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Full Paper

Identification of *Pfdhfr* mutant variants in *Plasmodium berghei* model

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Abstract: Parasite resistance to antimalarials is a major burden in controlling malaria disease. Genetic mutations within the parasites are found to be the factor in conferring resistance to drugs. In this study, the power of random mutant library and transgenic parasite systems were employed to identify mutations on the antimalarial drug target, viz. *Plasmodium falciparum* dihydrofolate reductase (DHFR), which could contribute to resistance, and to elucidate the functionality of resistant mutant parasites in *P. berghei*. Using the moderate drug-resistant *Pfdhfr*^{S108N} gene as template, we generated a library of *Pfdhfr* mutants by error-prone PCR followed by transfection and selection in *P. berghei*. Two clones of transgenic *P. berghei* expressing *Pf*DHFR of interest due to the position of mutations, i.e. *PbPf*DHFR3m1 (M55I+S108N+S189C) and *PbPf*DHFR3m2 (C50Y+S108N+F116S), were selected for drug sensitivity test. Although these transgenic parasite clones showed similar reproducibility with the parental transgenic *P. berghei*, expressing *Pf*DHFR with mutation at S108N (*PbPf*S108N) in response to antifolate pyrimethamine, this study reconfirms that this *P. berghei* model is effective in predicting the evolution of *Pfdhfr* mutations in vivo. This approach can be applied during the development of new antifolates with better effective properties against drug resistant parasites.

Keywords: *Plasmodium berghei*, transfection, malaria, dihydrofolate reductase, antifolate resistance

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INTRODUCTION

Malaria is one of the devastating diseases endemic to tropical and subtropical areas of the world. It is caused by parasites in the genus *Plasmodium*, of which *P. falciparum* is the most virulent species that infects humans. The current major issue for malaria control is the increasing resistance to the available antimalarial drugs. Although efforts are being made in the discovery of novel antimalarial drugs by research groups, understanding of current drug resistance mechanism developed by the parasites is necessary for the design of new compounds that are effective against drug resistant parasites and for averting further possible resistance in the future.

A bi-functional dihydrofolate reductase-thymidylate synthase (DHFR-TS) enzyme is a well-defined target of antimalarial drugs such as pyrimethamine, cycloguanil and methotrexate [1]. Dihydrofolate reductase (DHFR) catalyses the production of tetrahydrofolate from dihydrofolate while thymidylate synthase (TS) is in charge of transferring a methyl group from methylene-tetrahydrofolate to deoxyuridine monophosphate (dUMP), thereby generating deoxythymidine monophosphate (dTMP) and tetrahydrofolate [2]. Resistance to pyrimethamine, cycloguanil and methotrexate was reported to be associated with mutations in *dhfr* gene [3]. Despite the possibility to develop more mutations, thus becoming more resistant to the drugs, the availability of target-based screening models [4-5] and detailed crystal structure [6] makes DHFR still an attractive drug target for malaria.

Recently, our group has reported the use of transgenic *P. berghei* model to predict the evolution of drug-resistant *Pfdhfr* mutations [7]. The system utilised the power of *P. berghei* transfection technology to identify possible novel drug-resistant mutants that could arise against the antifolate drug. Using our system, we have successfully identified S108N (serine, S, at amino acid position 108 changed to asparagine, N) mutant of *Pf*DHFR, which was the first reported pyrimethamine-resistant mutation isolated from the field. It was reported that the accumulation of amino acid substitutions on DHFR enzyme at positions 51, 59, 164 and 108 increases the level of the parasites' resistance to the drug [8].

In this study, in order to predict possible evolutionary changes within the DHFR enzyme, which may increase the level of drug resistance, we have used the moderate drug-resistant *Pfdhfr*^{S108N} mutant as a template to generate a random mutant library of *Pfdhfr* and transfected the mutant library to the *P. berghei* parasite. After pyrimethamine selection, a number of *Pfdhfr*^{S108}-based variants were identified and tested for pyrimethamine resistant level. This study reconfirms the power of the random mutant library and transgenic *P. berghei* systems to predict the drug-resistant *Pfdhfr* mutations in an in vivo setting.

