รายงานวิจัยฉบับสมบูรณ์

โครงการ

การระบุเครื่องหมายชีวภาพดื้อยาจากเชื้อมาลาเรียดื้อยาอาร์ทิมิซินิน

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ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

กิตติกรรมประกาศ (Acknowledgement)

คณะผู้วิจัยขอแสดงความขอบคุณสำนักงานกองทุนสนับสนุนการวิจัยและศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติที่กรุณาให้การสนับสนุนการวิจัยนี้ ขอขอบคุณผู้ให้ความอนุเคราะห์พลาสมิดและเชื้อมาลาเรีย ดังนี้ พลาสมิด pFCEN1 จาก Professor Shiroh Iwanaga, Tokyo Medical and Dental University, Japan และเชื้อมาลาเรีย *Plasmodium falciparum* สายพันธุ์ K1, MRA-1236 (IPC 3445, Pailin Cambodia 2010), MRA-1237 (IPC 3663, Pailin Cambodia 2010), MRA-1238 (IPC 4884, Pursat Cambodia 2011), MRA-1239 (IPC 5188, Rattanakiri Cambodia2011), MRA-1240 (IPC 5202, Battambang Cambodia 2011), MRA-1241 (IPC 4912, Mondolkiri Cambodia 2011) จาก Malaria Research and Reference Reagent Resource Center (MR4)

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ABSTRACT

In this study, we applied Plasmodium Artificial Chromosome (PAC) in the identification of genetic markers conferring resistance to dihydroartemisinin (DHA). Six artemisinin-resistant *P. falciparum* reference lines from the parasite depository at Malaria Research and Reference Reagent Resource Center (MR4), USA, were re-assessed for their level of resistance to DHA using the Ring-stage Survival Assay (RSA). We confirmed that the parasites line number MRA1236, MRA1240, and MRA1241 exhibited various degrees of resistance to the DHA treatment whereby MRA1240 displayed the highest artemisinin resistance among the resistant isolates in accordance with the published report. Gene library of MRA1240 isolate has been constructed using PAC technology. Using modified protocol, matured schizont-stage of the artemisinin-sensitive *P. falciparum* laboratory strain 3D7 parasites were purified and transfected with the PAC plasmid containing the MRA1240 gene library. The transgenic parasites harboring the pPAC1240 gene library plasmids containing the DNA fragments (library) were obtained as confirmed by Southern analysis. These transgenic parasites were assessed for their artemisinin sensitivity using the RSA. The results showed that all of them were sensitive to DHA, at the similar level to the 3D7 parental strain. However, for selection of DHA resistant transgenic parasites, another selection strategy using SAAR method was employed. After 4 rounds of low dose DHA treatment, we could obtain DHA-resistant 3D7_PAC1240 transgenic parasites which were subsequently isolated to single clones. One of the transgenic clones, 2G2, was analyzed for its inserted MRA1240 gene library using genome walking technique. The Southern, BLAST and chromosome analyses showed that the inserted MRA1240 gene library include a genome fragment of approximately 20 kB down-stream of the PF3D7_1448300.1 gene (coding for conserved protein with unknown function), which includes PF3D7_148200.1 (coding for condensin-2 complex subunit G2, putative), PF3D7_148100.1 (coding for conserved protein, unknown function), PF3D7_148000.1 (U3 small nucleolar RNA-associated protein 12, putative), and interestingly PF3D7_147900.1 (coding for multidrug resistance protein 2). From all the results and analysis, multidrug resistance protein 2 (MDR2) is the most promising gene candidate that may contribute to drug resistance to DHA in our study. We are in the process of reconfirming the role of PfMDR2 in DHA resistance by over-expression of PfMDR2 in the DHA-sensitive 3D7 parasite. We hypothesize that over-expression of MDR2 will make sensitive parasite become more resistance to DHA comparing to its parental parasite.

Although not proposed in the original proposal, as another approach to study the artemisinin resistance mechanism, we decided to study the currently proposed molecular marker for artemisinin resistance, PfKelch13, in more details by generating transgenic parasites harboring double and triple mutations of the K13 proteins and examine whether they have an effect on the degree of artemisinin resistance. Plasmids for introduction of different K13 mutation combinations to the parasites, pCas.BSgK13F and a series of pL6.SgK13B.K13mut, have been constructed and transfected to *k13* wild-type K1 parasite. At present, we have obtained transgenic parasites transfected with the plasmids. We are in the process of confirming sequences of the *k13* locus and assessing artemisinin sensitivity of these transgenic parasites.

In conclusion, this study has proposed PfMDR2 as a promising candidate that may contribute to the artemisinin resistance, although more studies are needed to confirm this finding. We hypothesize that over-expression of PfMDR2 will make sensitive parasite become more resistance to DHA comparing to its parental parasite. Once confirmed, PfMDR2 would be proposed as the marker for monitoring the progress of drug resistant parasites that will allow the public health authorities to plan effective treatment and control the spread of resistant parasites in the endemic areas.

Keywords: Artemisinin, drug resistance, biomarkers, Plasmodium Artificial Chromosome (PAC), *Plasmodium falciparum*

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1. Introduction

Malaria is an important infectious disease that affects people living in tropical and subtropical regions of the world. The World Health Organization estimates that there were 219 million infected cases and 435,000 deaths in the year 2017 (World Malaria Report 2018). Although the highest burden of disease is in sub-Saharan Africa, countries in Southeast Asia, South America and South Pacific are still highly endemic to malaria including Thailand. Areas boarding neighboring countries, such as Cambodia and Myanmar are highly endemic with reports on drug tolerant/resistant *P. falciparum* parasites, particularly, to the front-line drug artemisinin derivatives. Recent report describing the possibility of artemisinin resistance developed by the parasites spreading in Southeast Asia (Ashley et al., 2014) has sent an alarming signal that the parasites are developing resistance to all the available antimalarial drugs and that the control and treatment of malaria parasites will be difficult. Therefore, there is an urgent need to monitor the resistant parasites and limit their prevalence by planning effective treatment and control the spread of resistant parasite in the endemic areas.

Although not all mechanisms of drug resistance developed by the malaria parasites are known, the common mechanisms include point mutations or increased copy numbers of the drug targets as well as their associated proteins. Resistance to the antifolate drugs pyrimethamine and cycloguanil is caused by point mutations of the enzyme dihydrofolate reductase (DHFR) (Cowman et al., 1988), while resistance to the sulfonamides and sulfones is by mutations in the enzyme dihydropteroate synthase (DHPS) (Triglia et al., 1997; Sibley et al., 2001). Chloroquine drug resistance is reported to be mediated by a food vacuole membrane transporter molecule, chloroquine-resistance transporter (CRT), which contains mutation at position 76 from lysine to threonine as well as other mutations (Fidock et al., 2000). The level of resistance is also modulated by a second transporter, multi-drug resistance protein-1 (MDR-1). Polymorphisms and increased copy numbers of the *Pfmdr-1* gene also affect responses to a number of drugs (Cowman et al., 1994). Recent studies described K13-propeller polymorphisms involves the resistance to artemisinins of *Plasmodium falciparum* in patients (Ariey et al., 2014; Ashley et al., 2014).

To identify mutations involving drug resistance, parasite DNA collected from infected patients was subjected to conventional DNA sequencing of the known target genes. Other experimental techniques used to determine the parasite genes responsible for resistance involved classical genetic approaches, such as laborious genetic crosses (of drug resistant and sensitive parasites) followed by ‘phenotypic typing’ of cloned progeny and subsequent RFLP analysis. However, this analysis is very complicated and has only resulted in the identification of a few drug resistance genes such as *Pfcrt* the gene that plays a key role in chloroquine resistance in *P. falciparum* (Fidock et al., 2000; Su et al., 1997; Wellems et al., 1990; 1991). Recently, the next generation high-throughput sequencing technology (Bennett et al. 2005) and DNA chip (Jiang et al., 2008) were applied to perform genome-wide association study to identify drug resistance genes (Jeffares et al., 2007; Volkman et al., 2007). These technologies could be used to identify

microsatellites and single nucleotide polymorphisms (SNPs) that differ between sensitive and resistant parasite lines. However, these approaches are limited in that most SNPs are not indicative of drug resistance (Miotto et al., 2013), and thus each identified candidate resistance gene must be subsequently functionally characterized in *Plasmodium* parasites to determine and confirm the drug resistance phenotype. The whole-genome sequencing of parasite lines from Africa and clinical isolates from Cambodia showing delayed parasite clearance after treament with artemisinins suggested mutations in the PF3D7_1343700 kelch propeller domain ('K13-propeller') are associated with artemisinin resistance (Ariey et al., 2014). Very recent study using CRISPR/Cas9 genome editing tool has introduced candidate mutation to K13-propeller protein and shown that the transgenic parasites were more tolerant to artemisinin (Ghorbal et al., 2014). Although K13-propeller protein was proposed as candidate marker for artemisinin resistance, some parasites collected from infected patients which harboured K13-propeller polymorphisms still have very rapid parasite clearance rate, when treated with artemisinins. And conversely some parasites that had delayed parasite clearance half-life did not harbor associated K13-propeller polymorphisms (Ashley et al., 2014). The use of K13-propeller protein as biomarker for monitoring the artemisinin resistance is then still in question. Other genetic biomarkers for artemisinin resistance are yet to be discovered. Therefore, the effective method to identify the functional drug resistance marker(s), not only detecting SNPs, is needed and used to identify the candidate functional drug resistance gene(s) from the drug resistant parasites, especially to artemisinins.

