base media was prepared with RPMI 1640 medium with HEPES containing 2 mM L-glutamine, supplemented with 4 g/L D-glucose, 360 µM hypoxanthine, 5 g/L Albumax II Lipid Rich BSA, 25 µg/mL Penicillin G potassium salt, and 0.5 µg/mL Cefquinome sulphate. Growth medium was supplemented with 10% (v/v) heat-inactivated horse serum. Parasites were cultured at 37°C in a mixed gas chamber (5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub>).

### Plasmid constructs

DNA constructs for CRISPR manipulation of *P. cynomolgi* were adapted from the plasmids pDC2\_vCam\_GFP\_hDHFR, and pDC2\_Cam\_Cas9\_hDHFR. A *Streptococcus pyogenes* Cas9 and a U6 promoter expressing a chimeric guide RNA from the plasmid pDC2\_Cam\_Cas9\_U6\_hDHFR were inserted into pDC2\_vCam\_GFP\_hDHFR through restriction cloning, following digestion with *XhoI* and *AvrII*, and *BamHI* respectively. The guide RNA g1 was constructed by annealing primers p1 and p2 (Supplementary Table 1) and inserted following digestion of the plasmid with *BbsI* as described by Ng and Fidock<sup>8</sup>.

An 859 bp donor template was PCR-amplified from *P. cynomolgi* Berok K4A7 genomic DNA using primers p3 and p4 (Supplementary Table 1) and was inserted into the cloning plasmid pGEM. The Y976F mutation was introduced at amino acid position 976 through site-directed mutagenesis using primers p5 and p6 (Supplementary Table 1). Silent mutations were also introduced at positions 986-988 using primers p7 and p8 (Supplementary Table 1) to restrict cleavage following successful donor insertion and mutation. The donor sequence was cloned into pDC2\_vCam\_Cas9\_hDHFR through restriction cloning following digestion with *SfoI* and *AatII* to generate the transfection plasmid pDC2\_vCam\_Cas9\_mdr1\_hDHFR (Figure 1A). A separate transfection plasmid was assembled containing the silent binding site mutations with a wildtype Y976.

### Transfection

Synchronous schizont stage parasites were enriched through gradient centrifugation on a 55% Nycodenz cushion, followed by a three-hour incubation with the drug 4-[7-[(dimethylamino)methyl]-2-(4-fluorophenyl)imidazo[1,2-a]pyridin-3-yl]pyrimidin-2-amine (compound 2). Transfections were carried out using the P3 Primary cell 4D Nucleofector X Kit L (Lonza), as described by Mohring et al.<sup>9</sup>. Successfully transfected parasites were selected through use of 2.5 nM WR99210 (Jacobus Pharmaceuticals) after 24 hours and were kept under drug pressure for 6 days with daily media changes. Editing was confirmed by PCR and Sanger sequencing.

### Drug assays

*P. cynomolgi* sensitivity to antimalarial drugs was measured by incubating synchronous ring-stage parasites with a serial dilution of drug concentrations at 0.8% parasitaemia and 1% haematocrit in 96-well plates. Assay plates were incubated at 37°C for 72 hours under normal culturing conditions. Final parasitaemia was determined through flow cytometry using a BD FACSCanto II following staining with 1x SYBR Green I nucleic acid, and 100 nM Mitotracker Deep Red. Experiments were carried out in duplicate, with three biological replicates. IC<sub>50</sub> values were calculated through nonlinear regression analysis, and statistical significance was determined through an ordinary two-way ANOVA and a Šidák's multiple comparisons test using GraphPad Prism version 9.3.1.

## Results

Pairwise comparisons between the *P. cynomolgi* Berok and *P. vivax mdr1* genes showed a similarity of 88.7% and 93.9% at the nucleotide and amino acid levels, respectively (Supplemental Figure 1). Interestingly, *P. cynomolgi* Berok, B, and M strains all carry the T958M and F1076L polymorphisms, which have previously been linked to chloroquine resistance in *P. vivax*<sup>2</sup>.