MATERIALS AND METHODS

Experimental Animals and *P. berghei* Parasite

Female BALB/c mice (National Laboratory Animal Centre, Mahidol University) 4-6 weeks old and weighing 20-25 grams were used in all experiments. The transgenic *P. berghei* parasite line MRA-867, stably expressing green fluorescent protein (PbGFP) and kindly provided by Drs. Andrew Waters and Chris Janse of Malaria Research Group, Leiden University Medical Centre, the Netherlands, was used [9]. The animal study protocol was approved by the Ethical Committee of

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Animal Experimentation at the Faculty of Medicine, Chiang Mai University (Protocol Number 18/2553). Animal experiments conformed to international and national guidelines for ethical conducts on the care and use of animals.

Transfection Plasmid

The plasmid for *P. berghei* transfection, designated pY005^{S108N}, was modified from the original plasmid pL0017 [10], which was kindly provided by Drs. Andrew Waters and Chris Janse (Leiden University Medical Centre, the Netherlands). The pY005^{S108N} plasmid contained *Pfdhfr*^{S108N}*-ts* gene flanked with 2.3 and 1.0 kilobases (kb) of 5' and 3' untranslated region (UTR) sequences of *Pbdhfr-ts* respectively, which also served as homologous recombination sites. *Bam*HI and *Afl*II restriction sites introduced at 5' and 3' ends of the *Pfdhfr* domain respectively served as cloning sites for the randomly mutated *Pfdhfr* library.

Random Mutagenesis of *Pfdhfr* Library

The *Pfdhfr* mutant library was generated by error prone PCR [11]. The PCR reaction contained 1 ng pY005^{S108N} [7], 10 µM of sense primer F1 (CGGTGGATCCATGATGGAACAAG; *Bam*HI site is underlined) and antisense primer R1 (CTTTGTCATCATTCTTAAGAGGC; *Afl*II site is underlined), 0.1 mM dGTP, 0.1 mM dATP, 0.5 mM dCTP, 0.5 mM dTTP, 1x mutagenesis buffer [12] and 5 units of GoTaq® DNA polymerase. The thermocycle condition was: 1 cycle at 95ºC for 3 min, 30 cycles at 95ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min. The PCR products of the random mutant *Pfdhfr* library of about 0.7 kb were cloned into *Bam*HI/*Afl*II sites of pY005^{S108N}. Variant clones were randomly picked and the mutant *Pfdhfr* genes were sequenced (BioDesign sequencing service, Thailand).

Transfection, Selection and Identification of *Pfdhfr* Random Mutant Library

The bacterial clones with plasmids containing *Pfdhfr* mutation library were pooled and grown in Luria Bertani broth containing 100 µg/ml ampicillin at 37ºC with shaking for 12-16 hr. Plasmids were extracted and purified using a Qiaprep Spin Miniprep kit (Qiagen) according to the manufacturer's protocol. Extracted plasmids were pooled, precipitated by isopropanol and resuspended in 10 µl TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). In vitro culture of PbGFP and transfection was performed as described [13]. Briefly, parasitised blood was collected from a donor animal and cultured overnight in RPMI 1640 medium containing 20% heat-inactivated fetal calf serum, 50 IU/ml neomycin and 25mM Hepes. Schizont stage parasites were purified from the culture by Nycodenz gradient centrifugation [13], transfected with plasmid DNA containing mutant library using the Amaxa Nucleofector protocol [13] and re-infected into the animals by intravenous injection. Twenty-four hr after transfection, the infected mice were daily treated by intraperitoneal (i.p.) injection with 0.25 mg/kg of pyrimethamine. When the parasitemia reached 3%, tail blood was drawn from the infected animals on alternate days for genomic DNA extraction until parasitemia reached 8-10%. The obtained genomic DNA was transformed into *E. coli* DH5α. The transformed bacterial colonies were picked, the plasmid DNA extracted and the sequence of *Pfdhfr* mutants obtained by DNA sequencing as described above.

