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Fitness of sulfadoxine-resistant *Plasmodium berghei* harboring a single mutation in dihydropteroate synthase (DHPS)

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ABSTRACT

Genetic changes conferring drug resistance are generally believed to impose fitness costs to pathogens in the absence of the drug. However, the fitness of resistant parasites against sulfadoxine/pyrimethamine has been inconclusive in *Plasmodium falciparum*. This is because resistance is conferred by the complex combination of mutations in dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*), which makes it difficult to separately assess the extent and magnitude of the costs imposed by mutations in *dhps* and *dhfr*. To assess the fitness costs imposed by sulfadoxine resistance alone, we generated a transgenic rodent malaria parasite, *P. berghei* clone harboring an A394G mutation in *dhps* (PbDHPS-A394G), corresponding to the causative mutation for sulfadoxine resistance in *P. falciparum* (PfDHPS-A437G). A four-day suppressive test confirmed that the PbDHPS-A394G clone was resistant to sulfadoxine. PbDHPS-A394G and wild-type clones showed similar growth rates and gametocyte production. This observation was confirmed in competitive experiments in which PbDHPS-A394G and wild-type clones were co-infected into mice to directly assess the survival competition between them. In the mosquitoes, there were no significant differences in oocyst production between PbDHPS-A394G and wild-type. These results indicate that the PbDHPS-A394G mutation alters the parasites to sulfadoxine resistance but may not impose fitness disadvantages during the blood stages in mice and oocyst formation in mosquitoes. These results partly explain the persistence of the PfDHPS-A437G mutant in the natural parasite populations.

1. Introduction

Malaria is a widely distributed infectious disease caused by *Plasmodium* parasites and transmitted by Anopheles mosquitoes. In 2018, there were 228 million cases and 405,000 deaths worldwide (WHO, 2019). *Plasmodium falciparum* is the most virulent malaria parasite (WHO, 2019). Malarial infection in pregnant women causes severe maternal anemia, abortion, low birth weight, and perinatal mortality (Desai et al., 2007). These risks can be substantially reduced by the successful prevention of malaria with intermittent preventive therapies for pregnant women (IPTp) (Desai et al., 2018; Desai et al., 2007; van Eijk et al., 2019). Sulfadoxine-pyrimethamine is the most used IPTp regimen, particularly in sub-Saharan Africa (WHO, 2019), due to its superior efficacy and fewer adverse events compared to other regimens (Desai et al., 2018). Sulfadoxine-pyrimethamine has also been used as a partner drug in artemisinin-based combination therapies (ACTs), in some endemic regions of the Middle East and some parts of India (WHO, 2019).

Sulfadoxine inhibits the parasite's dihydropteroate synthase (DHPS), an essential coenzyme in the folate synthesis pathway (Dieckmann and Jung, 1986), which acts synergistically with pyrimethamine, an inhibitor of dihydrofolate reductase (DHFR). Resistance to sulfadoxine and pyrimethamine is conferred by amino acid change(s) at particular position(s) in *dhps* (Brooks et al., 1994; Triglia et al., 1997) and *dhfr* (Lozovsky et al., 2009; Peterson et al., 1988), respectively. In *dhps*, an A437G mutation (*dhps* single-mutant) is an initial step for sulfadoxine resistance in *P. falciparum* (Brooks et al., 1994; Mita et al., 2014; Triglia et al., 1997). Thereafter, additional mutations accumulate at particular positions (436, 540, 581, and 613) in a stepwise manner (Brooks et al., 1994; Mita et al., 2014; Triglia et al., 1997). Recently, crystal structures of wild-type and mutant DHPS have been determined in *P. falciparum* (Chitnumsub et al., 2019). Structural analyses showed that all the above-mentioned sulfadoxine-resistant related amino acid changes are located in the para-aminobenzoic acid (pABA)-binding pocket (Chitnumsub et al., 2019).