We constructed a single-plasmid CRISPR vector expressing a *S. pyogenes* Cas9 gene with a *P. vivax* calmodulin promoter, a U6 driving gRNA, an *hdhfr* selection cassette, and a repair template (Figure 1A). Transfections with this system successfully introduced a Y976F mutation in the *Pcmdr1* gene (Figure 1B), as well as silent binding-site mutations to prevent re-cleavage following homology-directed repair. We also introduced the same silent binding site mutations into a line with wildtype Y976 to act as a control (the BSmut control) and to ensure that these silent mutations did not alter the phenotype. We genotyped transfected parasites with primers p9 and p10 (Supplementary Table 1), which were located outside of the repair template to avoid plasmid amplification.

No significant change was observed in the chloroquine sensitivity of *P. cynomolgi* for the recombinant Y976F line as compared to that of the BSmut control, with geometric mean (Geo. SD) IC<sub>50</sub> values of 10.9 nM (1.3nM), and 13.3 nM (1.2nM), respectively (Figure 1C). Similarly, the Y976F mutation did not result in with any significant changes to the sensitivity profile of *P. cynomolgi* to the antimalarial drugs artesunate, amodiaquine, or piperazine (Figure 1C). However, we observed that the Y976F mutant led to a significant sensitization to

mefloquine, with a mean IC<sub>50</sub> of 5.2 nM (1.7nM) compared to 40.2 nM (1.0nM) in the BSmut control (p < 0.0001) (Figure1C).

## Discussion

A fundamental understanding of resistance mechanisms notwithstanding, deciphering the genetic basis of drug resistance provides the ability to monitor and restrict the spread of resistant parasites, thus averting morbidity and mortality and optimising control measures. For *P. falciparum* this has been possible through the combined availability of routine *in vitro* cultivation and genetic manipulation, two avenues lacking for *P. vivax*. A combination of clinical studies, *in vitro* phenotyping, and reverse genetics provided the community with verified molecular markers of drug resistance for *P. falciparum*, which additionally enhanced target optimisation for drug development against resistant populations<sup>10,11</sup>. The emergence of *P. vivax* lines resistant to chloroquine, still the first line treatment for this parasite, requires similar investigations. However, these are limited to clinical drug efficacy trials and *ex vivo* sensitivity assays<sup>12</sup>, both of which are subject to biological and technical variability, hence the contradictory genetic associations obtained to date. *In vivo* non-human primate models are prohibitively expensive and cannot provide a tenable alternative to the extensive *in vitro* reverse genetic investigations conducted for *P. falciparum*.

We have exploited a cultured *P. cynomolgi* cloned line to introduce the *Pcmdr1* Y976F mutation, which is possibly associated with CR in *P. vivax*<sup>5</sup>, by an integrative genetic manipulation through CRISPR Cas9. To our knowledge, this is the first time this was achieved with this species and approach. We observed no significant change in chloroquine sensitivity, which strongly suggests that this mutation is not directly linked to a resistant phenotype. Similarly, no change in sensitivity to amodiaquine resulted from the introduction of the Y976F mutation, although it had previously been linked to amodiaquine and sulfadoxine-pyrimethamine in *P. vivax* alongside *Pvdhfr* mutations<sup>13</sup>. Interestingly, we did note an increase in sensitivity to mefloquine, of a magnitude similar to that reported earlier in *ex vivo* studies of Thai and Indonesian *P. vivax* isolates<sup>14</sup>: 7.7-fold reduction in mefloquine IC<sub>50</sub> values, from 40 nM to 5 nM for *P. cynomolgi*, which compares favourably with the 8.6-fold reduction, from 121 nM to 14 nM for *P. vivax*. It is important to note that the *P. cynomolgi* Berok K4A7 clone used here has a single copy of *Pcmdr1*. These observations suggest that the *Pvmdr1* Y976F mutation that is predominant in Papua

