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Long cryopreserved lab-adapted *Plasmodium falciparum* increases resistance to chloroquine but not its susceptibility

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ABSTRACT

Background: *Plasmodium falciparum* is a deadly protozoan that is accountable for malaria and chloroquine was the first-line antimalarial drug before its withdrawal and replaced by artemisinin. To date, several studies showed that *P. falciparum* had regained its sensitivity towards chloroquine after its withdrawal for decades. By understanding the basic principle and mechanism of chloroquine resistance in *P. falciparum*, at the molecular level, it would be valuable prior to the re-introduction of chloroquine as a first-line anti-malarial drug for malaria treatment. Thus, this study was conducted to determine the chloroquine resistance level of long preserved lab-adapted *P. falciparum* strain. **Methodology:** By using 14 years (2006-2020) cryopreserved chloroquine-sensitive (3D7) and chloroquine-resistant (W2) lab-adapted *P. falciparum* strains, the strains were subjected to continuous culture for three months before *in vitro* drug susceptibility assay and single nucleotide polymorphisms (SNP) analysis on Pfcrt and Pfmdr-1 gene for both strains. **Results:** This study shows the IC_{50} chloroquine of lab-adapted *P. falciparum* 3D7 and W2 strains were at 32.98 nM and 691.21 nM, respectively and both strains showed 3-fold higher IC_{50} when compared to their susceptibility before cryopreserved (3D7; 13.84nM and W2; 208.27 nM). The SNPs result showed a consistent amino acid substitution at position 76 (K to T) on PfCRT and 86 (N to Y) in Pfmdr-1 gene which concordance with other studies before preservation. **Conclusion:** Thus, this study shows that long cryopreserved of lab-adapted *P. falciparum* increases the chloroquine resistance level but not exhibited any change in susceptibility.

Keywords: *Plasmodium falciparum*; chloroquine resistance; SNPs, *PfCRT; PfMDR-1*

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INTRODUCTION

Malaria is a mosquito-borne infectious disease caused by parasites that are transmitted from human to human or from animal to human via the bites of infected female *Anopheles* mosquitoes. According to the World Malaria Report 2018 published by the World Health Organization (WHO), an estimated 219 million malaria cases were reported worldwide in 2017. Based on the report, the African region contributed the most to the cases (200 million or 92% in 2017), followed by Southeast Asia (11 million or 5%). Although there were an estimated 20 million malaria cases fewer from 2010 to 2017, the period of 2015 to 2017 highlighted that there was no significant progress in eliminating global malaria cases (World Health Organization, 2019). Nevertheless, malaria remains a major global health problem in the 21st century and more than half of the world's population is at the risk of acquiring malaria.

Malaria in humans is caused by the protozoan parasite of the genus *Plasmodium*, such as *Plasmodium falciparum* (*P. falciparum*), *Plasmodium malariae* (*P. malariae*), *Plasmodium ovale* (*P. ovale*) and *Plasmodium vivax* (*P. vivax*) (Perlmann & Troye-Blomberg, 2000; Sharma & Khanduri, 2009; Siswantoro *et al*., 2011; Sutherland *et al*., 2010). In 2000, *Plasmodium knowlesi* (*P. knowlesi*) was rectified as the fifth human malaria parasite that initially infected monkeys (Sabbatani *et al*., 2010; Tang *et al*., 2010).

Among the five human *Plasmodium*, *P. falciparum* has extensively been explored as it is the most prevalent malaria parasite in most of the world regions, the most clinically important infection due to its high risk of death and the parasite develop resistance towards chloroquine as its first-line therapy. Other than that, *P. falciparum* is among three human malaria parasites other than *P. vivax* (Mehlotra *et al.,* 2017) and *P. knowlesi* (Butcher *et al.,* 1971) that could only be continuously cultured to its complete life-cycle in the laboratory. Therefore, cryopreservation is the only procedure to banking the isolates and to have a sustainability supply of the parasite stock for *in vitro* experiment. Due to high frequency of freezing and thawing, there is a need to characterize the isolates for it variations such as drug-response, and virulent after long storage in liquid nitrogen. Other than that, studies on the genetic characteristics of drug resistance in the isolates provide essential information on the extent to which mutations involved (WHO memorandums, 1981).