Acquisition of drug-resistant mechanisms generally imposes a fitness cost to the parasites, which often causes a survival disadvantage under drug-free conditions (Hastings and Donnelly, 2005; Mackinnon and

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Hastings, 1998; Rosenthal, 2013; Walliker et al., 2005). This phenomenon has been widely observed in natural *P. falciparum* parasite populations, particularly in sub-Saharan Africa (Rosenthal, 2013). In these regions, chloroquine-resistant parasites have been outcompeted by chloroquine-susceptible parasites after the cessation of chloroquine use (Balikagala et al., 2020; Laufer et al., 2006; Mita et al., 2003). Laboratory experiments have provided direct evidence of fitness impairment as a result of not only chloroquine resistance (Dhingra et al., 2019; Petersen et al., 2015) but also pyrimethamine (Heinberg et al., 2013) and artemisinin resistance (Nair et al., 2018). In the case of sulfadoxine, however, previous molecular epidemiological observations are conflicting (Duah et al., 2012; Hailemeskel et al., 2013; Tessema et al., 2015; Wamae et al., 2019; Zhou et al., 2008). Furthermore, laboratory analysis using the field isolates is hardly available on the fitness cost associated with *dhps* mutations, because the resistance of the field isolates is conferred by the complex combination of mutations in both *dhps* and *dhfr*, making it difficult to solely assess the magnitude of the costs imposed by *dhps* mutation.

To clarify whether sulfadoxine resistance imposes a fitness disadvantage in the absence of the drug, we performed *in vivo* analyses using a rodent malaria model that could separately evaluate the parasite's fitness within both mice and mosquitoes. We first generated a sulfadoxine-resistant rodent malaria parasite, *P. berghei,* harboring an A394G mutation in *dhps* (PbDHPS-A394G), which is equivalent to a mutation (A437G) that confers sulfadoxine resistance in *P. falciparum* (Brooks et al., 1994; Mita et al., 2014; Triglia et al., 1997). The fitness of wild-type and *dhps* mutant parasites was then assessed in both the mouse and mosquito stages.

2. Materials and methods

2.1. Parasites, mosquitoes, and mice

The *P. berghei* ANKA strain was used for genetic modifications. The *Anopheles stephensi* SXK strain was maintained at 25 °C ± 1 °C, 80% humidity, and light: dark (12:12) cycle. Adults were fed with 10% (w/v) sucrose containing 0.05% 4-aminobenzoic acid (Beier et al., 1994; Matz et al., 2019; Peters and Ramkaran, 1980). To maintain their lifecycle, female mosquitoes were fed with mouse blood to induce egg-laying on a filter paper in a cup filled with distilled water containing 0.005% (w/v) mineral salt. Hatched larvae were reared in distilled water containing 0.005% (w/v) mineral salt and were given TetraMin®. BALB/c and ddY mice (female, 6-week-old, Sankyo Labo Service Corporation, JAPAN) were used in this study and maintained at 26 °C and light: dark (12:12) cycle.

2.2. Generation of PbDHPS-A394G mutant clones

The PbDHPS-A394G mutant was generated by genome editing using clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (CRISPR/Cas9). The pYC plasmid was originally generated for the genome editing of *P. yoelii* and was generously gifted by Dr. Yuan (Zhang et al., 2014). The U6 promoter of *P. yoelii* in the pYC plasmid was replaced by the corresponding gene of *P. berghei*, resulting in the pBC-1 plasmid. As a donor, a fragment encoding *the A394G* mutation and silent mutations of *P. berghei dhps* (PbDHPS) (PBANKA_1426700) was synthesized by GENEWIZ® (Fig. S1) and used as a template for PCR using a pair of primers, pbdhps-forward and reverse. The resultant PCR fragment was ligated into pBC-1 using the In-Fusion HD Cloning Kit (Takara Bio Inc.) after digestion with HindIII/AflII (pBC-2). A potential PAM site was searched using CHOP-CHOP (http://chopchop.cbu.uib.no) and double-stranded DNA coding for gRNA was ligated into a BsmBI site in pBC-2 using In-Fusion ligation. The final plasmid (pBC-pbdhps) was checked with Sanger sequencing. Primers and gRNAs are listed in Table S1.