Recently, our collaborator Iwanaga et al. has developed a novel system to identify drug resistance genes from *Plasmodium* parasites (Iwanaga et al., 2010). The system involves the steps of generation of *Plasmodium* gene library in the "*Plasmodium artificial chromosome* (PAC)", introduction of the gene library into sensitive parasites, selection of parasites acquiring resistance to antimalarials, recovering of PAC and identification of drug resistance gene(s). It has been shown that the PAC promotes high level of transfection efficiency, the bottle-neck of transgene technique in *Plasmodium* study, with improvement of 100-1,000 times better than existing transfection protocols (Iwanaga et al., 2012). With these improvements, this system enables the identification of a resistance gene much faster than other methods. Furthermore, the identification system using the PAC has greater advantages than other methods on: (1) shorter time is required for the identification of functional drug resistance gene from parasite and (2) the functional resistance genes can be directly identified by drug screening.

In this study, we aim to apply PAC technology, which was developed by Prof. Iwanaga, currently at Tokyo Medical and Dental University, Japan, in identification of novel genetic markers conferring resistance to dihydroartemisinin and its partner drug mefloquine from (artemisinin-resistant) reference *P. falciparum* lines obtained from the parasite depository at Malaria Research and Reference Reagent Resource Center (MR4), USA. The parasites have been re-assessed for their artemisinin resistance *in vitro* using Ring-stage Survival Assay (RSA) (Witkowski et al., 2013). The resistant parasite's gene library has been generated using the PAC technology and transfected to

the sensitive *P. falciparum* 3D7 strain. The artificially generated transgenic resistant parasites have been selected with dihydroartemisinin. The PAC gene library containing resistance genes have been recovered from transgenic resistant parasites and the introduced resistance gene has been identified by genome walking and DNA sequencing. Currently the selected candidate drug resistance gene is being confirmed by re-introduction to sensitive *P. falciparum* parasites. The results are yet to be reported by further communication to the TRF.

2. Objectives of the study

The main objective of this study is to identify novel genetic markers conferring resistance to dihydroartemisinin from artemisinin-resistant reference *P. falciparum* lines using *Plasmodium Artificial Chromosome* (PAC) technology. The identified markers will be used to monitor the progress of drug resistant parasites that will allow the public health authorities to plan effective treatment and control the spread of resistant parasites in the endemic areas.

3. Materials and Methods

3.1 Plasmids and *Plasmodium falciparum* parasites

A plasmid containing *P. falciparum* Artificial Chromosome (pFCEN1) (Iwanaga et al., 2012) was kindly provided by Professor Shiroh Iwanaga, currently at Tokyo Medical and Dental University, Japan. The laboratory *P. falciparum* strains 34D7 and K1 as well as the artemisinin-resistant (delayed parasite clearance) reference *P. falciparum* isolates, MRA-1236 (IPC 3445, Pailin Cambodia 2010), MRA-1237 (IPC 3663, Pailin Cambodia 2010), MRA-1238 (IPC 4884, Pursat Cambodia 2011), MRA-1239 (IPC 5188, Rattanakiri Cambodia2011), MRA-1240 (IPC 5202, Battambang Cambodia 2011), MRA-1241 (IPC 4912, Mondolkiri Cambodia 2011) were obtained from the parasite depository at Malaria Research and Reference Reagent Resource Center (MR4), USA.

3.2 Ring-stage Survival Assay (RSA)

The Ring-stage Survival Assay (RSA) (Witkowski et al., 2013) was performed by applying a high-dose of dihydroartemisinin (DHA) of 700 nM to the 0.5 - 1% parasitemia of highly synchronous young ring-stage parasites (0 - 3 hour-post-invasion) at 2% hematocrit for exactly 6 h. Dimethyl sulfoxide (DMSO) was used as a control treatment. After 6-hour treatment, the parasites were extensively washed with RPMI-1640 media to completely remove the drug. The treated parasites were then put back into the normal culture condition for an additional 66 hours and thin blood smears were prepared from all of the tested isolates as well as the laboratory strain as control. The survival parasites were then microscopically counted and calculated for the initial, DMSO-treated, and DHA-treated parasitemia of each parasite.

3.3 Generation of transgenic *P. falciparum* parasites harbouring gene library from DHA-resistant parasite using *P. falciparum* Artificial Chromosome technology

3.3.1 Construction of gene library from DHA-resistant parasite

Genomic DNA of DHA-resistant parasite was partially digested with restriction enzyme Sau3AI for 1 min and then was separated on agarose gel electrophoresis. The fragments of digested DNA with the size over 10 kB were then extracted, purified and ligated to the BamHI-digested pFCEN1 plasmid backbone, of which 5′ phosphates were treated with alkaline phosphatase to prevent self-ligation, to generate the gene library of those drug resistant parasites.

3.3.2 Transfection of gene library to drug-sensitive *P. falciparum* 3D7 parasites, and selection of transfected parasites

The generated gene library in pFCEN1 plasmid was transfected into the Percoll-purified schizonts of drug-sensitive *P. falciparum* 3D7 parasite using optimized and high efficiency *P. falciparum* transfection protocol and Amaxa™ Nucleofector™ II program U-33. Multiple transfection experiments were performed to ensure the coverage of whole gene library. The transfected

parasites bearing pFCEN1 plasmid containing gene library were firstly selected using 2 nM of WR99210 to exclude the background non-transfected 3D7 parasites. The transfected parasites were stably cultured in the presence of the WR99210 until they were used for further experiments.

3.4 *In vitro* Simple Assay for Artemisinin Resistance (SAAR) method

A modification of RSA method, named Simple Assay for Artemisinin Resistance (SAAR), for assessing the sensitivity to DHA and selection of artemisinin-resistant of *P. falciparum* parasites was developed by Professor Shiroh Iwanaga at Tokyo Medical and Dental University, Japan (submitted Japan patent application number: 2016-151355). The *P. falciparum* parasites at 0.1% mixed stages and 2% hematocrit were treated with final concentration of 5 nM DHA for 4 consecutive days (Day 0-3), with fresh complete media and DHA changed everyday. On day 4, the drug was removed and the culture was continued in complete media without drug. Thereafter, the parasite growth was observed by thin blood smear and Giemsa's staining. The % parasitemia was analysed by counting the viable parasite in 1,000 RBC at days 4 and 8 or every 4 days until the parasites reappeared in the culture. The drug resistant parasites would be more tolerate and reappear back in the culture faster than those that are more sensitive to the drug.

3.5 Genome Walking and Sequence Analysis

Five micrograms genomic DNA of the parasites were digested in 200 µl reaction volume with 50 U of SspI restriction enzyme for overnight at 37°C. After the digestion, 5 µl of each reaction was loaded onto a 1% agarose gel to determine digestion efficiency. The remaining DNA was extracted twice with phenol/chloroform (1:1; v/v) and then precipitated by addition of 0.5x volume of 3 M ammonium acetate, 2 µg yeast tRNA, and 2.5x volume of absolute ethanol. After mixing, the tubes were incubated for 20 minutes at -80°C. Each pellet was washed twice with 85% ethanol, air dried, and dissolved in 20 µl sterile deionized H_2O. The digested gDNA was ligated with 80 pmol adaptor for 20 hours at 16°C (20 µl total volume). The reaction was stopped at 75°C for 15 minutes and then diluted with 180 µl of sterile deionized H_2O. Unligated genomic DNA-adaptors were removed by 200 U of Exonuclease III (ExoIII) by incubation for 20 hours at 37°C. The reaction was terminated by 15 minutes incubation at 75°C and kept at 4°C until further analysis.

For amplification of the inserted end, each PCR (50 µl total volume) contained PCR buffer, 5 mM dNTPs, 5 µM each APN and PAC walk R1 primers, 4 µl (approximately 50 ng) library DNA, and 0.5 U GoTaq® DNA Polymerase. All reactions were carried out in thin-walled tubes with 7 cycles at 25 seconds at 93°C and 3 minutes at 72°C, followed by 32 cycles at 25 seconds at 93°C, 3 minutes at 67°C, and 4 minutes extension for a last cycle. PCR products were analyzed using agarose 1.5% gel electrophoresis.

Finally, the amplified DNA fragments were cloned into the pGEM®-T Easy Vector (Promega), and the plasmids in each clone were digested with EcoRI restriction enzyme for checking

the inserted fragments. The sequence of inserted fragments were determined by DNA sequencing using the T7 universal primer, and compared to the Plasmodium genome using BLAST analysis to identify the gene candidates.