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Generation and Cloning of Transgenic *P. berghei* Expressing *Pf*DHFR Mutants

Plasmids containing *Pfdhfr* mutants of interest were selected and re-introduced into *P. berghei*. The plasmids were linearised for double crossover recombination at the 5' and 3' UTR. In vitro culture of PbGFP and transfection was performed as described above. Twenty-four hr after transfection, 0.25 mg/kg of pyrimethamine was used to treat the infected mice by i.p. injection daily until the resistant parasites reappeared in the blood. The animals were sacrificed and infected blood was collected for genomic analysis. The integrated transgenic mutant parasite clones were obtained by the limiting dilution method [14].

Genomic Analysis of Transgenic *P. berghei* Parasites

To determine the correct integration of mutant vector into the genome of the transgenic parasites, a 4.0-kb DNA fragment spanning the endogenous 5' UTR *Pbdhfr-ts* gene and the introduced *Pfdhfr* in the vector was detected by PCR using F2 (TTGAGCTACATAACTTCCATACAT) and R1 primers (described above). A 3.0-kb DNA fragment spanning the *Pfdhfr-ts* in the vector and the endogenous 3' UTR *Pbdhfr-ts*, indicative of a 3' integration event, was detected by PCR using F1 (described above) and R2 (CGATCTACACCTCTTCAT) primers (Figure 2).

The correct integration of the mutant *Pfdhfr* transgenic parasites was further confirmed by Southern analysis [15]. Genomic DNA of the transgenic mutant parasites was digested with *Eco*RI restriction enzyme and hybridised with *Pfdhfr* probe labelled with Digoxigenin-11-dUTP (using DIG high prime DNA labelling and detection starter kit II, Roche Applied Science). The pattern of hybridisation was detected using alkaline phosphatase-conjugated anti-DIG antibody and CSPD reagent according the manufacturer's protocol (Roche Applied Science).

Sensitivity of Transgenic *P. berghei* Expressing *Pf*DHFR Mutant to Pyrimethamine

The 4-day suppressive test [16] was used to determine the level of susceptibility of the mutant parasites to pyrimethamine. Six groups of four BALB/c mice per group were infected intravenously with $1x10^7$ parasitised erythrocytes. The experimental groups were treated with different concentration of pyrimethamine by i.p. injection four hr after infection. The control group was treated with 5%(v/v) DMSO in PBS. The treated groups were administered daily with pyrimethamine through the same route for 3 days. Twenty-four hr after the last treatment (day 4), blood smears were made from all groups for microscopic screening after Giemsa staining to determine the percentages of parasitemia. The difference between the mean value of the control group (taken as 100%) and those of the experimental groups was calculated and expressed as per cent inhibition.

Statistical Analysis

The non-linear regression for sigmoidal dose-inhibition (variable slope) was used to calculate the 50% effective dose (ED_{50}) value. Per cent inhibition was calculated using the formula below. The unpaired *t*-test was used to compare the mean ED_{50} value. All data were subjected to statistical analysis using the SigmaPlot software.

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% Inhibition = 100 – {[mean parasitemia (treated) / mean parasitemia (control)] x 100}

RESULTS AND DISCUSSION

Generation of *Pfdhfr* Random Library Using *Pfdhfr*^{S108N} as Template

Approximately 8,300 bacterial colonies containing random mutant *Pfdhfr* library were generated. Twelve colonies were randomly picked from the pool of colonies and sequenced to check mutations within the *Pfdhfr* gene. Apart from the sequence at amino acid position 108 (serine, S; AGC changed to asparagine, N; AAC), which was used as template, up to 5 base substitutions per gene were found. The mutation frequency in this study was 0.27%, which is equivalent to approximately 2 base substitutions per 700 base pairs (bp) of *Pfdhfr* gene. It was within the criteria of functional mutation frequency of 2-5 base substitutions per gene [17].

Selection of Transgenic Parasite Lines Expressing Pyrimethamine-Resistant Phenotype

Plasmid DNA containing random mutant *Pfdhfr* library were purified and transfected to *P. berghei* parasite. Two mice were infected with the transfected parasite and treated with pyrimethamine to select transgenic parasite lines. Reappearance of pyrimethamine-resistant parasites was observed six days after transfection. The genomic DNA of the parasites were extracted and transformed to *E. coli*. Colonies from the transformation were picked, cultured and the extracted plasmids were subjected to sequencing analysis. Results from the sequencing analysis indicated the presence of *Pfdhfr* mutant genes that might contribute to pyrimethamine resistance of the transfected parasites. Many variations of *Pfdhfr* mutants were observed as shown in Table 1.