PbWT-GFP parasites are marker-free and express GFP constitutively driven by heat shock protein 70 promoter during the entire lifecycle, as reported previously (Kooij et al., 2012). PbWT-GFP was transformed with pBC-pbdhps to generate PbDHPS-A394G-GFP. PbWT-mRuby2 parasites are similar to PbWT-GFP, except that GFP is replaced by mRuby2. Parasite transfection and cloning were performed following previously reported methods (Janse et al., 2006) using ddY mice. The successful insertion of PbDHPS-A394G was confirmed in PbDHPS-A394G-GFP with Sanger sequencing.

2.3. Evaluation of sulfadoxine resistance

A four-day suppressive test (Peters, 1975) was performed to evaluate sulfadoxine resistance in PbDHPS-A394G clones. Five BALB/c mice were intravenously inoculated with 1×10^6 erythrocytes infected with either PbDHPS-A394G-GFP, PbWT, PbWT-GFP, or PbWT-mRuby2. Subsequently, 100 μL of sulfadoxine (3.0 mg/kg in 0.4% dimethylsulfoxide (DMSO), 0.5% hydroxypropylmethylcellulose (HPMC), 0.4% Tween 80, and 0.5% benzyl alcohol) (Yuthavong et al., 2012) was orally administrated at 4 h, 24 h, 48 h, and 72 h post-infection (Neerja and Puri, 2004; Songsungthong et al., 2016) (Fig. S2). Parasitemia was calculated between day 1 and day 7 post-infection by counting the number of infected erythrocytes per 3000 erythrocytes on Giemsa-stained thin blood smears under a light microscope (Olympus CX41) (1000×).

2.4. Parasite fitness in mice

To evaluate parasite fitness in mice, four to eight BALB/c mice were intravenously inoculated with 1×10^4 erythrocytes infected with either PbDHPS-A394G-GFP, PbWT, PbWT-mRuby2, or PbWT-GFP parasites, and the growth rate of each parasite line was monitored. Parasitemia and gametocytemia were calculated between day 2 and day 8 post-infection on 10% Giemsa-stained thin blood smears. Fitness was also assessed in mice in a survival competition experiment between PbDHPS-A394G and wild-type parasites. A total of 1×10^4 erythrocytes infected with an equal ratio of PbDHPS-A394G-GFP to PbWT-mRuby2 parasites were intravenously inoculated into four BALB/c mice. The ratio of green (PbDHPS-A394G-GFP) to red (PbWT-mRuby2) fluorescent signals was calculated by counting ten fields, which were equivalent to 6×10^4 erythrocytes between day 2 and day 10 post-infection.

2.5. Survival competition between PbDHPS-A394G and wild-type parasites in mosquitoes

BALB/c mice were intravenously inoculated with 1×10^5 erythrocytes infected with PbDHPS-A394G-GFP and PbWT-mRuby2 at a ratio of 1:1. On day 4 post-infection, 100–150 female mosquitoes fed on each mouse directly. After feeding, only fully engorged females were picked up and cultured. On day 12 post-blood-feeding, the mosquitoes were picked up randomly and dissected, and the number of green- and red-fluorescent oocysts in the midgut were counted under a fluorescent microscope. Self-fertilization of the PbDHPS-A394G-GFP and PbWT-mRuby2 clones produced green- and red-fluorescent oocysts, respectively, while cross-fertilization between two transgenic clones produced a parasite expressing orange fluorescence. To investigate the survival competition between PbDHPS-A394G and wild-type parasites in mosquitoes, the ratio of green to red oocysts was calculated.

2.6. Statistical analysis

The significance of discordance of parasitemia, gametocytemia, and green/red- fluorescent oocysts was measured using the Tukey-Kramer test, one sample t-test, Student's t-test or Wilcoxon rank sum test. All statistical analyses were performed using R version 3.6.0. (r-project.org) $P < 0.05$ was considered statistically significant.

2.7. Ethical approval

The experiments using mice and mosquitoes were approved by the Animal Care and Use Committee of Juntendo University, and the experiments were performed according to the committee's guidelines. The transgenic *P. berghei* clone was generated in accordance with the guidelines of the recombinant DNA experiments committee of Juntendo University. The assigned approval numbers for animal and recombinant DNA experiments were 310043 and 25–115, respectively.