There are two genes associated with the chloroquine-resistant in *P. falciparum*, which is *Pfcrt* (*Plasmodium falciparum* chloroquine resistance transporter) and *Pfmdr-1* (*Plasmodium falciparum* multiple drug resistance 1) (Cui *et al*., 2015). The protein encoded from both genes that found at the food vacuole membrane of *P. falciparum*, which is also the site of chloroquine actions (Antony *et al.,* 2016). Although the involvement of PfMDR-1 protein in chloroquine-resistant is not very clear, several studies indicate that the existence of these two markers causes more resistance to chloroquine than just a single marker. SNPs that occur at both genes result in the encoding of different amino acids that cause the protein to be inactive and led to chloroquine resistance. The mutant Pfcrt alleles found in chloroquine-resistant parasites contain several point mutations, and the pattern of mutations depending on the region where its originated (Sindhu et al. 2002, Basco and Ringwald 2001). Chloroquine-resistant parasite from South East Asia and Africa carry point mutation at codon 74,75,76,220,271,326,356 and 371. Meanwhile, South America strain, carry point mutation at position codon 76, 220 or 72, 326 and 356 or 75,97 and 371. However, a point mutation at codon 76 plays an important role in conferring chloroquine resistant (Basco and Ringwald 2001, Fidock et al. 2000). For Pfmdr-1, a point mutation at codon 86, 184, 1034, 1042 and 1246 have been proposed to be associated with chloroquine-resistant (Reed et al. 2000). Among those genes, the mutation at codon 86 from asparagine to tyrosine has been reported widely. Thus, this present study is aimed to characterize drug susceptibility of long cryopreserved *P. falciparum* in our biobank as this may help us inventories and establishing chloroquine drug-baseline standard for future work.

MATERIALS AND METHODS

Cultivation of malaria parasites

Fourteen years old cryopreserved *P. falciparum* chloroquine-sensitive 3D7 (MRA-102; Amsterdam) and chloroquine-resistance W2 (MRA-157; Indochina) strains obtained through BEI Resources, NIAID, NIH in 2006 were cultured by referring to Frederick L. Schuster's method (2002). Both strains were cultivated continuously in RPMI-1640 media, supplemented with 10% AlbuMAX (Thermo Fisher Scientific, USA) and 10% Type O^+ washed RBCs. Culture flask was maintained in a CO_2 incubator at 37±0.5°C under a regulated gas mixture of N_2 (90-92 %), CO_2 (5 %) and O_2 (3-5 %) for optimal parasite growth. The culture was continuously maintained at <10% parasitaemia in a 2-4% haematocrit for 3 months and synchronized every 2 weeks with 5% sorbitol Lambros and Vanderberg, 1979).

In vitro drug susceptibility assay

Drug susceptibility assay was conducted for detection of *P. falciparum* susceptibility to chloroquine diphosphate (molecular weight of base: 319.5; Sigma St. Louis, Mo., USA) by measuring the growth of the parasite using SYBR green 1-based fluorescence detection (Dery et al. 2015). The *in vitro* drug susceptibility assay was performed for at least 3 hours after synchronization of *P. falciparum.* A 180 µL of *P. falciparum* culture (triplicate for each strain) was treated with chloroquine at different concentrations. The chloroquine concentration was diluted 2-fold serial reduction of its concentrations from its working stock (1000 ng/mL) across the 96-well plate. There was also a well-prepared control group (without chloroquine), and only washed RBCs (background calibrator). Each well was resuspended and incubated for at least 72 hours in 37°C, CO_2 incubator chamber (regulated gas contains 5% CO_2) prior to the addition of SYBR Green I (Thermo Fisher Scientific, United Scientific) for fluorescence spectrometry analysis. Analysis using SpectraMax® M5e Microplate Reader (Molecular Device) with excitation and emission wavelength bands cantered at 485 and 530 nm, respectively. The fluorescence value was plotted against the chloroquine concentration and the 50% inhibitory concentration (IC_{50}) was determined by using dose–response curve by non-linear regression analysis using PRISM Version 8.0.2 as described by Le Nagard and Kaddouri (Kaddouri *et al,* 2006) .