4. Results and Discussion

4.1 Reassessment of the artemisinin resistance phenotype of the reference *P. falciparum* Cambodian isolates from the MR4 depository

Prior to the generation of PAC plasmid containing gene library of the artemisinin-resistant isolate, it was of interest to confirm whether these isolates remain resistant to DHA in our culture system. Six *P. falciparum* isolates from Cambodia were obtained from the MR4 depository, four of which were reported to exhibit artemisinin-resistant characteristics and the other two, served as controls for this experiment, were defined as artemisinin-sensitive isolates. The assessment of the artemisinin resistance phenotype was done following the Ring-stage Survival Assay (RSA) protocol specifically established to evaluate the artemisinin resistance (Witkowski et al., 2013). The major characteristic of artemisinin-resistant parasites is the delayed clearance after treatment with the drug compared to the sensitive parasites. It was suggested that the resistant parasites managed to tolerate the effect of artemisinin by quiescence at the ring stage (Witkowski et al., 2010). As shown in **Figure 1** and **Table 1**, MRA1236, MRA1240, and MRA1241 exhibit various degrees of resistance to the DHA treatment in accordance with the MR4 information sheets whereby MRA1240 displayed the highest artemisinin resistance among the resistant isolates. A slight resistance, 1.9% survival rate, to DHA treatment of MRA1237, reported as artemisinin-sensitive isolate, was observed. On the other hand, the reported mild artemisinin-resistant MRA1238 appeared sensitive in our study. It is important to note that the survival percentages of resistant isolates in our hands are lower than those reported in the MR4 information sheets, e.g. 88% survival rate in the MR4 documentation vs 30.6% survival rate in this study for the MRA1240 (**Table 1**). Nonetheless, gDNA of MRA1240 was then used to generate a gene library for transfection using PAC technology in an attempt to identify the gene(s) responsible for the artemisinin resistance phenotype.

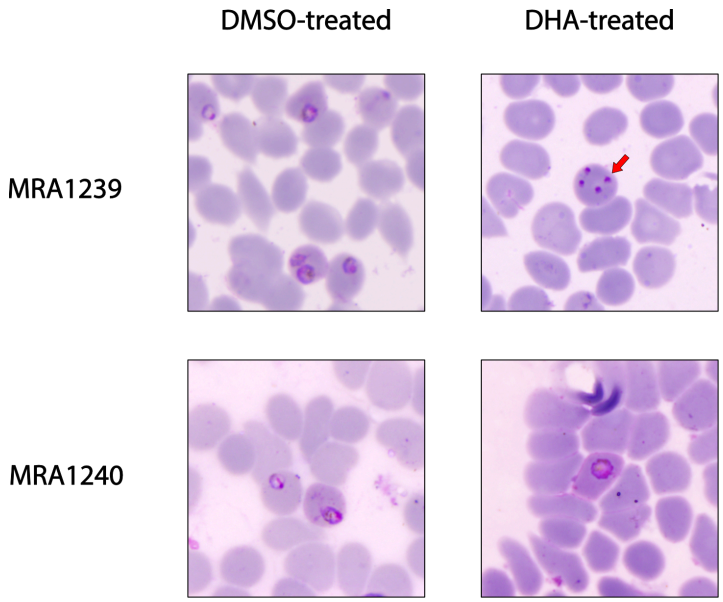

Figure 1. **Thin blood smears of Cambodian *P. falciparum* isolates demonstrating the resulting phenotype of artemisinin-sensitive and artemisinin-resistant parasites after dihydroartemisinin treatment in the Ring-stage Survival Assay.** Samples of thin blood smears showing the RSA results of the artemisinin-sensitive isolate, MRA1239, and the artemisinin-resistant isolate, MRA1240, are shown. After 72 hours since the beginning of the assay, the DMSO-treated controls of both MRA1239 and MRA1240 reached the trophozoite stage as expected. When they were treated with 700 nM DHA for 6 hours at young ring stage, the artemisinin-sensitive MRA1239 became pyknotic (red arrow), a characteristic of DHA killing effect on the *Plasmodium* parasites. However, 30.6% of the artemisinin-resistant MRA1240 remained visually normal looking ring- and trophozoite-stage parasites (example shown for the trophozoite stage only) confirming that this isolate has reduced susceptibility to the dihydroartemisinin treatment.

Table 1. Survival percentages of the *P. falciparum* isolates and laboratory strain in Ring-stage Survival Assay.

Parasites	Initial parasitemia (%)	DMSO-treated parasitemia (%)	DHA-treated parasitemia (%)	Survival rate (%)	Reported survival rate (%)
K1 (lab strain)	0.19	1.23	0	0	-
MRA1236	1.47	2.46	0.15	6.2	27.3
MRA1237	0.42	1.73	0.03	1.9	0.1
MRA1238	0.21	0.82	0	0	6.2
MRA1239	1.46	5.61	0	0	0.1
MRA1240	0.71	1.08	0.33	30.6	88.2
MRA1241	2.52	6.80	1.53	22.5	49.3

4.2 Selection of *P. falciparum* MRA1240 parasite population that are highly resistant to artemisinin

We hypothesised that we would be able to obtain highly artemisinin-resistant parasites, as monitored by an increasing survival rate, following multiple treatments with a high dose of DHA. MRA1240 was selected for this experiment as it initially demonstrated the highest resistance to DHA among the parasite isolates providing a better chance to select for the highly resistant parasites. The parasites were initially synchronised by sorbitol treatment to achieve 1-2 % parasitaemia of ring-stage parasites, approximately 0 – 8 hour-post-invasion. They were then treated with 700 nM of DHA for 6 hours followed by washing the drug off. The survived parasites were cultured and prepared for the subsequent round of DHA treatment. MRA1240 that had been exposed to DHA for 5 and 10 times were evaluated for their resistance by means of survival rate following the RSA. As presented in **Table 2**, the survival rates of DHA-exposed MRA1240, both 5 times and 10 times, were comparable to the unexposed control MRA1240. It could be that 10 times DHA exposures of 700 nM of DHA were still not enough, but higher dose of DHA with more rounds of DHA treatments might be required to select for exceptionally artemisinin-resistant parasite population. It is also important to note that the survival rate for the MRA1240 isolate in this experiment was lower than the result in section 4.1. This presumably dues to the difference in the parasite growth rates, being around 1.5 - 2 folds per cycle in the former experiments and around 3 - 4 folds per cycle in this experiment. With the hypothesis regarding the artemisinin resistance mechanism involves having slower growth and undergoing quiescence at ring-stage, the result can be implied that the active parasites are more prone to the DHA activity.

Table 2. Survival rates of the untreated and multiple times DHA-treated parasites following Ring-stage Survival Assay.

Parasites	Survival Rate (%)
MRA1240	13.05
5× DHA-treated MRA1240	14.96
10× DHA-treated MRA1240	13.65

4.3 Preparation of the gDNA from the artemisinin-resistant *P. falciparum* isolate, MRA1240, and the PAC plasmid for gene library construction

The artemisinin-resistant *P. falciparum* isolate, MRA1240, was harvested by saponin lysis and subjected to gDNA extraction using phenol/chloroform-based method. Fifty micrograms of the gDNA were aliquoted and precipitated in ethanol until use. For the PAC plasmid, pFCEN1, five micrograms were prepared per normal plasmid extraction method. The strategy to construct the gene library for parasite transfection was described in the Materials and Methods section. pFCEN1 plasmid backbone was carefully and completely digested with BamHI, and treated with alkaline

phosphatase to prevent self-ligation. For the preparation of gene library from MRA1240 isolate, the total MRA1240 DNA was carefully digested (1 minute with Sau3AI) and gel-purified. The digested MRA1240 gene library was then ligated to BamHI–digested pFCEN1 plasmid backbone to obtain the pPAC1240 library plasmids. In order to obtain gene library that covers the whole genome of the parasite, a large amount of genomic DNA fragments that successfully ligated to the PAC plasmid were needed for multiple transfection experiments.

4.4 Optimization of protocol to obtain the highly synchronous schizonts for transfection

The preparation of highly synchronous schizonts is a key step for successful transfection of *P. falciparum*, which is known to be at low efficiency. We have spent quite a period of time to to optimize the protocol to obtain the highly synchronous schizonts. This method resembles the strategy used to transfect a rodent malaria parasite, *P. berghei*, which proves to be highly efficient. In this study, the Percoll-purified schizont-stage parasites, approximately 42-48 hour-post-invasion, were initially treated with a cysteine protease inhibitor, E64, for around 6 hours to allow complete maturation of the merozoites inside the cells without egress of the parasites. This method allowed us to obtain fully matured schizonts, with mature merozoites, ready for highly efficient transfection experiments (**Figure 2**).