3. Results

3.1. Generation of a PbDHPS-A394G clone and evaluation of sulfadoxine resistance

We generated a PbDHPS-A394G-GFP clone using the CRISPR/Cas9 system and confirmed successful gene editing (Fig. S3). To confirm that the PbDHPS-A394G clone was sulfadoxine-resistant, we performed a four-day suppressive test with oral administration of 3.0 mg/kg of sulfadoxine at 4, 24, 48, and 72 h after parasite inoculation (Fig. S2). Wild-type parasite clones (PbWT, PbWT-mRuby2, and PbWT-GFP) were cleared between day 3 and day 7 following sulfadoxine administration (Fig. 1). In contrast, parasitemia continuously increased to over 5% on day 5 in the mice infected with PbDHPS-A394G-GFP clones. These results indicate that the A394G mutation in *Pbdhps* confers sulfadoxine resistance to *P. berghei*.

3.2. Fitness of sulfadoxine-resistant parasites in mice

To assess the fitness of sulfadoxine-resistant parasites within mice, the growth rate of the sulfadoxine-resistant (PbDHPS-A394G-GFP) clone was compared to that of susceptible ones (PbWT, PbWT-mRuby2, and PbWT-GFP) during the course of infection in the absence of sulfadoxine (Fig. 2). No significant differences in growth rates were observed among the four groups (the Tukey-Kramer test: $p > 0.05$); parasitemia in all mice similarly increased from day 4, which reached 5 to 8% on day 8 post-infection.

Next, we investigated the competition between sulfadoxine-resistant (PbDHPS-A394G-GFP) and susceptible (PbWT-mRuby2) clones within the mice. After inoculation of an equal number of these clones into mice, the parasites expressing green (PbDHPS-A394G-GFP) and red (PbWT-mRuby2) fluorescent signals were counted under a fluorescence microscope until day 10 (Fig. S4). Parasitemia in both clones similarly increased from day 4, reaching 5 to 8% on day 8 post-infection (Fig. 3A). No statistically significant difference in parasitemia was observed between the two clones (Student's t-test: $p > 0.05$). These ratios remained close to 1.0 throughout the monitored period (one sample t-test, $p > 0.05$) (Fig. 3B). These results indicate that the A394G mutation did not affect the growth rate of the parasite in the erythrocyte stage.

3.3. Gametocyte production rate

The gametocyte production rate (gametocytemia) was compared between PbDHPS-A394G and three *Pbdhps* wild-type clones (PbWT-mRuby2, PbWT-GFP, and PbWT) (Fig. 4). The results showed that the patterns of gametocytemia were similar in all parasite clones; they started to rise on day 5 and peaked at 0.75% on day 6. There was no statistically significant difference in parasitemia between each clone (the Tukey-Kramer test: $p > 0.05$).

3.4. Fitness of sulfadoxine-resistant parasites in mosquitoes

Equal numbers of gametocytes taken from the PbDHPS-A394G-GFP and the PbWT-mRuby2 clones infected mice were co-infected into mosquitoes to assess the relative fitness of the sulfadoxine-resistant clone in the mosquito stage. A total of 48 mosquitoes were obtained from five mice (Table S2). The number of oocysts observed varied widely among mosquitoes from 1 to 368 (Median 15). The number of green oocysts (PbDHPS-A394G-GFP) per midgut was not significantly different from that of red oocysts (PbWT-mRuby2) (10 *vs.* 11, $p = 0.990$, Wilcoxon rank sum test) (Fig. 5A and B). This result demonstrates that the PbDHPS-A394G clone was fertilized and differentiated into oocyst with an efficiency similar to that of the wild-type parasite.

4. Discussion

The aim of this study is to elucidate sulfadoxine resistance imposes a fitness cost on *Plasmodium* parasites. The results showed that introducing this mutation did not change the parasite fitness in mice (growth speed in the erythrocyte stage and gametocyte production) and mosquitoes (the number of oocysts). This is the first in vivo study of how mutations conferring resistance to sulfadoxine affect parasite fitness.