DNA extraction

The extraction of genomic DNA was performed by QIAamp DNA Blood Mini Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA extraction was carried out during the trophozoite-predominant developmental stage for both strains. The DNA concentration was measured using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and the DNA concentration was recorded using Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, United States) with quality ranges 1.8 to 2.0 under absorbance 260/280 (Abs260/280).

SNPs determination on Pfcrt and Pfmdr-1 gene

A set of primers were designed via Vector NTi ver 9 by targeting single nucleotide polymorphisms (SNPs) at different codon sites of the gene using sequence obtained from gene bank (Pfcrt accession no: AF030694 and *Pfmdr-1* accession no: S53996) as shown in Table 1 and Table 2, respectively. The conventional PCR was performed with PCR master mix contains of 9.3 µL TE buffer, 4.0 µLPCR reaction buffer, 1.2 µL $MgCl_2$, 1.0 µL forward primer, 1.0 µL reverse primer, 0.4 µL dNTPs and 0.2 µL DNA polymerase. The PCR was performed using Bio-Rad MyCycler™ Thermal Cycler System (Bio-rad, Hercules, California, United States) using the following programs; initial denaturation temperature of 95°C for 15 minutes, followed by 30 cycles of denaturation, annealing and extension at 95°C for 30 seconds, 48-72°C for 45 seconds and 72°C for 60 seconds per kb and final extension at 72°C for 7 minutes. All the PCR products were subjected for purification before sent for sequencing using QIAEX II Gel Extraction Kit (Qiagen, Germany) by following the manufacturer's protocol. The sequencing results were analysed using Bioedit software (version 7.2.5) by aligning the sequences for both parasite strain (3D7 and W2 strain) with gene bank (Pfcrt; AF030694 and Pfmdr-1; S53996).

Ethics approval

Ethical clearance for the study was granted by the Universiti Sains Malaysia, Human Research Ethics Committee with approval number USM/JEPEM/1500049. Informed consent was obtained from all participants, or their parents or legal guardians in case of children and adolescents.

Table 1: List of primer for detection of specific SNPs occurs at position 72, 73, 74, 76, 219, 220, 326, 356 and 371 on the *Pfcrt* gene

Point mutation	Primer sequence	Product size (bp)	Position in genomic (AF030694)
72,73,74,76	**Pfcrt76F** = TGT GCT CAT GTG TTT AAA CTT **Pfcrt76R** = CAA AAC TAT AGT TAC CAA TTT	145	23786-23800 23908-23930
219,220	**Pfcrt219F** = CTC GGA GCA GTT ATT ATT GT **Pfcrt219R** = ATG TTT GAA AAG CAT ACA GG	295	24541-24560 24816-24835
271	**Pfcrt271F** = ATT GTT CAC TTC TTG TCT TA **Pfcrt271R** = GAA AAT CCT ATT TTA CCT CT	260	25031-25050 25271-25290
326	**Pfcrt326F** = ACG GAG CAT GGG TAA GAA GC **Pfcrt326R** = CCC ATA TTT ATT TCC TCT TG	260	25441-25460 25681-25700
356,371	**Pfcrt356F** = TTT CTA CCA TGA CAT ATA C **Pfcrt356R** = CCA AAG TTA CGA AAT CTA AT	319	25801-25819 26101-26120

Table 2: List of primers for detection of specific SNPs occurs on the *Pfmdr1* gene