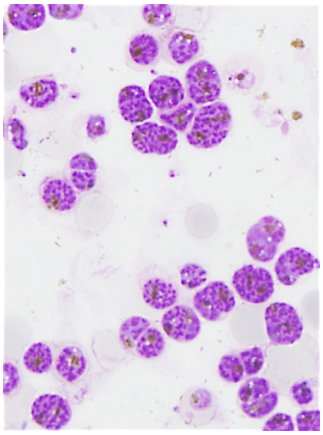

Figure 2. Improvement of the purification of schizont-stage parasites for highly efficient transfection experiments. The Percoll-purified schizont-stage parasites, approximately 42-48 hour-post-invasion, were initially treated with a cysteine protease inhibitor, E64, for around 6 hours to allow complete maturation of the merozoites inside the cells without egress of the parasites.

4.5 Generation of transgenic *P. falciparum* carrying gene library of the artemisinin-resistant isolate MRA1240 using PAC technology

The PAC plasmid containing gene library of MRA1240, the highest artemisinin-resistant isolate among the three resistant lines, was constructed and used to transfect the highly synchronized schizont stages of artemisinin-sensitive 3D7 parasites using Amaxa™ Nucleofector™ II program U-33. Following the positive drug selection using 2 nM WR99210, we successfully obtained the transfected parasites, named 3D7_PAC1240, which harbor the gene library of MRA1240. The control transfected parasites carrying the original pFCEN1 plasmid without the inserted gene library of MRA1240, named 3D7_pFCEN1, were also achieved. The transgenic parasites were further verified by Southern blot analysis to detect whether the transgenic parasites harbor library plasmids.

Figure 3 showed the pFCEN1 plasmid map with restriction digestion sites and the Southern analysis results. The digestion of total DNA of the control transgenic 3D7_pFCEN1 parasite with AflII (and SpeI) enzymes yield a DNA band at 8 kB as expected when probed with hDHFR probe, while digestion of total DNA from transgenic 3D7_PAC1240(1) with AflII and SpeI enzymes showed bands at around 2 and 3 kB, respectively. Total DNA from another batch of transgenic parasite 3D7_PAC1240(2), digested with AflII, showed multiple bands at around 3, 4.5, >10 and >48.5 kB. These results confirmed that a diverse population of transgenic parasites harbouring PAC1240 library plasmids containing inserted DNA fragment at the designated BamHI site have been obtained (**Figure 3**).

In our study, another choice of artemisinin-sensitive parasite served as a recipient for the PAC library plasmids were other artemisinin-sensitive Cambodian parasite isolates, either MRA1252 or MRA1253, which have mutations in multiple genes responsible for drug resistance, i.e. dihydrofolate reductase (*dhfr*), dihydropteroate synthase (*dhps*), chloroquine resistance transporter (*crt*), and multidrug resistance 1 (*mdr1*). However, we found that these parasite isolates did not grow well in our laboratory. Therefore, they were not used in our transfection experiments that require high recovery rate after transfection.

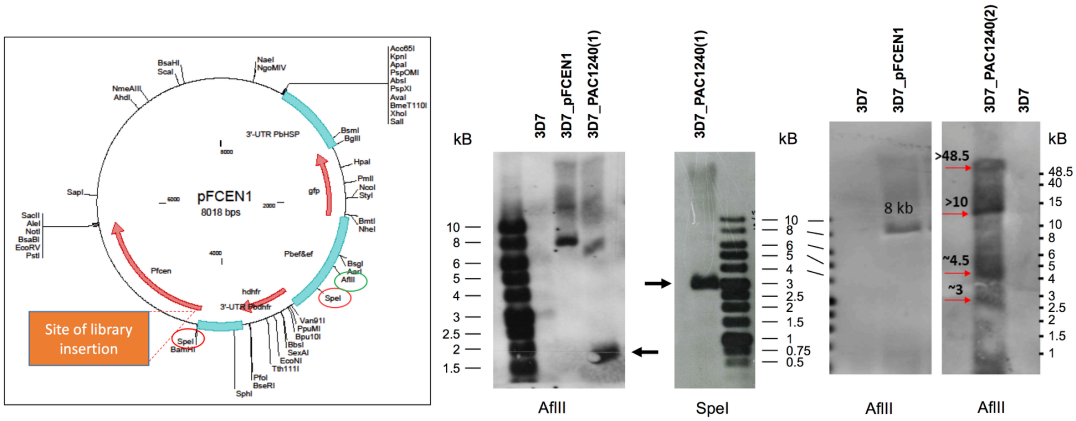

Figure 3. Southern analysis examining the genetic contents of transgenic parasites.

AflII- and SpeI-digested total DNA from the parental and transgenic parasites; 3D7, 3D7_pFCEN1, 3D7_PAC1240(1) and 3D7_PAC1240(2) were subjected to Southern blot analysis probing for the human dihydrofolate reductase (hDHFR) gene presented in the transfection plasmids. No band was detected in the wild-type 3D7 as expected. The control transgenic 3D7_pFCEN1 showed a band around 8 kilobases (kB) representing the size of original pFCEN1 plasmid without the insertion of gene library. Digestion of total DNA from transgenic 3D7_PAC1240(1) showed band of 2 and 3 kB when digested with AflII and SpeI, respectively (arrows), while of total DNA from transgenic 3D7_PAC1240(2) showed bands of 3, 4.5, >10 and >48.5 kB when digested with AflII.

4.6 Assessment of the sensitivity to DHA of the transgenic parasites using RSA method

The sensitivity to DHA of generated transgenic 3D7_PAC1240(1) and 3D7_PAC1240(2) harbouring the gene library of MRA1240, were assessed using the RSA as described in the Materials and Methods. Interestingly, the results showed that both batches of transgenic 3D7_PAC1240 parasites were sensitive to DHA, at the similar level to the 3D7 parental strain (**Table 3**), suggesting that the transfected gene library of MRA1240 did not introduce DHA resistance phenotype to the 3D7 parasites. However, we explored further whether we can use other phenotypic assays to assess for DHA resistance and select for those drug resistant transgenic parasites.

Table 3. Sensitivity to the DHA of the transgenic parasites using Ring-stage Survival Assay.

Parasites	INI	DE	DHA	Growth rate	% Survival
3D7	0.72	5.85	0.041	8.12	0.70
Transgenic 3D7_PAC1240(1)	0.58	4.25	0.027	7.33	0.64
Transgenic 3D7_PAC1240(2)	0.47	4.13	0.33	8.78	0.79

Note: **INI** = Initial parasitaemia at 0 h, **DE** = DMSO-exposed parasitaemia at 72 h, **DHA** = DHA-exposed parasitaemia at 72 h, **Growth rate** = DE/INI, % **Survival** = (DHA/DE) x 100

4.7 Assessment of the sensitivity to DHA using Simple Assay for Artemisinin Resistance (SAAR) method

A modification of RSA method, Simple Assay for Artemisinin Resistance (SAAR), for assessing the sensitivity to DHA and selection of artemisinin-resistant *P. falciparum* parasites was developed by Professor Shiroh Iwanaga (See Materials and Methods). This method reproduces the clinical treatment of malaria patients with DHA for 4 consecutive days, with fresh DHA treatment everyday. Instead of 1 high dose of DHA (at 700 nm) treatment for 6 hours as in the RSA method, this method employs everyday re-supplying and maintaining of low-dose 5 nM DHA to parasite culture. Those parasites that are more resistant to DHA would re-appear back in the culture faster than those that are more sensitive to DHA. **Table 4** showed the testing results of artemisinin-sensitive *P. falciparum* lab strain 3D7 parasites, and MRA1240, artemisinin-resistant Cambodian isolate, by SAAR method. The artemisinin-resistant MRA1240 isolate re-appeared in the culture as soon as day 4 (0.02 ± 0.01% parasitemia) and reached 1.11 ± 0.54% parasitemia at day 8, while artemisinin-sensitive 3D7 reached only 0.11 ± 0.01% parasitemia at day 8. These results confirmed that MRA1240 isolate is resistant to DHA, and this SAAR could be used to select DHA resistant parasites.

Table 4. Assessment of the sensitivity to DHA of the transgenic parasites using Simple Assay for Artemisinin Resistance (SAAR) method.

Parasites	type	parasite growth (10 replicates) (mean of % parasitemia ± SD)	
		Day 4	Day 8
3D7	ART sensitive	0	0.11 ± 0.01
MRA1240	ART resistant	0.02 ± 0.01	1.11 ± 0.54

4.8 Selection of artemisinin-resistant transgenic 3D7_PAC1240 parasites using SAAR method

SAAR method was employed to select for those transgenic parasites that were resistant to DHA. *P. falciparum* lab strain 3D7, and transgenic 3D7_PAC1240(1) and 3D7_PAC1240(2) parasites at 0.1% mix stage and 2% hematocrit were treated with 5 nM DHA for 4 days (drug and media changed everyday). The parasite growth was observed by thin blood smear and Giemsa's staining every 2-4 days. In our study, once the parasites reappeared in the culture, the SAAR method was repeated 3 more times. As shown in **Figure 4**, at round 4 of SAAR selection, transgenic 3D7_PAC1240(2) well_1 showed high tolerance to DHA treatment, but not for transgenic 3D7_PAC1240(2) well_2. The laboratory strain 3D7 also showed slight resistance to DHA since the parasites reappeared around day 11 post drug treatment. The slight resistance to DHA of 3D7 was also observed in Iwanaga Lab at TMDU (personal communication). The transgenic 3D7_PAC1240(2) well_1 parasites were then used for identification of the (DHA-resistance) gene introduced in these transgenic parasites.