Table 1. Variation of *Pfdhfr* mutation compared with S108N template after transfection to *P. berghei* parasite and selection with 0.25 mg/kg

Clone #	Amino acid #																			
	4	6	11	22	24	29	31	36	50	55	80	97	102	**108**	116	117	157	164	189	192
Template	Q	C	I	S	N	N	V	T	C	M	Y	K	V	N	F	K	N	I	S	E
1														N						
2								*T*		I				N					C	
3						F	F							N						
4	*Q*	S												N						
5				C					S					N						
6			T		D									N						
7														N		R	D			
8											C			N				*I*		
9														N						G
10									Y					N	S					
11													A	N						
12												*K*		N						

Note: Silent mutations are in italics. Mutations of interest are in red.

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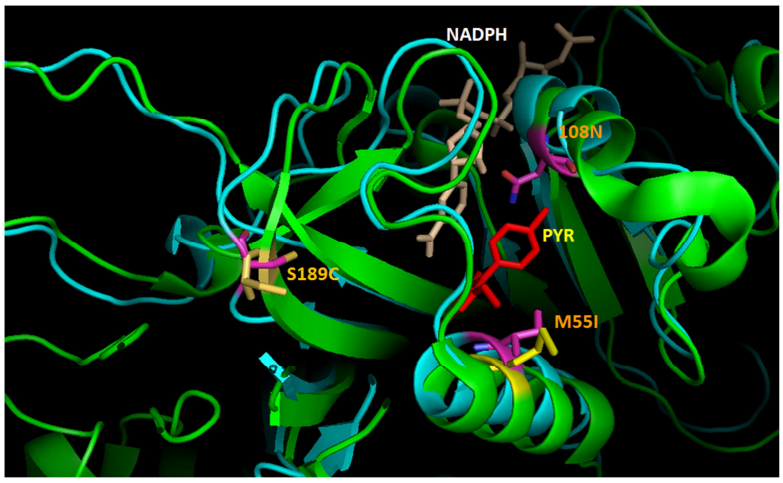
From the list, clone #2 (M55I+S108N+S189C) and clone #10 (C50Y+S108N+F116S), designated *Pf*DHFR3m1 and *Pf*DHFR3m2 respectively, were selected for further study since their positions of mutation might be associated with drug resistance. The *Pf*DHFR3m1 mutant contained mutation at Met55, which was an amino acid at the entry of catalytic cleft [18] and corresponded to Phe31 of human DHFR that was reported to account for resistance to antifolate methotrexate in human colon cancer cell lines [19]. The *Pf*DHFR3m2 mutant contained mutations at Cys50, which was previously reported to be associated with a high level of pyrimethamine resistance in field isolates [20-21], and at Phe116, which was positioned around the entry of catalytic cleft of the enzyme [18]. Figures 1A and 1B show the positions of those mutations on the models of *Pf*DHFR3m1 and *Pf*DHFR3m2 superimposed on the double mutant *Pf*DHFR-TS (C59R+S108N) complexed with pyrimethamine. These two mutants were further assessed for their level of pyrimethamine resistance in the transgenic *P. berghei* model.

(A)



(B)