To compare the fitness of PbDHPS-A394G and wild-type clones in the absence of sulfadoxine, we performed competitive experiments co-infecting these two types of clones in the same host. This experiment is important because the fitness of one organism can be potentially affected by other organisms coexisting in the same ecosystem, such as the interplay between PbDHPS-A394G and wild-type clones in the current study (Gordo et al., 2011). We first expected that PbDHPS-A394G would eventually succumb to the wild-type, as has previously been seen for other drug-resistant malaria parasites (Balikagala et al., 2020; Laufer et al., 2006; Mita et al., 2003). However, no significant difference in parasite growth speed was detected between PbDHPS-A394G and wild-type in mice. No in vivo experiment to assess the fitness of sulfadoxine-resistant parasites had been performed so far. Although some have been reported for pyrimethamine (Rosario et al., 1978; Shinondo et al., 1994), the results were, however, not consistent; one study reported a similar fitness between sulfadoxine-resistant and -susceptible *P. berghei* parasites in the absence of pyrimethamine (Shinondo et al.,

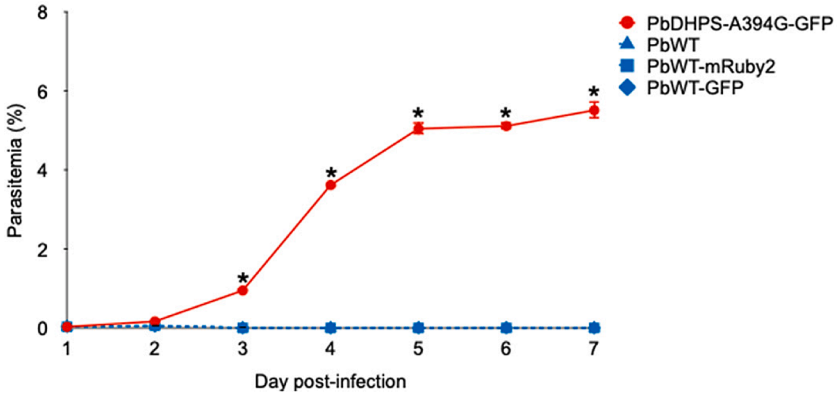

Fig. 1. Sulfadoxine resistance of PbDHPS-A394G-GFP, PbWT, PbWT-mRuby2, and PbWT-GFP *Plasmodium berghei* clones. PbDHPS-A394G-GFP (red circle), PbWT (blue triangle), PbWT-mRuby2 (blue square), or PbWT-GFP (blue rhombus) were intravenously injected into five BALB/c mice. Subsequently, sulfadoxine (3.0 mg/kg) was orally administrated at 4 h, 24 h, 48 h, and 72 h post-infection. Means and standard errors of the mean are shown. Statistically significant differences in parasitemia were observed between the PbDHPS-A394G-GFP clone and each susceptible clone (PbWT, PbWT-mRuby2, and PbWT-GFP) (the Tukey-Kramer test, *$p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

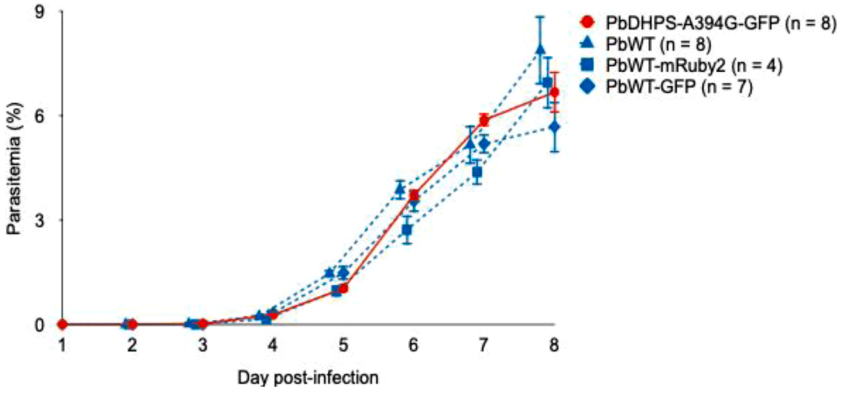

Fig. 2. Growth of PbDHPS-A394G-GFP, PbWT, PbWT-mRuby2, and PbWT-GFP *P. berghei* clones in mice without sulfadoxine. Four to eight mice were used per group. Means and standard errors of the mean are shown. No statistically significant difference was observed in parasitemia between each clone (the Tukey-Kramer test: $p > 0.05$).