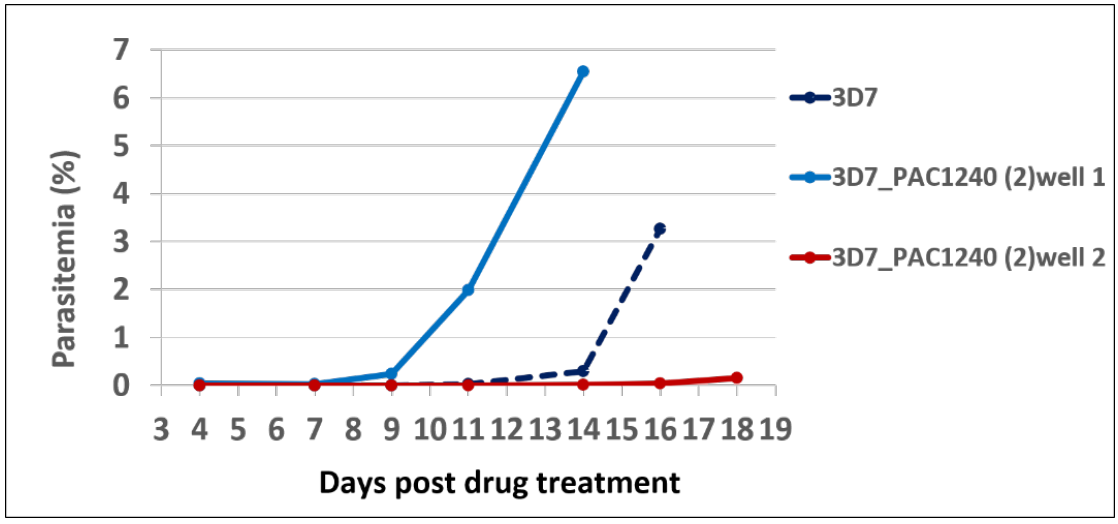

Figure 4. Selection of artemisinin-resistant transgenic 3D7_PAC1240 parasites using SAAR method. *P. falciparum* lab strain 3D7, and transgenic 3D7_PAC1240 (1) and 3D7_PAC1240(2) parasites were treated with 5 nM DHA for 4 days (drug and media changed everyday). The parasite growth was observed by thin blood smear and Giemsa's staining every 2-4 days. The treatment process was repeated 4 times in total. The growth curve showed the results after the last treatment.

In order to identify the (DHA-resistance) gene introduced in the transgenic 3D7_PAC1240(2) well_1, single parasite clones were then isolated by limiting dilution method. The transgenic parasite clones were further verified by Southern analysis to detect whether they harbored gene library plasmids. **Figure 5** showed the pFCEN1 plasmid map with restriction digestion sites and the Southern analysis results. The digestion of total DNA of the control transgenic 3D7_pFCEN1 parasite with AflII enzyme yielded a DNA band at 8 kB as expected when probed with hDHFR probe, while digestion of total DNA from transgenic 3D7_PAC1240(2) well_1 clones with AflII enzyme showed major bands at around 10 kB, 15 kB and over 40 kB. These results confirmed that all transgenic parasite clones still contained the inserted library plasmid containing candidate DNA fragments.

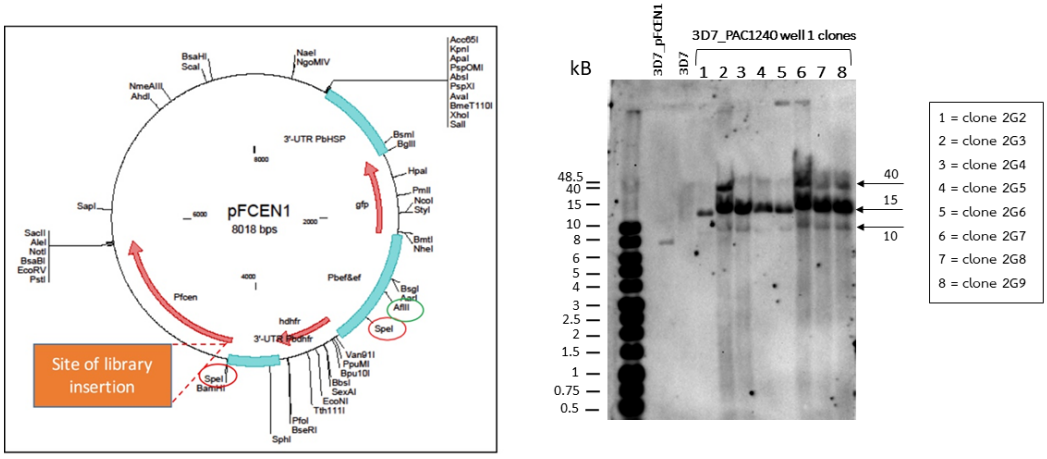

Figure 5. Southern blot analysis examining the insertion of gene library of the 3D7_PAC1240(1) transgenic parasite clones. AflII-digested total DNA from the parental and transgenic parasites; 3D7, 3D7_pFCEN1, 3D7_PAC1240(1) clones 2G2 – 2G9 were subjected to Southern blot analysis probing for the human dihydrofolate reductase (hDHFR) gene presented in the transfection plasmids. No band was detected in the wild-type 3D7 as expected. The control transgenic 3D7_pFCEN1 showed a band around 8 kilobases (kB) representing the size of original pFCEN1 plasmid without the insertion of gene library. Digestion of total DNA from transgenic 3D7_PAC1240(2) well_1 clones with AflII enzyme showed major bands at around 10 kB, 15 kB and over 40 kB.

4.9 Identification of drug resistance genes from transgenic 3D7_PAC1240(2) well_1 clone 2G2

As shown in **Figure 5**, all of the transgenic 3D7_PAC1240(2) well_1 clones showed similar Southern analysis pattern, transgenic clone 2G2 was then selected for further analysis to identify inserted (resistance) gene(s) by Genome Walking analysis as described in the Materials and Methods and **Figure 6**. The PCR-amplified genome walked fragments were cloned to pGEM-T Easy plasmid, which were subsequently analyzed for the DNA fragment and its sequence by DNA sequencing. Identification of the candidate resistance gene(s) in other transgenic 3D7_PAC1240 clones, ie 2G3-2G9, is also being performed.

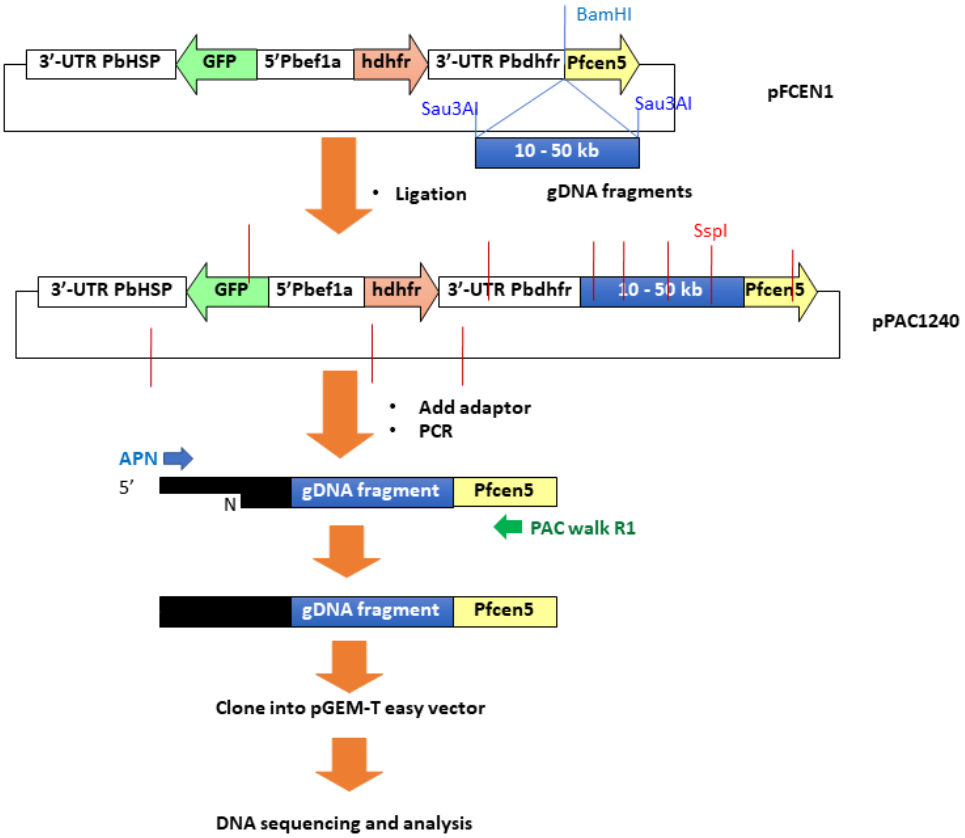

Figure 6. The maps of pFCEN1 and the pPAC1240 library plasmids, and the strategy for Genome Walking analysis. Genomic DNA of the transgenic 2G2 clone, which contain pPAC1240 library plasmid, was digested with SspI and ligated to adaptor. The ligated genomic DNA-adaptor was PCR amplified using PAC walk R1: 5' AATAGTATTGGGAATTCCAGCACACTGGCGGC 3', APN (Adaptor specific primer) 5' CAGCAGAACGACGCCCCGCCGACAA 3' primers. The PCR products were cloned to pGEM-T Easy vector and subjected to DNA sequencing analysis.

pGEMT-2G2 plasmid from 10 bacterial colonies were purified and digested with EcoRI restriction enzyme for checking the inserted fragments. As shown in **Figure 7**, only colony 1, 2, 7, 8 and 9 showed the inserted DNA at around 200-400 bp. The pGEMT_2G2_cl1, pGEMT_2G2_cl8 and pGEMT_2G2_cl9 was then selected and analyzed by DNA sequencing using T7 universal primer.