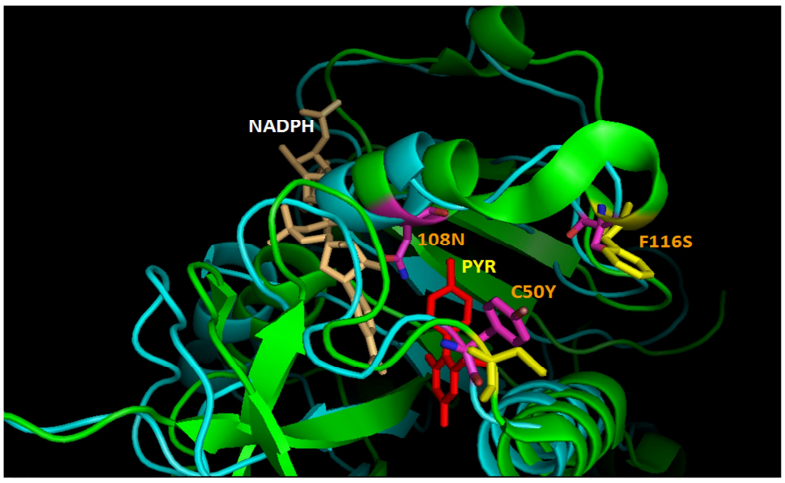


Figure 1. Superimposition of *Pf*DHFR(C59R+S108N)/NADPH/pyrimethamine complex (green - structure; yellow - amino acids) on (A) *Pf*DHFR3m1 (M55I+S108N+S189C) or (B) *Pf*DHFR3m2 (C50Y+S108N+F116S) (cyan – structure; pink - amino acids). Each image presents a clear visualisation, in different views, of the locations of amino acid of interest. The numbers indicate positions with mutation. The models were created by Geno3D program (http://geno3d-pbil.ibcp.fr). Figures were generated with PyMOL program [22].

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Generation of Transgenic *P. berghei* Expressing *Pf*DHFR3m Mutants

Transgenic *P. berghei* parasites stably expressing *Pf*DHFR3m1 and *Pf*DHFR3m2, designated *PbPf*DHFR3m1 and *PbPf*DHFR3m2 respectively, were generated. Endogenous *Pbdhfr-ts* was replaced with either *Pfdhfr*3m1 or *Pfdhfr*3m2 mutant genes by double homologous recombination. The allelic replacement strategy is shown in Figure 2. Correct integration was investigated by PCR analysis on genomic DNA of the transgenic parasites using specific primer pairs. The expected 4.0- and 3.0-kb PCR products were obtained, thus confirming 5' and 3' integration respectively of the introduced *Pfdhfr*3m mutants replacing *Pbdhfr-ts* in the transgenic *P. berghei* (Figure 3A). Additional evidence for proper genomic integration was investigated by Southern analysis of digested genomic DNA of the transgenic parasites using a *Pfdhfr* probe (Figure 2). As shown in Figure 3B, a 4.8-kb band of *Eco*RI digested fragment confirmed the correct integration of *Pfdhfr*3m gene in the transgenic parasites. The digested plasmid DNA pY005^{S108N} control produced a 7.5-kb product while the digested genomic DNA of transgenic parasite PbGFP was used as negative control.