Point mutation	Primer sequence	Product size (bp)	Position in genomic (S53996)
86,184	**Pfmdr86F** = TAC CTG CAC AAC ATA GAA AAA TTA TT **Pfmdr86R** = TAA AGC CTC TTC TAT AAT GGA CAT	569	146-170 691-714
1034,1042	**Pfmdr1034F** = TGT AAT TTG ATA GAA AAA GCT ATT G **Pfmdr1034R** = TAA ATA AGG ATT TCA TAA AGT CAT C	191	3016-3040 3181-3206
1246	**Pfmdr1246F** = AAT GAA TTT TCA AAC CAA TCT GGA T **Pfmdr1246R** = TTG GTA ATG ATT CGA TAA ATT CAT C	266	3631-3656 3871-3896

RESULTS

Investigation of chloroquine susceptibility was determined by establishment dose-response of two *P. falciparum* lab-adapted strain (3D7; chloroquine sensitive and W2; chloroquine resistant) as shown in Figure 1. The curve shows that the IC_{50} obtained for 3D7 is 32.98nM (10.55ng/mL) whilst for W2 is 691.21nM (221.10ng/mL). Meanwhile, the SNPs analysis shows the amino acid sequence of PfCRT at positions 72, 73, 74, 75 and 76 of 3D7 strain is CVMNK and CVIET for W2 strain (Table 3). The alignment result for *PfMDR-1* gene shows the amino acid sequence at positions 83, 84, 85, 86 and 87 of 3D7 strain is KNMNL while for W2 strain is KNMYL (Table 4).

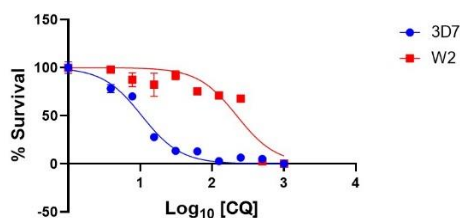

Figure 1: Dose-response curve of survival (%) against Log_{10} [CQ] chloroquine susceptibility for *P. falciparum* 3D7 and W2 strain

DISCUSSION AND CONCLUSION

Continuous exposure of parasite cultures to a chemotherapeutic agent has contributed to the development of drug resistant parasite in the lab. However, the possibility that the susceptibility changes developed in the laboratory after long cryopreservation of the parasites has never been reported. Thus, this study is aimed to investigate the susceptibility of two common lab-adapted *P. falciparum* strains (chloroquine sensitive;3D7 and chloroquine resistant; W2) after 16 years preservation in liquid nitrogen towards chloroquine. Based on Khairul's group study backdated in 2006-2007 showed that chloroquine IC_{50} of 3D7 was 13.84nM (Khairul *et al,* 2006)) and W2 was 208.27 nM (Min *et al,* 2007)). Meanwhile, this recent study shows that the susceptibility of the chloroquine concentration is 3-fold higher of both strains when compared to the IC_{50} before cryopreservation. However, the mechanism or how this happen is not fully understand. One of possible reason is that due to the method applied for the susceptibility test. It happens that last 16 years ago the susceptibility assay was done using hypoxanthine technique where this study using fluorescence-based technique which is reported to be more sensitive.

Radioactively labelled hypoxanthine ($3H^+$-Hypoxanthine) uptake assay was once replaced by fluorescence dye method, as the former assay is cumbersome, expensive and often limited to well-sourced radioactive laboratories and equipment. There are several fluorescence dyes that can be used to stain the DNA, such as DAPI, PicoGreen and SYBR Green I. SBYR Green I was selected for this study due to its low signal-to-noise ratio of 3:1 if compared to DAPI which is 9:1 (Baniecki *et al.*, 2007). SYBR Green I is the preferred dye because it is convenient, relatively rapid, reproducible and less costly than radioisotope (Dennull *et al.*, 2009). The dye has gained popularity it also reduced some hurdles associated with in vitro *P. falciparum* drug susceptibility assay.