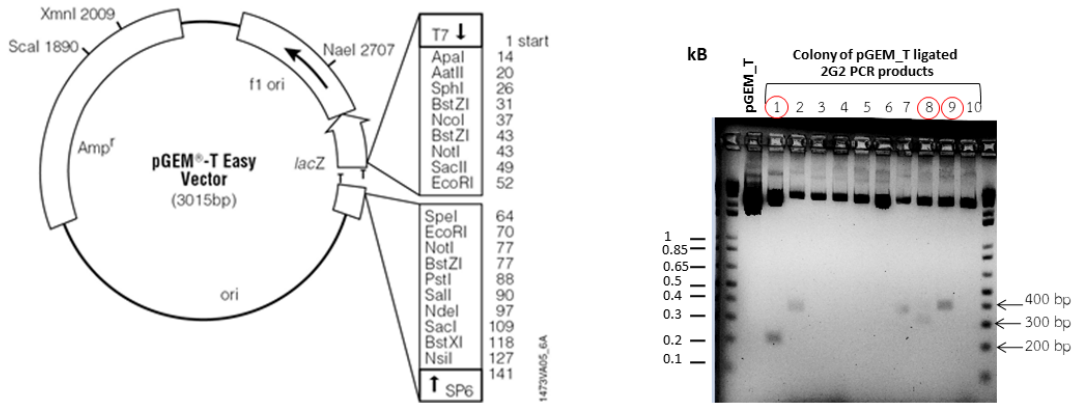

Figure 7. The map of pGEM-T Easy vector and the analysis of inserted DNA fragments in the pGEM vector.

As shown in **Figure 8**, the sequencing analysis showed that the approximately 200 bp of DNA insert of pGEMT_2G2_cl1 was identified as a portion of Plasmodium rRNA_17. The sequencing result for pGEMT_2G2_cl8 did not identify any Plasmodium gene (data not shown). Therefore, we did not pursue further on these 2 DNA inserts.

For pGEMT_2G2_cl9, the inserted DNA was identified as 3' end of PF3D7_1448300.1 gene on chromosome 14. This gene codes for a conserved protein with unknown function (**Figure 9**). Since only the 3' end of PF3D7_1448300.1 was confirmed to be inserted to the pFCEN1 plasmid, this protein does not contribute to the DHA resistance. The genes down-stream of PF3D7_1448300.1 are those that would be the candidate genes that may contribute to the drug resistance. From our Southern analysis in **Figure 5** showing that, digestion of total DNA from transgenic 3D7_PAC1240(2) well_1 2G2 with AflII enzyme showed major bands at around 15 kB. And from the chromosome 14 sequence analysis, we found that the inserted gene library could have the size up to approximately 20 kB. The genes that position down stream of PF3D7_1448300.1 are PF3D7_148200.1 (coding for condensin-2 complex subunit G2, putative), PF3D7_148100.1 (coding for conserved protein, unknown function), PF3D7_148000.1 (U3 small nucleolar RNA-associated protein 12, putative), and interestingly PF3D7_147900.1 (coding for multidrug resistance protein 2) (**Figure 10**). From all the results and analysis, multidrug resistance protein 2 (MDR2) is the most promising gene that may contribute to drug resistance to DHA in our study. We are in the process of reconfirming the role of PfMDR2 in DHA resistance by over-expression of PfMDR2 in the DHA-sensitive 3D7 parasite. We hypothesize that over-expression of MDR2 will make sensitive parasite become more resistance to DHA comparing to its parental parasite.

The genome-wide association analysis of parasites from different areas of the world has identified T484I mutation in PfMDR2 as a genetic background marker for kelch13 mutations that is statistically significant association with artemisinin resistance (Miotto et al., 2015). This supports our finding that PfMDR2 may contribute to drug resistance to DHA in our study. Briolant et al. (2012) have also reported that F423Y in PfMDR2 is associated with resistance to pyrimethamine. van der Velden et al. (2015) described that *Pfmdr2* is dispensable for blood stage development in *P. falciparum* and *P. falciparum* mutants lacking expression of MDR2 showed reduced oocyst formation and reduced sporozoite production (Rijpma et al. 2016). Although, *Pfmdr2*-knockout mutant did not alter sensitivity to artemisinin, chloroquine, DHA and lumefantrine, our plan to over-express PfMDR2 may give different insight into its contribution to artemisinin resistance.

A. Sequencing result

>pGEMT_2G2_cl1_T7F

ATGCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATTCAGCAGAACGACGCCCCGCCGACAA**GGACAGG**
TATTTCAAGTTACCGACATCTGCCCGGCATCAATGATAAACGGCGGCTGTATTTTAAACGGTCCTAAGG
TAGCAAAATTCCTTGTCGGGTAATCTCCGTCCTGCATGAACGGTGTAACGACTTCCCCATTGCCGCCAG
TGTGCTGGAGTTCCCAATACTATTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGA
GAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCA

B. BLAST Analysis

```
> mal_rna_17:rRNA | gene=mal_rna_17 | organism=Plasmodium_falciparum_3D7
| gene_product=unspecified product | transcript_product=unspecified
product | location=Pf_M76611:-(-) | length=195
| sequence_SO=mitochondrial_chromosome | SO=rRNA_encoding
| is_pseudo=false
Length=195

 Score =  176 bits (194),  Expect = 2e-43
 Identities = 97/97 (100%), Gaps = 0/97 (0%)
 Strand=Plus/Plus

Query  41   TGATAAACGGCGGCTGTATTTTAAACGGTCCTAAGGTAGCAAAATTCCTTGTCGGGTAAT  100
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGATAAACGGCGGCTGTATTTTAAACGGTCCTAAGGTAGCAAAATTCCTTGTCGGGTAAT  60

Query  101  CTCCGTCCTGCATGAACGGTGTAACGACTTCCCCATT  137
            |||||||||||||||||||||||||||||||||||||
Sbjct  61   CTCCGTCCTGCATGAACGGTGTAACGACTTCCCCATT  97
```

Figure 8. (A) Sequencing analysis of inserted DNA fragment in pGEMT_2G2_cl1 plasmid and (B) BLAST analysis of the identified sequence. (A) Identified DNA sequence was underlined and bolded. The sequences highlighted in grey were primers used to amplify the genomic DNA-adaptor ligated product. (B) The sequenced DNA fragment was subjected to BLAST analysis to identify its gene. The sequence matched a part of Plasmodium rRNA_17.

A. Sequencing result

>pGEMT_2G2_cl9_T7F

ATGCTNNGGCCGCCATGGCGGCCGCGGGAATTCGATTCAGCAGAACGACGCCCCGCCGACAA**GACAGGT**
ATTTTTTTATGGTTAAGTTATATTTTACATGATATTTTTATTTTACGTTTTAATATGTTAACAACCATT
TTCCTCTTCCGATGATATAATACATATAAAATTATGCGTCTTTTGTATCTTATTATTTGAATCCATAGA
TTTTGTTTTATTCTTTTCAAGATATTTATTATTTTTGTTATATATATTTGGTAGACACTCATTTTTTAA
TTTTCTCTTTTTAAATAAATTATTTTGTTCATTTAAATTATAACTTGAATTAATATGAGGTTTGTCATC
TGATACGATAAAATTCTGATCCACTAGTAACGGCCGCCAGTGTGCTGGATTCCCAATACTATAATCACT
AGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGA

B. BLAST analysis

```
> PF3D7_1448300.1 | gene=PF3D7_1448300 | organism=Plasmodium_falciparum_3D7
| gene_product=conserved protein, unknown function
| transcript_product=conserved protein, unknown function
| location=Pf3D7_14_v3:1972498-1977423(-) | length=4926 | sequence_SO=chromosome
| SO=protein_coding | is_pseudo=false
Length=4926

 Score =  434 bits (480),  Expect = 1e-120
 Identities = 240/240 (100%), Gaps = 0/240 (0%)
 Strand=Plus/Minus

Query  65    TTAACAACCATTTTCCTCTTCCGATGATATAATACATATAAAATTATGCGTCTTTTGTAT  124
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4926  TTAACAACCATTTTCCTCTTCCGATGATATAATACATATAAAATTATGCGTCTTTTGTAT  4867

Query  125   CTTATTATTTGAATCCATAGATTTTGTTTTATTCTTTTCAAGATATTTATTATTTTTGTT  184
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Sbjct  4866  CTTATTATTTGAATCCATAGATTTTGTTTTATTCTTTTCAAGATATTTATTATTTTTGTT  4807

Query  185   ATATATATTTGGTAGACACTCATTTTTTAATTTTCTCTTTTTAAATAAATTATTTTGTTC  244
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Sbjct  4806  ATATATATTTGGTAGACACTCATTTTTTAATTTTCTCTTTTTAAATAAATTATTTTGTTC  4747

Query  245   ATTTAAATTATAACTTGAATTAATATGAGGTTTGTCATCTGATACGATAAAATTCTGATC  304
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4746  ATTTAAATTATAACTTGAATTAATATGAGGTTTGTCATCTGATACGATAAAATTCTGATC  4687
```
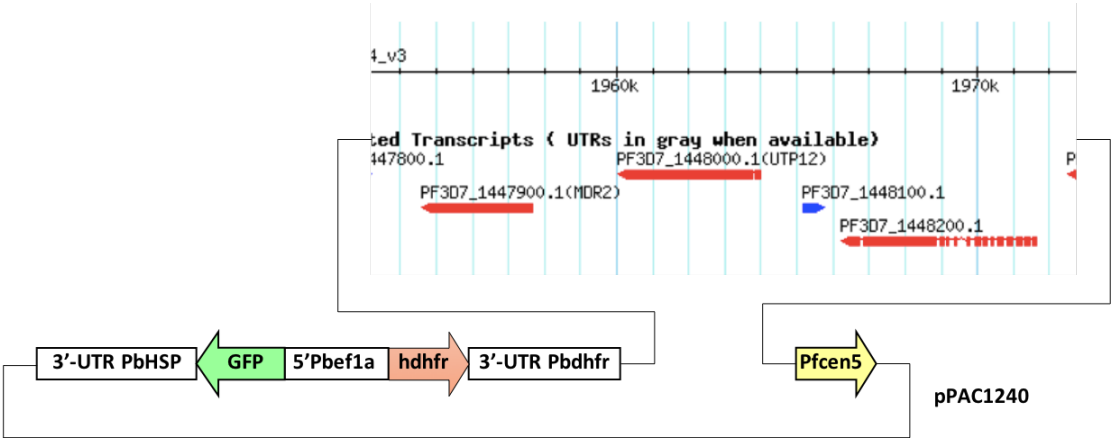

Figure 9. (A) Sequencing analysis of inserted DNA fragment in pGEMT_2G2_cl9 plasmid and (B) BLAST analysis of the identified sequence. (A) Identified DNA sequence was underlined and bolded. The sequences highlighted in grey were primers used to amplify the genomic DNA-adaptor ligated product. (B) The sequenced DNA fragment was subjected to BLAST analysis to identify its gene. The sequence matched 3' end of PF3D7_1448300.1 gene that codes for conserved protein with unknown function.

A.



B.

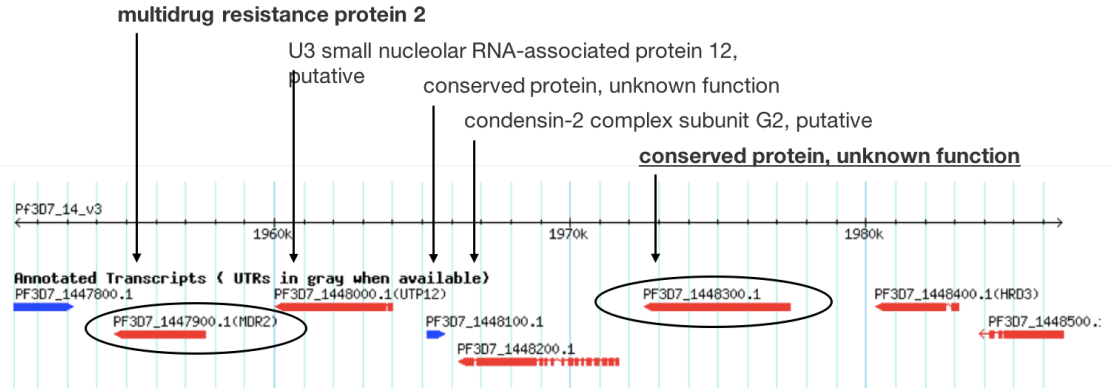


Figure 10. Proposed insertion of the PAC1240 gene library fragment to pFCEN1 plasmid.

(A) The DNA sequencing, Southern and chromosome 14 sequence analyses suggested that the inserted gene library could have the size up to approximately 20 kB. **(B)** The genes that position down stream of PF3D7_1448300.1 are PF3D7_148200.1 (coding for condensin-2 complex subunit G2, putative), PF3D7_148100.1 (coding for conserved protein, unknown function), PF3D7_148000.1 (U3 small nucleolar RNA-associated protein 12, putative), and interestingly PF3D7_147900.1 (coding for multi drug resistance 2). Multidrug resistance protein 2 (MDR2) is the most promising gene that may contribute to drug resistance to DHA in this study.

4.10 Characterisation of double mutant PfKelch13

Although the following study was not proposed in the original proposal and work plan, we were interested to explore other directions to identify molecular markers that may contribute to the artemisinin resistance.

While the identification of other molecular markers contributing to the artemisinin resistance using PAC technology reported in the early part of this report was underway, it was also of interest to better characterise the current marker, PfKelch13 (PfK13), whether it indeed plays an important role in artemisinin resistance. One aspect of the several PfK13 mutations reported for the artermisinin-resistant parasites is that those mutations are a variety of single mutations. The common PfK13 mutations found in the Cambodian and Thai resistant parasite isolates are M476I, Y493H, R539T, I543T, and C580Y (Ménard et al., 2016; Straimer et al., 2014; Talundzic et al., 2015). It is then intriguing to explore the other possible mutations that might be able to contribute to higher artemisinin resistance. Since the reported single mutations each contributes to artemisinin resistance at various degrees, we proposed to study them in combination to evaluate if these combinations could confer greater level of artemisinin resistance. Since C580Y appears to be the most widespread mutation across Thailand and Cambodia, we planned to firstly examine the double mutant PfK13 based on the combination of other mutations with C580Y. The transfection approach was to use the CRISPR-Cas9 gene editing technology to replace the endogenous PfK13 with the created double and triple mutant PfK13 in different *P. falciparum* strains and isolates. This will allow us to examine whether the introduction of double or triple mutant PfK13 facilitates higher resistance to artemisinin in the transgenic parasites when compared with the parental parasite lines by the RSA.

4.11 Generation of plasmids for the modification of k13 in P. falciparum using CRISPR-Cas9 system

K13 mutated transgenic parasites will be generated using CRISPR-Cas9 system targeting at the *k13* locus with two single-guide RNAs flanking the region planned to modify. The combinations of mutations intended to create in the K13 protein are R539T/C580Y, I543T/C580Y, and R539T/I543T/C580Y. The C580Y substitution is included in all mutation combinations as it is a dominant mutated allele found in the Southeast Asian region, while the other point mutations are also individually associated with artemisinin resistance (Straimer et al., 2014).

To generate transgenic parasites harbouring various mutations of *k13* gene, two plasmids will be co-transfected into the parasites to modify endogenous *k13* with various mutation combinations. The first plasmid, pCas.BSgK13F (**Figure 11A**), contains gene expression cassettes for the Cas9, single-guide RNA targeting *k13* front locus (SgK13 F), and blasticidin-S-deaminase (BSD) for positive selection, while another plasmid, pL6.SgK13B.K13mut (**Figure 11B**), contains mutated *k13* template for homology directed repair (HRK13mut), another single-guide RNA targeting *k13* back

locus (SgK13 B), and human dihydrofolate reductase (hDHFR) for positive selection. Four variants of pL6.SgK13B.K13mut plasmids are constructed corresponding to the four types of mutations planned to modify, i.e. C580Y alone, R539T/C580Y, I543T/C580Y, and R539T/I543T/C580Y. Both SgK13 F and SgK13 B DNA fragments were prepared by PCR and incorporated into the corresponding plasmids by Gibson assembly technique. For the HRK13mut homology directed repair fragment, we began by preparing 3 DNA fragments of *k13* by PCR, around 400 base pairs each, covering the C580Y single mutation from gDNA of MRA1236 *P. falciparum* isolate, known to have this *k13* genotype (Straimer et al., 2014). These *k13* fragments were then incorporated into the pGEM-3Zf plasmid by Gibson assembly technique. Other mutation variants were subsequently generated by site-directed mutagenesis from this plasmid to obtain the other 3 mutation combinations as mentioned earlier. Once the correct sequences of each HRK13mut were confirmed, they were individually subcloned to obtain various versions of pL6.SgK13B.K13mut plasmids. The sequences of the primers used in this study are shown in the **Table 5** and **6**.