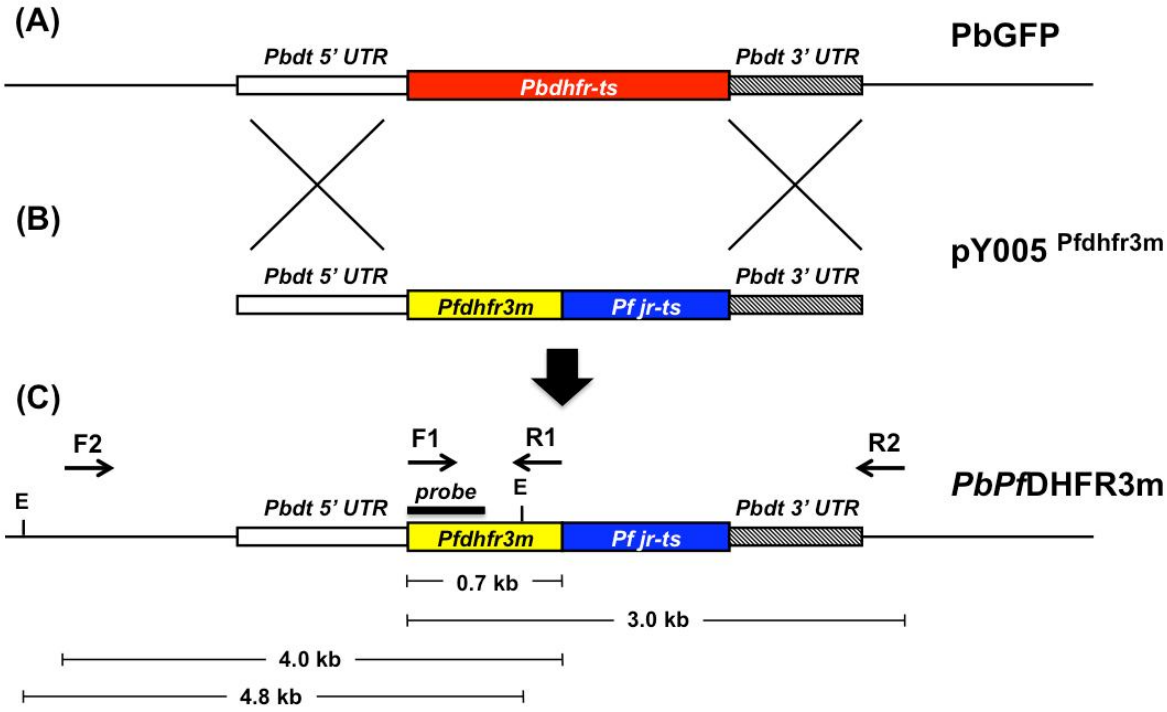

Figure 2. Double-crossover homologous recombination of *Pfdhfr-ts* into *Pbdhfr-ts* locus: (A) endogenous *Pbdhfr-ts* gene in PbGFP parasite; (B) linearised plasmid containing mutant *Pfdhfr3m* gene; (C) correct integration of the construct contributing to the replacement of mutant *Pfdhfr3m* gene. Positions of the primers used for PCR analysis are indicated by arrows. *Pfdhfr* probe is shown as solid bar. E represents *Eco*RI restriction site.

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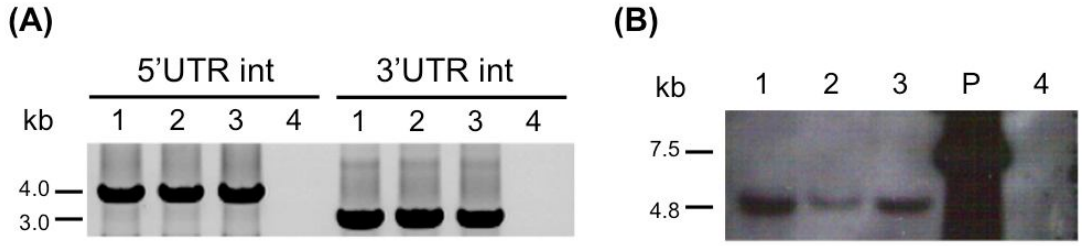

Figure 3. Genotype analysis of transgenic *P. berghei* expressing *Pf*DHFR3m mutants: (A) PCR analysis of 5' and 3' UTR integrations; (B) Southern analysis of transgenic parasites: *PbPf*S108N (1), *PbPf*DHFR3m1 (2) and *PbPf*DHFR3m2 (3), and genomic DNA of control parasite, PbGFP, as negative control (4). The pY005^{S108N} plasmid (P) serves as positive control in Southern analysis.

Growth Rate and Susceptibility to Pyrimethamine of Transgenic Parasites

The transgenic *PbPf*DHFR3m1 and *PbPf*DHFR3m2 parasites were infected into naive mice and the growth rates were compared with the parental PbGFP parasite. As shown in Figure 4, the growth rates of both transgenic parasites were not significantly different from each other or from the parental PbGFP parasite. Accumulation of 3 mutations in *Pf*DHFR expressed in the newly generated transgenic parasites in this study did not cause a significant defect to the function of *Pf*DHFR-TS in complementing endogenous *Pb*DHFR-TS. The ED_{50} values of pyrimethamine against the transgenic *PbPf*S108N [7], *PbPf*DHFR3m1 and *PbPf*DHFR3m2 parasites were 1.08 ± 0.23, 1.61 ± 0.70 and 1.07 ± 0.39 mg/kg respectively (Figure 5). The results showed that the combination of S108N mutation with M55I and S189C, or with C50Y and F116S mutations did not significantly confer a higher resistance to pyrimethamine compared with the starting S108N mutant as expected. Using the unpaired *t*-test to compare the means of the transgenic parasite clones with that of the parental *PbPf*S108N parasite clone, the *P*-values of *PbPf*DHFR3m1 and *PbPf*DHFR3m2 against *PbPf*S108N were 0.28 and 0.99 respectively.