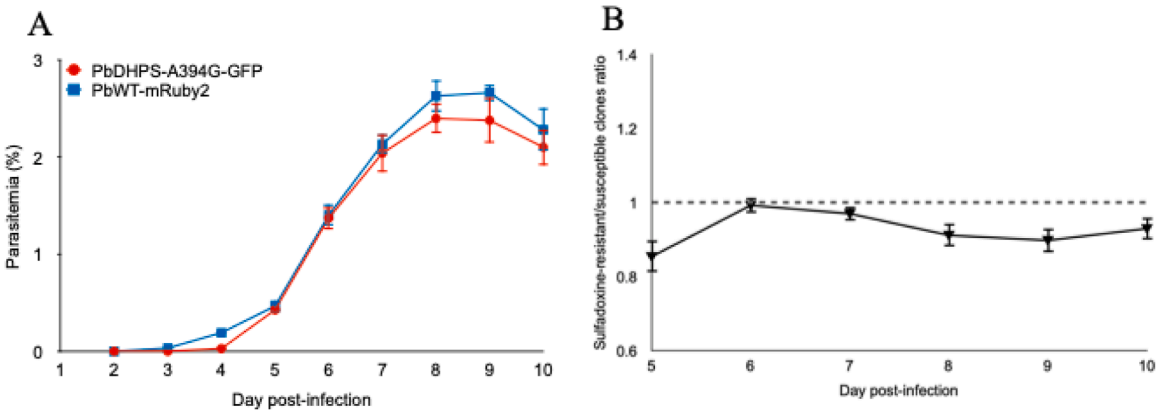

Fig. 3. Within-mouse growth competition analysis between sulfadoxine-resistant (PbDHPS-A394G-GFP) and -susceptible (PbWT-mRuby2) *P. berghei* clones without sulfadoxine. (A) The parasitemias of PbDHPS-A394G-GFP (resistant) and PbWT-mRuby2 (susceptible) clones are expressed as the mean ± standard error of the mean. No statistically significant difference in parasitemia was observed in any sampling day (Student's t-test: $p > 0.05$). Four mice were used per group. (B) The ratios (resistant (GFP)/susceptible (mRuby2)) are expressed as the mean ± standard error of the mean. No statistically significant difference was observed (one sample t-test: $p > 0.05$).

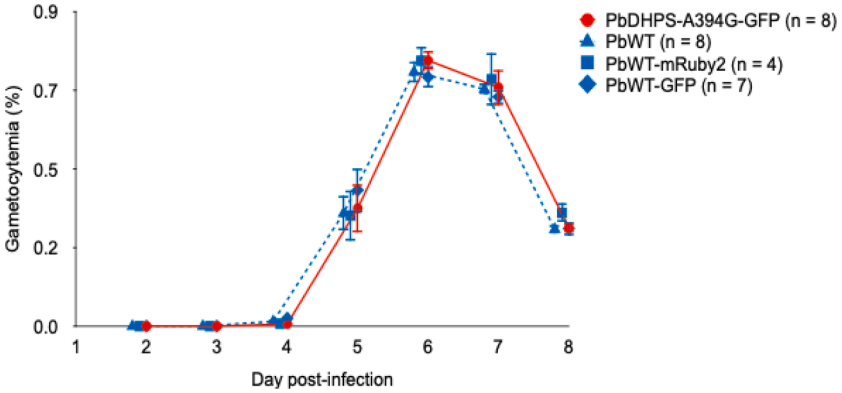

Fig. 4. Gametocytemia between sulfadoxine-resistant and -susceptible (PbWT-mRuby2) *P. berghei* clones without sulfadoxine. Gametocytemia is expressed as the mean ± standard error of the mean; PbDHPS-A394G-GFP (red circle), PbWT (blue triangle), PbWT-mRuby2 (blue square), or PbWT-GFP (blue rhombus). Four to eight mice were used per group. No statistically significant difference was observed (the Tukey-Kramer test: $p > 0.05$).