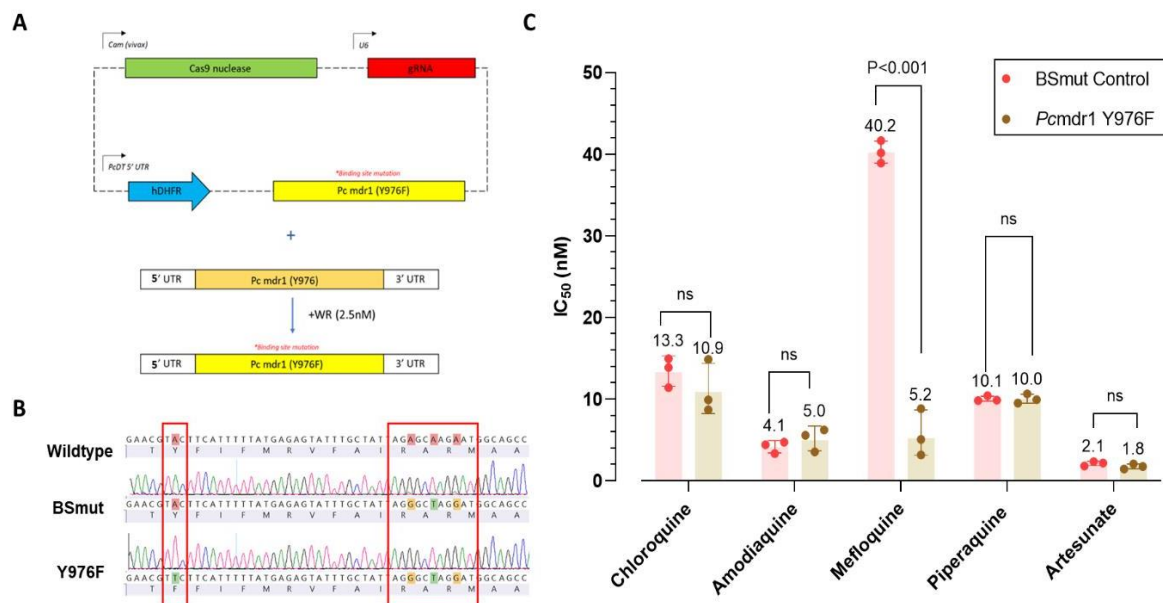


Indonesia, but also present in *P. vivax* populations spanning the globe<sup>2,15</sup>, is not being selected for by exposure to chloroquine.

This study demonstrates that *P. cynomolgi* cultivation associated to genetic manipulation offers an excellent opportunity to investigate the contribution of various candidate genetic mutations, alone or in defined combinations, to the susceptibility of *P. vivax* to a given anti-malarial drug.

## References

- 1 Price, R. N. *et al.* Global extent of chloroquine-resistant *Plasmodium vivax*: a systematic review and meta-analysis. *The Lancet Infectious Diseases* **14**, 982-991, doi:10.1016/S1473-3099(14)70855-2 (2014).
- 2 Buyon, L. E., Elsworth, B. & Duraisingh, M. T. The molecular basis of antimalarial drug resistance in *Plasmodium vivax*. *Int J Parasitol Drugs Drug Resist* **16**, 23-37, doi:10.1016/j.ijpddr.2021.04.002 (2021).
- 3 Tjitra, E. *et al.* Multidrug-resistant *Plasmodium vivax* associated with severe and fatal malaria: a prospective study in Papua, Indonesia. *PLoS Med* **5**, e128-e128, doi:10.1371/journal.pmed.0050128 (2008).
- 4 Pava, Z. *et al.* Expression of *Plasmodium vivax* Is Related to Parasite Stage but Not *Ex Vivo* Chloroquine Susceptibility. *Antimicrobial Agents and Chemotherapy* **60**, 361-367, doi:10.1128/aac.02207-15 (2016).
- 5 Suwanarusk, R. *et al.* Chloroquine resistant *Plasmodium vivax*: *In vitro* characterisation and association with molecular polymorphisms. *PLoS ONE* **2**, 1-9, doi:10.1371/journal.pone.0001089 (2007).
- 6 Tachibana, S. *et al.* *Plasmodium cynomolgi* genome sequences provide insight into *Plasmodium vivax* and the monkey malaria clade. *Nat Genet* **44**, 1051-1055, doi:10.1038/ng.2375 (2012).
- 7 Chua, A. C. Y. *et al.* Robust continuous *in vitro* culture of the *Plasmodium cynomolgi* erythrocytic stages. *Nature Communications* **10**, 3635, doi:10.1038/s41467-019-11332-4 (2019).
- 8 Ng, C. L. & Fidock, D. A. *Plasmodium falciparum* *In Vitro* Drug Resistance Selections and Gene Editing. *Methods Mol Biol* **2013**, 123-140, doi:10.1007/978-1-4939-9550-9\_9 (2019).
- 9 Mohring, F. *et al.* Rapid and iterative genome editing in the malaria parasite *Plasmodium knowlesi* provides new tools for *P. vivax* research. *eLife* **8**, e45829, doi:10.7554/eLife.45829 (2019).
- 10 Yuthavong, Y. *et al.* Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target. *Proc Natl Acad Sci U S A* **109**, 16823-16828, doi:10.1073/pnas.1204556109 (2012).
- 11 Kim, J. *et al.* Structure and drug resistance of the *Plasmodium falciparum* transporter PfCRT. *Nature* **576**, 315-320, doi:10.1038/s41586-019-1795-x (2019).
- 12 Russell, B. *et al.* Human *ex vivo* studies on asexual *Plasmodium vivax*: The best way forward. *International Journal for Parasitology* **42**, 1063-1070, doi:https://doi.org/10.1016/j.ijpara.2012.08.010 (2012).
- 13 Marfurt, J. *et al.* Molecular markers of *in vivo* *Plasmodium vivax* resistance to amodiaquine plus sulfadoxine-pyrimethamine: mutations in *pvdhfr* and *pvmr1*. *The Journal of infectious diseases* **198**, 409-417, doi:10.1086/589882 (2008).
- 14 Suwanarusk, R. *et al.* Amplification of *pvmr1* Associated with Multidrug-Resistant *Plasmodium vivax*. *The Journal of Infectious Diseases* **198**, 1558-1564, doi:10.1086/592451 (2008).
- 15 Ferreira, M. U. *et al.* Monitoring *Plasmodium vivax* resistance to antimalarials: Persisting challenges and future directions. *International Journal for Parasitology: Drugs and Drug Resistance* **15**, 9-24, doi:https://doi.org/10.1016/j.ijpddr.2020.12.001 (2021).