The active site region of *Pf*DHFR contains amino acid residues that have earlier been identified by studies in other species as important in the activity of DHFR [6]. Methionine at position 55 of *Pf*DHFR corresponds to Phe31 of *h*DHFR and has been shown to be present in the active site region of DHFR [6, 23]. It was reported that the mutation of this Phe31 residue of *h*DHFR to Ser or Arg confers resistance to methotrexate [19] while variant isolates with mutations of F31L, F31V and F31T were not resistant to methotrexate [24-25]. Another report in yeast complementation system [4] demonstrated that an additional mutation of N51I+S108N with S189R in *Pf*DHFR increases resistance to pyrimethamine and a prodrug WR99210 at a level higher than a double mutant template. This effect may be related to the fact that Ser189 is within a region in close proximity to the key substrate or to the drug [26].

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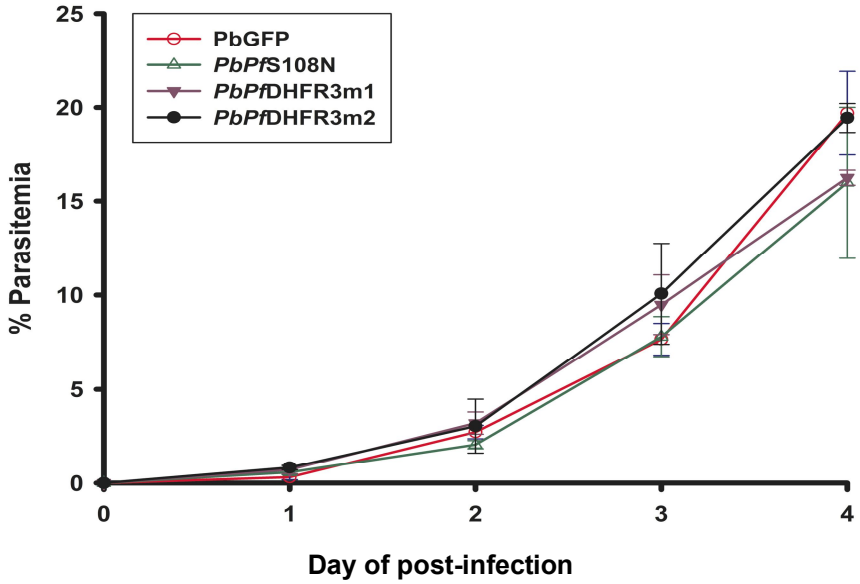

Figure 4. Growth profile of transgenic parasites expressing *Pf*DHFR mutant enzymes: parental PbGFP (red line), *PbPf*S108N (green line), *PbPf*DHFR3m1 (M55I+S108N+S189C) (purple line) and *PbPf*DHFR3m2 (C50Y+S108N+F116S) (black line). The experiments were performed in mice in three independent experiments and the data represent mean ± SD values.