1994), whereas this was not observed in another study using *P. chabaudi* (Rosario et al., 1978). Apart from the difference in *Plasmodium* species used for the experiments, one reason for this inconsistency could be the potential occurrence of additional genetic changes that compensate for the fitness impairment imposed by drug resistance. In contrast, our study used a sulfadoxine-resistant clone that was only one amino acid different (A394G in PbDHPS) from the susceptible clone. Therefore, we were able to exclude the effects of other gene mutations and directly examine the change in fitness when acquiring a resistance gene to sulfadoxine.

We also found that the introduction of PbDHPS-A394G did not change either the number of gametocytes or oocyst formation in mosquitoes, suggesting that the relative fitness did not change during

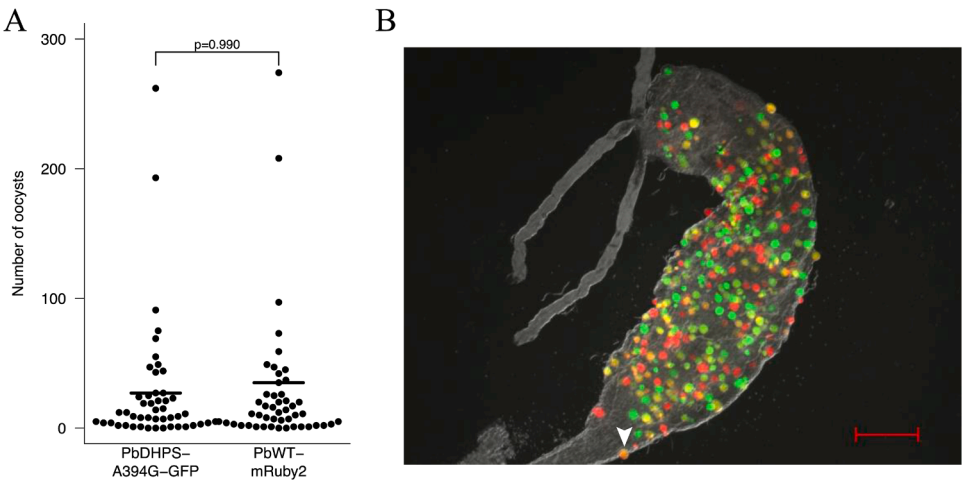

Fig. 5. Within-mosquito competition between sulfadoxine-resistant (PbDHPS-A394G-GFP) and -susceptible (PbWT-mRuby2) *P. berghei* clones. (A) The numbers of oocysts in the infected mosquitoes are plotted. A total of 48 infected mosquitoes were obtained from five mice. Horizontal bars indicate the median. No statistically significant difference was observed (Wilcoxon rank sum test: $p > 0.05$). (B) Overlay image of the mosquito midgut carrying oocysts. Each oocyst from self-fertilization of PbDHPS-A394G-GFP and PbWT-mRuby2 shows green and red signals, respectively, whereas oocysts from cross-fertilization are orange (arrowhead). The scale bar represents 200 μm.

the studied developmental stages. However, since several thousands of sporozoites are produced in one oocyst, substantial DNA synthesis is required for sporozoite formation at this stage. It is conceivable that fitness elevation may appear after the oocyte stage, such as the number of sporozoites per oocysts and salivary gland sporozoites. However, we did not assess parasite fitness after the oocyst stages. This is because sexual recombination occurs in mosquito stages, which may segregate *PbDHPS* and *GFP* separately. Thus, the GFP fluorescent signal was not used as a marker for PbDHPS-A394G after the oocyst stages.

In our study, we did not see significant difference in the fitness between PbDHPS-A394G and wild-type parasites during examined developmental stages. In vitro enzymatic study revealed that PfDHPS-A437G protein exhibited a higher affinity to pABA than wild-type did (Chitnumsub et al., 2019). This suggests that PfDHPS-A437G parasites may utilize a limited amount of pABA more efficiently than wild-type. It is thus conceivable that the fitness advantage of PbDHPS-A394G, a corresponding mutation of PfDHPS-A437G, could be visible under pABA-restricted conditions. However, this scenario is unlikely because *P. berghei* can synthesize sufficient amount of pABA for normal development in mice that are fed with pABA-free diet (Matz et al., 2019). Besides, the mutant parasite lacking aminodeoxychorismate synthase, one of two pABA synthesis enzymes, could produce oocysts with a similar number to that of wild-type under pABA-free conditions, suggesting that pABA is not required for oocyst formation (Matz et al., 2019). Overall, it can be concluded that the externally supplied pABA in our experiment does not interfere with the proper evaluation of parasite fitness.