Other than that, it is found that prolonged freezing of the parasite might give a stress environment which probably cause changes on the genetic material. Thus, our group tried to investigate this hypothesis by analysing single nucleotide polymorphism (SNP) to two important genes associated with chloroquine resistance in *P. falciparum* which is *Plasmodium falciparum* chloroquine resistance transporter protein (PfCRT) and *Plasmodium falciparum* multiple drug resistance 1 protein (PfMDR-1) that encoded by the *PfCRT* and *PfMDR-1* gene respectively. Moers suggested that *PfCRT* and *PfMDR-1* genes played an important role in the development of chloroquine-resistance (Moers *et al.,* 2015). Besides, Vathsala claimed that *PfMDR-1* that encoded the p-glycoprotein was initially proposed as a determinant of the chloroquine-resistance phenotype (Vathsala *et al.,* 2004). These proteins can be found

at the food vacuole membrane of *P. falciparum*.

From the alignment result, we indicate that CQ resistant *P. falciparum* Dd2 strain encoded an amino acid pattern CIETSETI at positions 72, 74, 75, 76, 219, 220, 271, 356 and 371 on *Pfcrt* gene and CMNKAQIR respectively in 3D7, CQ-sensitive *P. falciparum* strain. Meanwhile, FYSND for resistance and NYSND for sensitive strain at positions 86, 184, 1034, 1042 and 1246 on *Pfmdr1*. Our *Pfcrt* results are consistently similar as reported by other studies. However, Shilea A. Peel, reported that the putative mutation at codon position 86 of W2 in *Pfmdr1*, referred to Thailand strain K1, which resulting in asparagines (AAT) to tyrosine (TAT) changes at amino acid 86 which can cause the W2 strain resistant to other drug such Mefloquine (Mef) (Peel *et al.*, 1994).

Even though our finding shows that the level of chloroquine susceptibility increases to 3-fold for each strain after being revived from cryopreservation, but the SNPs results reveal no changes at gene level of two proteins associated with the chloroquine resistant and our SNP finding is consistent with other studies (Antony *et al.*, 2016, Cui *et al.*, 2015).

Table 3: SNPs analysis of Pfcrt gene

Codon position	AFO30694 (resistant type)	Sequencing Results	
		W2	3D7
72	TGT (Cys)	TGT	TGT
74	ATT (Ile)	ATT	ATG (Met)
75	GAA (Glu)	GAA	AAT (Asn)
76	ACA (Thr)	ACA	AAA (Lys)
219	AGT (Ser)	AGT	AGT (Ser)
220	TCC (Ser)	TCC	GCC (Ala)
271	GAA (Glu)	GAA	CAA (Gln)
326	AGC (Ser)	ND	ND
356	ACA (Thr)	ACA	ATA (Ile)
371	ATA (IIe)	ATA	AGA (Arg)

ND; not detected, Cys; Cysteine, Ile; Isoleucine, Glu; Glutamic acid, Thr; Threonine, Ser; Serine, Met; Methionine, Asn; Asparagine, Lys; Lysine, Ala; Alanine, Gln; Glutamine, Arg; Arginine

Table 4: SNPs analysis of Pfmdr-1 gene

Codon position	S53996 (resistant type)	Sequencing Results	
		W2	3D7
86	TAT (Tyr)	TAT	AAT (Asn)
184	TTT (Phe)	TAT	TAT (Tyr)
1034	TGT Cys)	AGT	AGT (Ser)
1042	GAT (Asp)	AAT	AAT (Asn)
1246	TAT (Tyr)	GAT	GAT (Asp)

Tyr; Tyrosine, Phe; Phenylalanine, Cys; Cysteine, Asp; Aspartic acid, Asn; Asparagine, Ser; Serine, Asp; Aspartate

DISCLOSURES

All authors declare that there is no conflict of interest regarding the publication of this article.

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