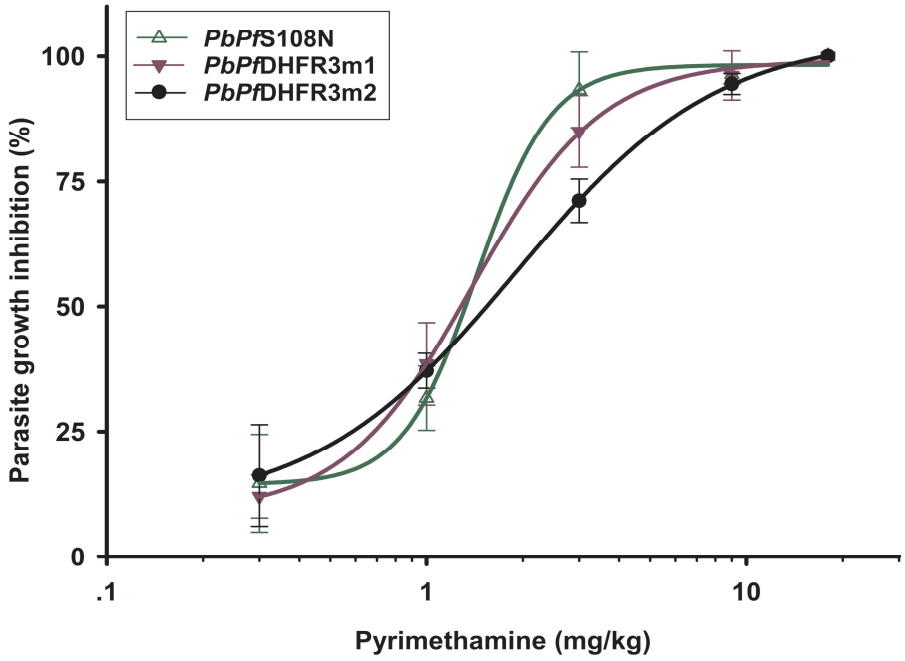

Figure 5. Pyrimethamine susceptibility profile of transgenic *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2 parasites. The data represent mean ± SD of percentage of growth inhibition for 4 mice per group from three independent experiments. The average ED_{50} values of pyrimethamine against *PbPf*S108N [7], *PbPf*DHFR3m1 and *PbPf*DHFR3m2 are 1.08 ± 0.23, 1.61 ± 0.70 and 1.07 ± 0.39 mg/kg respectively. Using the unpaired *t*-test, the *P*-values of *PbPf*DHFR3m1 and *PbPf*DHFR3m2 against *PbPf*S108N are 0.28 and 0.99 respectively.

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Cysteine at position 50 of *Pf*DHFR is located around the active site region of the enzyme [6]. Previous study on *Pf*DHFR random library in yeast complementation system [27] using a *Pf*DHFR triple mutant (N51I+C59R+S108N) as a template has identified a quadruple mutant C50S+N51I+C59R+S108N that confers resistance to chlorcycloguanil at a level higher than the quadruple mutant N51I+C59R+S108N+I164L, a common antifolate-resistant allele found in field isolates. In *E. coli* complementation system [11, 28], C50R mutation was identified from *Pf*DHFR quadruple mutation library selected by WR99210. The C50R mutation was also reported to confer resistance to sulfadoxine-pyrimethamine in Venezuela and Brazil [20-21]. There was no previous report revealing any mutations at Phe116 of *Pf*DHFR located on the entry of catalytic cleft of the enzyme in the field or any complementation systems. In this study, both transgenic *PbPf*DHFR3m1 and *PbPf*DHFR3m2 lines showed comparable pyrimethamine resistance level with the transgenic *P. berghei* line expressing *Pf*DHFR with mutation at S108N (*PbPf*S108N) previously reported by our group [7]. The results confirm that although additional mutations do not affect the function of the enzyme, a proper combination of mutations is required to confer resistance to the drug, which shows that the *Pf*DHFR mutant library expressed by our *P. berghei* surrogate model can be used as an appropriate model to study the drug resistance level and the evolution of *Pfdhfr* mutations in vivo. The only limitation of this system is host tolerance to the drug. If the animals could tolerate a high dose of drug without toxicity, selection of mutant clones with a higher level of drug resistance would be possible.

CONCLUSIONS

The power of random mutant library and transgenic parasite system to elucidate the functionality of resistant mutant parasites in real situation of *Plasmodium* species has been demonstrated. The approach adopted in this system sets to find and predict such mutations at different positions not only around or at the active sites, but also away from the enzyme catalytic region. Using a moderate drug-resistant $Pfdhfr^{S108N}$ mutant gene as template, a library of *Pfdhfr* mutants was generated and subsequently transfected and selected in *P. berghei* parasite. Two clones of major interest of transgenic *P. berghei* parasites *PbPf*DHFR3m1 (M55I+S108N+S189C) and *PbPf*DHFR3m2 (C50Y+S108N+F116S), expressing *Pf*DHFR with mutations at such positions, showed a similar level of growth rate and resistance to pyrimethamine with the parental transgenic *P. berghei* expressing *Pf*DHFR with mutation at S108N (*PbPf*S108N). The study has reconfirmed that this transgenic *P. berghei* model expressing a random mutant library is an effective model for studying the accumulation of *Pfdhfr* mutations in vivo. The approach of our system is very adaptable and can serve as a better tool than the complementation systems in mimicking possible mutations in nature. The approach can be applied during the development of new antifolates with better effective properties against drug-resistant parasites.

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