**Figure 1.** The genetic modification of MDR1 Y976F in *P. cynomolgi* Berok K4A7 using CRISPR Cas9 and its corresponding association with *in vitro* sensitivity to five antimalarial drugs. **A.** An all-in-one approach<sup>8</sup> was used for the CRISPR Cas9 strategy for editing the *mdr1* locus, consisting of a *S. pyogenes* Cas9 gene with a *P. vivax* calmodulin promoter, a U6 driving gRNA, a human *dhfr* selection cassette, and an *mdr1* specific donor template for homology-directed repair. Donors coding for MDR1 Y976F and silent binding site mutations were generated by site-directed mutagenesis of the wildtype *P. cynomolgi mdr1* donor sequence. **B.** Sequencing results showing the introduction of individual *Pcmdr1* mutations in recombinant parasites (Y976F), with wildtype amino and nucleic acid sequences for reference. The mutation Y976F was introduced into *Pcmdr1*, along with silent binding site mutations, and a separate control BSmut line was produced with the same silent binding site mutations and a wildtype Y976. **C.** The geometric mean (three biological replicates) of  $IC_{50}$  values are shown for chloroquine, artesunate, mefloquine, piperazine and amodiaquine against *P. cynomolgi* K4A7 *Pcmdr1* Y976F and the BSmut control. Significance was determined by ordinary two-way ANOVA and a Šidák's multiple comparisons test.

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**Supplementary Table 1. Primers used in the site directed mutagenesis (SDM) of PcMDR1 Y976F**

<b>Primer</b>	<b>Description</b>	<b>Sequence</b>
p1	Guide oligo F	TATTATTTGCTATTAGAGCAAGAA
p2	Guide oligo R	AAACTTCTTGCTCTAATAGCAAAT
p3	Donor template F	GCCATTATAGCCCTGAGCATT
p4	Donor template R	TACCGGTGAATAAAAA
p5	Y976F SDM F	CCGGAACGTtCTTCATTTTTATGAGAGTATTGCTATTAGAGC
p6	Y976F SDM R	TAAAAATGAAGaACGTTCCGGTCAACACAGCCGCTA
p7	Bsmut SDM F	CTATTAGgGcTAGgATGGCA
p8	Bsmut SDM R	cCTaGCcCTAATAGCAAATACTCTC
p9	Sequencing F	AAACCCAAAGCTCCAAACAACCT
p10	Sequencing R	TGCATTTTCTGAGTCTCCCTTTAG