Each variant of pL6.SgK13B.K13mut plasmid have already been transfected into the artemisinin-sensitive K1 parasites and we have successfully obtained transgenic parasites harbouring the mutated *k13* template for homology directed repair. These parasites are in the process of transfecting the second plasmid pCas.BSgK13F, to express Cas9 as well as single guide RNA targeting K13 locus for editing to introduce the desired mutations.

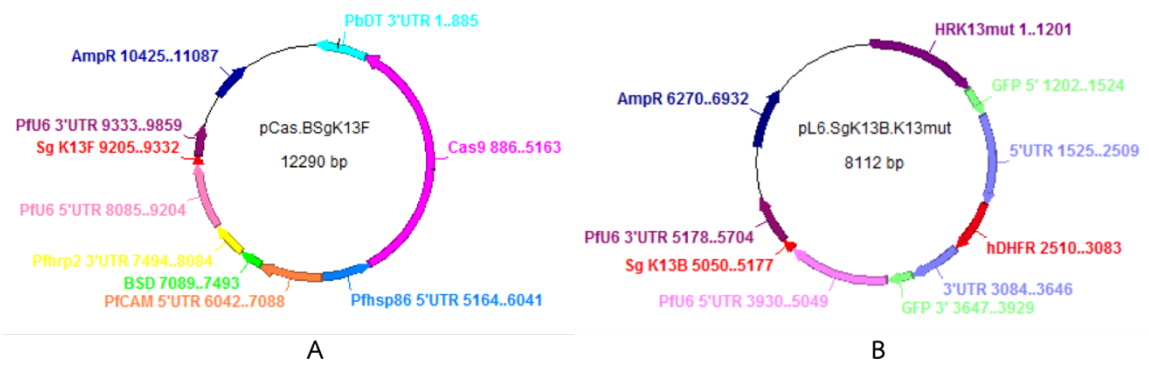


Figure 11. Illustration of the genetic components in the transfection plasmids, pCas.BSgK13F (A) and pL6.SgK13B.K13mut (B).

Table 5. List of primers for PCR.

DNA fragments	Primers	Sequences (5′ - 3′)
SgK13F	SgK13F F1	GCACATATTTCATATTAAGTATATAATATTTAGATATTAGTCAACAATGC
	SgK13F R1	ATTTTAACTTGCTATTTCTAGCTCTAAAACGCATTGTTGACTAATATCTA
SgK13B	SgK13B F1	GCACATATTTCATATTAAGTATATAATATTTATGTCATTGGTGGAACTAA
	SgK13B R1	ATTTTAACTTGCTATTTCTAGCTCTAAAACTTAGTTCCACCAATGACATA
K13F	pGemK13F	TTGTAATACGACTCACTATAGGGCGAATTGTTGATGCAAATATTGC
	K13F R	CGATGGAATTATTAGATATTTCGCAGCAGTGTTGGCGTAT
K13M	K13M F	ATATTTCGCAGCAGTGTTGGCGTATGTGTACACCTATGTC
	K13M R	TCTCACCATTCGTCCCGCCGATGACATAAATTTTATTATCAAAAGC
K13B	K13B F	GTCATCGGCGGGACGAATGGTGAGAGATTAAATTCTATTG
	K13B pGem R	CATGCCTGCAGGTCGACTCTAGAGGAGTGACCAAATCTGGGAAC
HRK13mut	PL6K13NotI F	TTAGGTGACACTATAGAATACTCGCGGCCGCAATTGTTGATGCAAATATTGC
	PL6K13SpeI R	TCAGGGTCAGCTTGCCGTAGGTGGACTAGTGAGTGACCAAATCTGGGAAC

Table 6. List of oligonucleotides for site-directed mutagenesis.

Mutations	Primers	Sequences (5’ – 3’)
R539T	R539T F	GTTACGTCAAATGGTACAATTTATTGTATTGG
	R539T R	CCAATACAATAAATTGTACCATTTGACGTAAC
I543T	I543T F	GTAGAATTTATTGTACTGGGGGATATGATGG
	I543T R	CCATCATATCCCCCAGTACAATAAATTCTAC
R539T + I543T	R539T + I543T F	GTTACGTCAAATGGTACAATTTATTGTACTGGGGGATATGATGG
	R539T + I543T R	CCATCATATCCCCCAGTACAATAAATTGTACCATTTGACGTAAC

5. Summary and Conclusion

In this study, we applied Plasmodium Artificial Chromosome (PAC) in the identification of genetic markers conferring resistance to dihydroartemisinin (DHA). We have obtained six artemisinin-resistant *P. falciparum* reference lines from the parasite depository at Malaria Research and Reference Reagent Resource Center (MR4), USA. These parasite lines were re-assessed for their level of resistance to DHA using the Ring-stage Survival Assay (RSA). We confirmed that the parasites line number MRA1236, MRA1240, and MRA1241 exhibited various degrees of resistance to the DHA treatment whereby MRA1240 displayed the highest artemisinin resistance among the resistant isolates in accordance with the published report. Gene library of MRA1240 isolate has been constructed using PAC technology with special attentions on the steps of preparation of PAC-containing plasmid backbone, the digestion and purification of total DNA from the artemisinin-resistant parasite isolate, and ligation protocol. Using modified protocol, matured schizont-stage of the artemisinin-sensitive *P. falciparum* laboratory strain 3D7 parasites were purified and transfected with the PAC plasmid containing the MRA1240 gene library. The transgenic parasites harboring the gene library plasmid(s) containing the DNA fragment (library) were obtained as confirmed by Southern analysis. These transgenic parasites were assessed for their artemisinin sensitivity using the RSA. The results showed that all of them were sensitive to DHA, at the similar level to the 3D7 parental strain. However, for selection of DHA resistant transgenic parasites, another selection strategy using SAAR method was employed. After 4 rounds of low dose DHA treatment, we could obtain DHA-resistant 3D7_PAC1240 transgenic parasites which were subsequently isolated to single clones. One of the transgenic clones, 2G2, was analyzed for its inserted MRA1240 gene library using genome walking technique. The Southern, BLAST and chromosome analyses showed that the inserted MRA1240 gene library include a genome fragment of approximately 20 kB down-stream of the PF3D7_1448300.1 gene (coding for conserved protein with unknown function), which includes PF3D7_148200.1 (coding for condensin-2 complex subunit G2, putative), PF3D7_148100.1 (coding for conserved protein, unknown function), PF3D7_148000.1 (U3 small nucleolar RNA-associated protein 12, putative), and interestingly PF3D7_147900.1 (coding for multidrug resistance protein 2). From all the results and analysis, multidrug resistance protein 2 (MDR2) is the most promising gene candidate that may contribute to drug resistance to DHA in our study. We are in the process of reconfirming the role of PfMDR2 in DHA resistance by over-expression of PfMDR2 in the DHA-sensitive 3D7 parasite. We hypothesize that over-expression of MDR2 will make sensitive parasite become more resistance to DHA comparing to its parental parasite.

Although not proposed in the original proposal, as another approach to study the artemisinin resistance mechanism, we decided to study the currently proposed molecular marker for artemisinin resistance, PfK13, in more details by generating transgenic parasites harboring double and triple mutations of the K13 proteins and examine whether they have an effect on the degree of artemisinin resistance. Plasmids for introduction of different K13 mutation combinations to the parasites, pCas.BSgK13F and a series of pL6.SgK13B.K13mut, have been constructed and transfected

to *k13* wild-type K1 parasite. At present, we have obtained transgenic parasites transfected with the plasmids. We are in the process of confirming sequences of the *k13* locus and assessing artemisinin sensitivity of these transgenic parasites.

In conclusion, this study has proposed PfMDR2 as a promising candidate that may contribute to the artemisinin resistance, although more studies are needed to confirm this finding. We hypothesize that over-expression of PfMDR2 will make sensitive parasite become more resistance to DHA comparing to its parental parasite. Once confirmed, PfMDR2 would be proposed as the marker for monitoring the progress of drug resistant parasites that will allow the public health authorities to plan effective treatment and control the spread of resistant parasites in the endemic areas.

6. References

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Output จากโครงการวิจัยนี้

ที่ผ่านมา ยังไม่มี output จากโครงการวิจัยนี้ เนื่องจากผลการทดลองยังไม่ครบถ้วน อย่างไรก็ตาม ในปัจจุบันได้ผลการทดลองที่น่าสนใจมาก จึงอยู่ระหว่างรวบรวมผลการทดลองทั้งหมดและกำลังอยู่ระหว่างการร่างต้นฉบับเพื่อตีพิมพ์ในวารสารวิชาการนานาชาติ เช่น PloS One หรือ Malaria Journal ในหัวข้อ

1. *Plasmodium falciparum* Multidrug Resistant Protein 2 contributes to Artemisinin Resistance
2. Contribution of PfKelch13 Mutations on Artemisinin Resistance Level in *Plasmodium falciparum*