This study investigated the role of the *Pbdhps* A394G mutation on parasite fitness. However, as mentioned earlier, malaria parasites can accumulate various genetic changes to compensate for fitness. One example is the *gch1* gene, which encodes a GTP cyclohydrolase that acts upstream of *dhps* in the folate synthesis pathway (Nair et al., 2008). Since an increased copy number of *gch1* has been strongly associated with a large number of mutations in *dhps* and *dhfr*, it has been suggested that this genetic change compensates for the fitness impairment caused by *dhfr* and/or *dhps* (Nair et al., 2008). However, a transfection study revealed that the compensation effects varied among parasites with different genetic backgrounds (Heinberg et al., 2013). Importantly, this finding suggests that parasite fitness is also determined by the complex combination of background genetic changes. Similar observations have also been described in artemisinin (Straimer et al., 2017) and chloroquine resistance (Dhingra et al., 2019). As mentioned above, the potential effect of background mutations was excluded in the present study. This is an appropriate experimental system to clarify the change in fitness that occurs when only resistance gene mutations are acquired. However, to estimate fitness in the actual malaria parasite population, it is necessary to create an experimental system considering additional gene mutations that may compensate for fitness changes. Forward genetics is a promising approach to elucidate this. Under the continuous progressive sulfadoxine pressure, parasites accumulate background mutations as well as mutations in *dhps*, which enables us to elucidate the effect of background mutations on fitness compensation. We generated a transgenic *P. berghei* parasite with defective proofreading exonuclease activity in DNA polymerase, which displayed a 37-fold higher mutation rate than that of wild-type parasites (Honma et al., 2014; Honma et al., 2016). The utilization of this mutator parasite will accelerate the isolation of sulfadoxine-resistant parasites with various background mutations (Hirai and Mita, 2015), as we have successfully identified the gene associating with the reduced susceptibility to piperaquine by using this transgenic parasite (Ikeda et al., 2021).

In conclusion, we found that a single mutation for sulfadoxine resistance was not disadvantageous in the absence of the drug. Our results support the observation that the A437G mutation was largely distributed even after the use of sulfadoxine-pyrimethamine for first-line malaria treatment was discontinued (Basuki et al., 2018; Conrad et al., 2017; Jiang et al., 2019; Kaingona-Daniel et al., 2016; Mandoko et al., 2018; Osarfo et al., 2018; Ravenhall et al., 2016; Voumbo-Matoumona et al., 2018; Yaqoob et al., 2018; Zeng et al., 2016). The spread of sulfadoxine-pyrimethamine resistant parasites threatens the effectiveness of IPTp, particularly the combination of *dhps* triple-mutant (SGEGA) and *dhfr* triple-mutant (CIRNI, at amino acid positions 50, 51, 59, 108, and 164 with mutations underlined), known as sextuple mutants, in some endemic regions of Africa (van Eijk et al., 2019). Further studies to assess the effect of sextuple mutants on fitness will provide useful information for future IPTp strategies. In this regard, this study is the first step in clarifying changes in fitness caused by resistance to sulfadoxine in mouse and mosquito hosts.

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Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author contributions

M.Y. performed all the experiments, analyzed the results, and wrote the manuscript. M.H. supervised the experimental procedures, provided resources, and wrote the manuscript. S-I.T. supervised the experimental procedures and provided resources. T. Mori supervised the experimental procedures and provided resources. T. Mita conceived the original idea, provided resources, and wrote the manuscript.

CRediT authorship contribution statement

Masato Yamauchi: Investigation, Formal analysis, Writing – original draft, Visualization. **Makoto Hirai:** Methodology, Supervision, Resources, Writing – review & editing. **Shin-Ichiro Tachibana:** Supervision, Resources. **Toshiyuki Mori:** Supervision, Resources. **Toshihiro Mita:** Conceptualization, Resources, Writing – review & editing.

Declaration of Competing Interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2021.106049.

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